

PC1

Characterisation of KCNQ expression and functional impact in the cerebral vasculatureJ.D. Moffatt², A. Hatch², S.M. Yeung¹ and I. Greenwood¹¹Basic Medical Sciences, St George's, University of London, London, UK and ²The Royal Veterinary College, London, UK

Voltage-dependent potassium channels are crucial determinants of smooth muscle membrane potential and ipso facto vascular contractility. Recent work by the authors revealed that Kv7 channels encoded by KCNQ genes influenced vascular reactivity in mouse portal vein (Ohya et al., 2003; Yeung & Greenwood, 2005). The aim of the present study was to ascertain whether KCNQ genes were expressed in rodent cerebral vasculature and to determine if the expression products influenced vascular reactivity.

Total RNA was extracted from SD rat and BALB/c mouse basilar arteries (rBA / mBA) and rat middle cerebral arteries (MCA) using the RNeasy mini spin columns (Qiagen, UK) and following DNase I treatment was reverse-transcribed to cDNA using M-MLV (Invitrogen, UK). KCNQ gene expression products were detected using primers specific for the 5 different isoforms. RNA derived from mouse heart and brain was used as positive controls. Isometric tension experiments were performed using segments of rat basilar artery mounted on a wire myograph and bathed in Krebs' solution aerated with 95% O₂ / 5 % CO₂ at 37°C.

KCNQ1 and KCNQ4 were expressed in the basilar arteries and MCA at sufficiently high levels to be detected easily after 35 PCR cycles (n=3 each). Nested PCR was employed to explore the expression of the isoforms further. Whilst this technique revealed some KCNQ2 to be present KCNQ3 and KCNQ5 were not detected. Appropriate amplicons were detected in the brain and heart samples. In the functional studies, the selective Kv7 channel blocker XE991 (10 µM) contracted rBA from baseline tension to 4.93 ± 0.69 mN in 14 of 15 preparations from 8 animals. This equated to 103 ± 31 % of the contraction produced by 60 mM KCl. The Kv7 channel activator flupirtine (1 and 10 µM) relaxed contractions produced by 1 µM 5HT with a mean relaxation of 72 ± 10 % (n=7 from 4 animals) at 10 µM. These were reversed completely by 10 µM XE991.

These studies show that cerebral arteries express KCNQ1 and KCNQ4 prominently and modulation of the ion channels alters markedly cerebral artery basal or 5-HT-induced tone. As flupirtine does not activate Kv7.1 channels these data combined suggest that ion channels encoded by KCNQ4 are important determinants of cerebral blood flow.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC2

Mechanism of activation by carbon monoxide of the recombinant human large conductance potassium (BK_{Ca}) channel *in vitro*

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Carbon monoxide (CO) is a potent activator of large conductance, calcium-dependent potassium (BK_{Ca}) channels when heterologously expressed in HEK293 cells (Williams et al., 2004; Jaggar et al., 2005) or when natively expressed in carotid body glomus (Riesco-Fagundo et al., 2001) and vascular smooth muscle cells (Jaggar et al., 2002). Cellular CO production emanates primarily from the enzymatic catalysis of heme by hemeoxygenases, a process which has the strict co-requirement for nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (O₂). Hemeoxygenase-2 (HO-2) is a protein partner closely associated with the BK_{Ca} channel complex and co-application of its substrates, NADPH, heme and O₂, evoke channel activation which is rapidly reversed upon removal of O₂ (Williams et al., 2004). Such data suggest that HO-2 functions as an O₂ sensor in tissues where BK_{Ca} and HO-2 are co-expressed. Here, using the BK_{Ca} channel α -subunit (KCNMA1 - BK α) stably expressed in HEK293 cells, we investigated the mechanism of channel activation by CO in inside-out excised membrane patches. CO was introduced into the bathing solution by using the well-characterised chemical CO-donor, tris-carbonyldichlororuthenium (II) dimer ([Ru(CO)₃Cl₂]); the breakdown product of this donor was utilised as a control. CO evoked a rapid activation of BK α which was concentration-dependent ($EC_{50} = 37.9 \pm 4.3$ µM, mean $\pm$ S.E.M., 35 observations from 7 patches) and freely reversible. Single channel conductance (in symmetrical 140 mM potassium solutions, $[Ca^{2+}]_i = 336$ nM) was unaltered upon treatment with CO (187.0 ± 4.8 pS vs. 173.9 ± 6.9 pS (n = 7, P > 0.1, Student's paired t-test)). However, CO evoked an increase in the rate of channel activation which was reflected in a reduction in the activation time constants from, for example, 11.82 ± 3.70 ms to 3.7 ± 0.95 ms (at +50 mV, n = 7, P < 0.01) without affecting the deactivation time constants. CO activation demonstrated a dependence on $[Ca^{2+}]_i$ with a window of between 100 nM and 10 µM. Importantly, application of CO within this Ca²⁺ window was able to superstimulate BK α activity to levels not achieved by saturating $[Ca^{2+}]_i$. These data show that CO robustly stimulates BK_{Ca} channels principally by increasing the rate of channel opening. The β subunit is not necessary for this regulatory event. Although a pivotal event in this CO-evoked stimulation is clearly an increase in the intrinsic Ca²⁺-sensitivity of the α -subunit, a major component of this activation occurs independently of Ca²⁺, via a hitherto unidentified mechanism.

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Jaggar JH, Li A, Parfenova H, Liu J, Umstot ES, Dopico AM, & Leffler CW (2005). *Circ. Res.* **97**, 805-812.

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Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C, & Kemp PJ (2004). *Science* **306**, 2093-2097.

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PC3

Structural determinants of BK_{Ca} channel modulation by carbon monoxide: Where does CO bind?

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Regulation of large conductance, Ca²⁺-activated potassium (BK_{Ca}) channels by O₂-dependent enzymatic heme catalysis is primarily mediated by CO (Williams et al., 2004). However, the mechanism by which CO modulates BK_{Ca} channel activity remains elusive. Using HEK 293 cells stably and transiently expressing the α 1 subunit (KCNMA1) of the BK_{Ca} channel, we have investigated the structural and molecular determinants of channel regulation by CO in excised, inside-out membrane patches. CO was introduced into the bathing solution by addition of 30 μ M of a CO-releasing molecule or its stable, inactive analogue as a control. CO treatment resulted in a rapid and reversible activation of the BK_{Ca} whereas the control compound had no effect. Activation by CO was identical whether the β 1 channel subunit (KCNMB1) was co-expressed with the α 1-subunit or not. The effect of redox state of the channel was examined by treating patches with hypoxic (pO₂ ~ 40 mmHg) or hyperoxic (500 mmHg) solutions. Hypoxic conditions significantly reduced mean patch current from 0.40 ± 0.14 to 0.03 ± 0.01 pA ($n=10$, $P<0.03$). Hyperoxia had no significant effect on BK_{Ca} channel activity ($P>0.3$). Application of CO during either hypoxia or hyperoxia robustly increased mean patch current to 1.95 ± 0.80 pA ($n=10$, $P<0.05$) and 1.18 ± 0.54 pA ($n=6$, $P<0.05$), respectively. It has been proposed (Jaggar et al., 2005) that CO regulates BK α channels via binding to reduced heme which associates with the α subunit at a consensus heme binding domain containing a conserved histidine (H611). Our data are not consistent with this hypothesis and we found that the redox state of exogenously added heme was unimportant to CO activation. Thus, in its oxidised form, heme (10 μ M) was able to inhibit activity as predicted (from 0.35 ± 0.11 to 0.06 ± 0.02 ($n=14$, $P<0.05$)), but concurrent CO treatment overcame this inhibition to increase mean patch current to 0.84 ± 0.36 pA ($n=14$, $P<0.05$). Moreover, heme itself (either reduced or oxidised) was not required for the CO effect since the channel activity of a H611R channel mutant (which does not bind heme, and was not inhibited by heme application – not shown) was still activated by CO. Finally, following the mutation of another histidine which has been implicated in CO regulation of the channels (H259R), singly and in combination with H611R, CO still activated both H259R (not shown) and H259R/H611R from 0.003 ± 0.002 to 0.178 ± 0.08 pA, $n=9$, $P<0.05$). These data show that, contrary to previous suggestions, CO binds to and activates BK_{Ca} channels by a mechanism which is independent of redox state, does not require heme and is independent of heme binding at conserved histidine residues.

Jaggar JH, Li A, Parfenova H, Liu J, Umstot ES, Dopico AM, & Leffler CW (2005). *Circ. Res.* **97**, 805-812.

Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C, & Kemp PJ (2004). *Science* **306**, 2093-2097.

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PC4

Blood free radical levels changes and myocardium apoptosis at the experimental heart failure

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Aims: Oxidative stress in the myocardium may play an important role in the pathogenesis of heart failure (HF) [1]. The aim of study was to examine the levels of free radicals (superoxide anion (O₂⁻), hydroxyl radical (OH), peroxynitrite (ONOO⁻), nitric oxide (NO) and superoxide dismutase (SOD) at the experimental HF, to detect the cardiomyocyte apoptosis and to calculate the apoptotic index (AI).

Methods: Wistar 16 male rats were divided at 2 groups. First group consisted from 12 rats, was subjected to post – myocardial infarction HF by 8 hours daily immobilizing rats in supine position [2] during 14 days. Second group was control and consisted from 4 animals. First group was divided at 4 subgroups, each consisting of 3 animals. At the 3rd, 7th, 11th and 14th day of experiment subgroups were subjected to decapitation in turn. Pairs of control animals were decapitated before and at the end of experiment. Experimental work was conducted according to [3;4]. Free-radicals, SOD and NO levels were detected by electron-paramagnetic resonance [5]. Apoptosis was registered by TUNEL method. The analysis of microscopic section was carried out at the microscope with semiachromatic Fluotar lens. AI was calculated in 20 arbitrary chosen vision fields at 400 multiple magnification. AI was calculated as ratio of positive stained cardiomyocyte nucleus to total quantity of cardiomyocytes. All concentrations were presented in nmole/ml.

Results:

(i) Blood concentrations of free radicals were not only authentically higher of analogous control group levels (O₂⁻ 7.86 ± 0.13 , OH 2.82 ± 0.16 , ONOO⁻ 0.96 ± 0.02), $p<0.001$, but dynamical growth was registered during 3rd, 7th, 11th, and 14th days of experiment (O₂⁻ – 15.73 ± 0.24 , 19.93 ± 0.72 , 19.39 ± 0.45 , 19.56 ± 0.12 ; OH 5.15 ± 0.29 , 6.62 ± 0.51 , 7.18 ± 0.86 , 6.91 ± 0.10 ; ONOO⁻ 2.75 ± 0.18 , 4.45 ± 0.17 , 4.36 ± 0.12 , 4.31 ± 0.25), $p<0.05$.

(ii) HF development at 3rd, 7th, 11th, and 14th days of experiment led to authentic decreasing of blood levels of NO (4.28 ± 0.18 , 6.44 ± 0.82 , 7.42 ± 0.69 , 7.44 ± 0.68 , $p<0.05$) and SOD (6.84 ± 0.37 , 7.79 ± 0.27 , 8.35 ± 0.83 , 7.93 ± 0.44 , $p<0.05$), in comparison with analogous dates of control group (NO 35.47 ± 3.14 , SOD 15.64 ± 0.29), $p<0.05$.

(iii) TUNEL registered single apoptotic cardiomyocytes in control group microscopic sections. AI was $6.3 \pm 3.1\%$. At experimental group animals the AI variation were from 18% to 61%.

At 3rd, 7th, 11th, 14th experimental day AI were $21.4 \pm 3.5\%$, $36.2 \pm 2.4\%$, $56.3 \pm 3.8\%$, $47.4 \pm 3.6\%$, ($p < 0.05$).

Conclusion: Experimental HF led to activation of free-radical system and reduction of NO and SOD levels, which probably promote apoptosis. Quick increasing of apoptotic cardiomyocyte quantity in periinfarct area was registered at first days of experiment and run to maximum at 11th day up. Massive apoptotic damaging led to decreasing of viable cardiomyocytes quantity and quick HF progression.

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PC5

Antioxidant status in diabetic, non-diabetic and senile patients with cataract and cardiovascular complications

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A common denominator in pathogenesis of most chronic diseases is the involvement of oxidative stress. The antioxidant defense system has many components. A deficiency in any of these components can cause a reduction in overall antioxidant status of an individual. It was aimed to assess the total antioxidant status in diabetic, non-diabetic and senile patients with, cataract, cardiovascular complications and without complications.

One hundred eighty-six patients and control subjects were selected. Among them thirty three were diabetic patients without any clinical evidence of chronic diabetic complications, thirty two were diabetic patients with cardiovascular complications, thirty were non-diabetic patients with cardiovascular complications, thirty were diabetic patients with cataract, thirty were non-diabetic patients with cataract and thirty-one apparently normal, age, sex and weight matched control subjects were investigated. All these patients were selected on clinical grounds from National Institute of Cardiovascular Disease, Karachi and Eye Ward Jinnah Postgraduate Medical Centre, Karachi, Pakistan. Total antioxidant status were significantly decreased ($P < 0.001$) in all diabetic patients with and without complication and non-diabetic patients with same complications as compared with control subjects. Fasting plasma glucose was increased ($P < 0.001$) in

all diabetic patients with and without complications and correlated significantly with glycosylated hemoglobin and serum fructosamine concentrations. Fasting plasma glucose, glycosylated hemoglobin and serum fructosamine were not different in diabetic patients with and without complications. Fasting plasma glucose, glycosylated hemoglobin, serum fructosamine and total serum protein were not different in non-diabetic patients with same complications as compared with control subjects.

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PC6

Hyperbaric oxygen preconditioning enhances myocardial eNOS and Hsp72 expression and improves myocardial function following ischemic reperfusion injury

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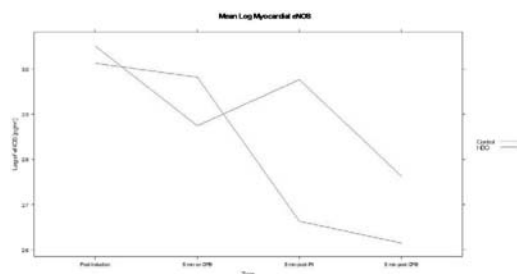
Experimental evidence has demonstrated that hyperbaric oxygen (HBO) preconditioning prior to ischemic reperfusion injury (IRI), limits myocardial infarct size(1). This cardioprotective mechanism involves the expression of myocardial endothelial nitric oxide synthase (eNOS) and heat shock protein 90 (Hsp90)(2). IRI also occurs in patients during coronary artery bypass graft surgery (CABG). This study clinically assessed the effects of HBO preconditioning prior to IRI on the myocardial eNOS and Hsp72 expression and, myocardial function following IRI.

This randomised control study of patients experiencing IRI during CABG involving cardiopulmonary bypass (CPB), consisted of 40 and 41 patients in Groups A (Control Group) and B (HBO Group), respectively. HBO preconditioning started 4 hours prior to CABG for 90 minutes at 2.4 ATA using 100% oxygen. Right atrial biopsies were sampled post anaesthetic induction, 5 minutes following the onset of CPB, 5 post ischemic reperfusion and 5 minutes post CPB. Atrial protein extracts were analysed for eNOS and Hsp72 via a quantitative sandwich ELISA. Pulmonary artery catheters measured haemodynamic parameters post induction, 5 minutes, 2, 4, 8, 12 and 24 hours post CPB.

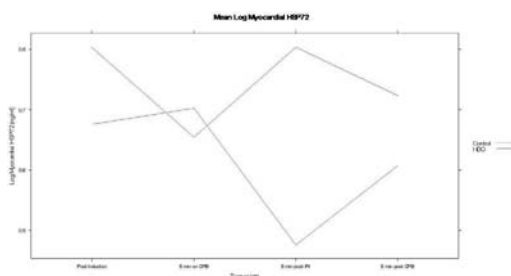
Post CPB, the stroke volume (SV) ($p = 0.01$), left ventricular stroke work (LVSW) ($p = 0.005$), left ventricular stroke work index (LVSWI) ($p = 0.02$) and cardiac output (CO) increased in Group B. Post induction, eNOS and Hsp72 in Group B were higher. Following IRI, eNOS and Hsp72 in Group A decreased while in Group B it increased. Post CPB, eNOS and Hsp72 in Group B remained higher. In Group B, eNOS post IRI positively

correlated with LVSW ($p=0.05$) and LVSWI ($p=0.006$) 4 hours post CPB. Furthermore, in Group B, Hsp72 post IRI correlated positively with LVSWI post CPB ($p=0.01$) while Hsp72 post CPB correlated positively with LVSWI post CPB ($p=0.01$). Post CPB, in Group B, the increase in eNOS correlated with Hsp72 ($p>0.05$).

This study clinically demonstrated that HBO preconditioning prior to IRI improves myocardial function following IRI. This cardioprotection occurs in association with increased myocardial expression of eNOS and Hsp72. This clinical study supports experimental findings which suggest that the mechanism for this cardioprotection may involve a dependent linkage between the expressions of eNOS and Hsp such as Hsp72.



Changes in myocardial eNOS expression



Changes in myocardial Hsp72 expression

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PC7

Increase of S-nitrosylation of protein tyrosine phosphatase by nitric oxide in endothelial cells under flow

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Vascular endothelial cells (ECs) are constantly exposed to blood flow-induced shear stress (laminar flow). Laminar shear flow,

which is atheroprotective, increases nitric oxide (NO) production via the activation of endothelial nitric oxide synthase (eNOS). Recent study suggests that NO tends to react on catalytic Cys residue of protein tyrosine phosphatase (PTP) that transiently inhibits PTP's activity and alters signaling pathways. In the present study, we investigate the NO-mediated S-nitrosylation of PTP in ECs exposed to flow.

Cultured ECs were exposed to laminar flow by a parallel-plate flow chamber system. Laminar flow to ECs leads to an elevated and sustained eNOS activation. Shear flow to ECs, similar to SNAP (NO donor) treatment, transiently inhibited PTPs activities via S-nitrosylation by eNOS-derived NO. This S-nitrosylation of PTP appears to be time- and shear force- dependent. Utilizing biotin switch method, a sustained increase of S-nitrosylation was detected in endogenous PTPs including SHP2. ECs over-expressed SHP2 mutant on catalytic site (SHP2C459S), but not other cysteine residue, showed an emphatic decrease of S-nitrosylation indicating that catalytic Cys residue in PTP is essential for S-nitrosylation in ECs under flow. Our results indicate that an elevated NO levels in ECs under flow predominantly S-nitrosylated the catalytic Cys residue in PTP. S-nitrosylation of catalytic Cys residue in PTP may protect it from H₂O₂-induced irreversible oxidative modification. This reversible S-nitrosylation of PTP may play a crucial role in modulating endothelial responses to various stimuli under physiological flow condition.

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PC8

Anthropometric measurements of obesity and MC4R gene mutation screening in Saudi Arabia

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Obesity and overweight are well known risk factors for most chronic diseases. Recent studies show that these are highly prevalent among people of the Kingdom of Saudi Arabia. MC4R mutations constitute the most common monogenic form of human obesity, which accounts for 5% of morbid obesity shown to be a constrictive factor among them. The prevalence of overweight and obesity in Saudi Arabia is 13.8% and 20.5% among male and female respectively. This number has increased to 35.5% as reported by Al-Nozha (2005). Previously, I have screened the MC4R gene in a UK population and identified two 'paucimorphisms' mutations (V103I in 27 subjects and -178A>C in 22 subjects). Anthropometric studies of these variants enable the detection of BMI effects as little as 0.5 units. Two rare variants were

also identified, one previously described (T112M) and one unknown (A87D). BMI of 31.5 in the latter might point to mild functional effect (Alharbi et al, 2006). The study of anthropometric measurements and genetics of obesity will be unique among of Saudi population.

Khalid K. Alharbi¹, Spanakis 1E, Karen Tan 3, Smith MJ, M. A. Aldahmesh O'Dell 1S D, Sayer AA, Lawlor DA, Ebrahim S, Smith GD, O'Rahilly S, Farooqi S, Cyrus Cooper 2, Phillips DI, I N M Day¹. Prevalence and functionality of paucimorphic and private MC4R mutations in a large, unselected European British population, Scanned by meltMADGE. Human Mutation, 2006 Oct 27. PMID: 17072869

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was measured simultaneously and non-invasively by oscillometry (SciMed). In the remaining 5 subjects, forearm blood flow (FBF) was measured using strain gauge plethysmography. AMS increased FBF, and decreased TPR, confirming the expected vasodilatation of the peripheral vasculature. However, increased B-R PWV, and arterial load (EA), demonstrate increased conduit and central arterial stiffness during AMS. To our knowledge, these results provide the first concurrent measurements of differential vascular responses to AMS and they support a mechanistic link between AMS and increased risk factors for cardiovascular disease.

Table 1. Central and peripheral vascular responses to AMS.

	Baseline	Stress
B-R PWV (m/s)	4.6 ± 0.1	5.2 ± 0.3 *
FBF (ml/100ml tissue/min)	2.3 ± 0.3	3.6 ± 0.4 *
EA (mmHg/ml)	1.03 ± 0.05	1.14 ± 0.07 *
TPR (mmHg/l/min)	17.3 ± 0.5	14.8 ± 0.7 *

Data are mean ± SEM. and * indicates significant difference ($P > 0.05$, post hoc Students paired t-test, with Bonferroni correction.) from baseline value.

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PC9

Central and peripheral vascular responses to acute mental stress

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The central and peripheral vascular responses to acute mental stress (AMS) have been studied independently in a number of studies. Through this approach AMS has been shown to elicit vasodilation in the resistance beds of the forearm, but not the calf (Halliwill et al. 1997), and has been shown to increase aortic stiffness and pulse wave reflections (Vlachopoulos et al. 2006). Given the important role that vascular dysfunction plays in the development of risk factors for cardiovascular disease, it is perhaps surprising that concurrent measurement of central and peripheral vascular responses to AMS has not been reported. 10 healthy volunteers (5 male; age 27 ± 2 yrs, height, 170 ± 2 cm; weight, 70 ± 3 kg) underwent 10 minutes of baseline recordings before performing a six minute mental arithmetic task. Haemodynamic measurements were obtained using a non-invasive bioimpedance monitor (TaskForce Monitor). Radial artery pressure waveforms were acquired using applanation tonometry and central end-systolic pressure (ESP) was derived from the generated central waveforms using pulse wave analysis (Sphygmocor, AtCor Medical). Effective arterial elastance (EA), an index of arterial load, was calculated as ESP/stroke volume. Total peripheral resistance (TPR) was calculated as mean arterial pressure/cardiac output. In 5 subjects, brachial-radial pulse wave velocity (B-R PWV)

PC10

VEGF activates AMP-activated protein kinase in endothelial cells via calcium/calmodulin-dependent protein kinase kinase

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AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade important in the regulation of cellular and whole body metabolism. Phosphorylation of Thr172 on the catalytic α subunit by an upstream AMPK kinase is essential for AMPK activity. The tumour suppressor LKB1 and Ca^{2+} /calmodulin dependent protein kinase kinase (CaMKK) have both been identified as upstream kinases that phosphorylate this residue. LKB1 responds to an increase in the cellular AMP:ATP ratio whereas CaMKK acts in a Ca^{2+} -dependent, AMP-independent manner.

Evidence suggests endothelial dysfunction, largely characterised by reduced nitric oxide (NO) bioavailability, is an important feature in regards to the development of type 2 diabetes and cardiovascular disease. AMPK has been shown to mediate some physiological responses in the endothelium and its activation may have beneficial effects in the dysfunctional endothelium. Vascular endothelial growth factor (VEGF) is an important regulator of endothelial cell function. VEGF stimulates growth and promotes survival of endothelial cells, stimulates NO production and vasodilation and induces angiogenesis. VEGF has been shown to increase intracellular Ca^{2+} levels in endothelial cells, an effect dependent on PLC γ activation. Subsequent to PLC activation DAG and IP₃ are produced. DAG increases Ca^{2+} influx

through non-selective cation channels, whereas IP_3 promotes the release of Ca^{2+} from intracellular stores.

Previous studies in our laboratory have shown that VEGF is a novel activator of AMPK. The aim of the present study was to investigate the signalling mechanisms leading to AMPK activation and the functional relevance of AMPK in endothelial cells stimulated with VEGF. As VEGF can increase intracellular Ca^{2+} levels we wanted to investigate whether CaMKK plays a role in VEGF mediated AMPK activity.

Human aortic endothelial cells (HAECs) treated with VEGF are phosphorylated at Thr172 resulting in a 2-fold increase in AMPK activity. These effects are dependent on CaMKK as demonstrated by the selective inhibitor STO-609. High potassium induced depolarisation used to reduce the driving force for Ca^{2+} entry through non-selective cation channels in HAECs abolished VEGF mediated AMPK activity. Inhibition of IP_3 sensitive intracellular stores with 2-APB had no effect.

We propose that VEGF increases Ca^{2+} influx through non-selective cation channels resulting in stimulation of CaMKK and subsequently an increase in AMPK activity.

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PC11

Ultrasound imaging of secondary flows in blood vessels

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The pattern of blood flow in arteries influences their biology and development of disease. Arterial geometry is commonly non planar¹ generating blood flow fields that often include secondary flows. Model studies show that mixing, resulting from such geometries, can render the distribution of wall shear stress more uniform and enhance blood-wall mass transport of lower molecular weight fluid phase controlled materials².

Doppler ultrasound is widely used clinically to visualize axial flows. By setting the ultrasound probe in a plane perpendicular to the vessel, Frazin et al³ detected secondary flows in a model and also in-vivo, showing the presence of swirling flow in the thoracic aorta. Hoskins et al^{4,5} detected swirling flow in the lower limb arteries and also a Dean flow pattern in a model bifurcation. Following the same approach, we have investigated the potential of using an ultrasound scanner to image secondary flows in arterial models with planar and non-planar geometries. The scanner employed was a Philips HDI 5000 colour Doppler with linear array transducers. We visualised both Dean vortices and swirling flow, depending on geometries and flows.

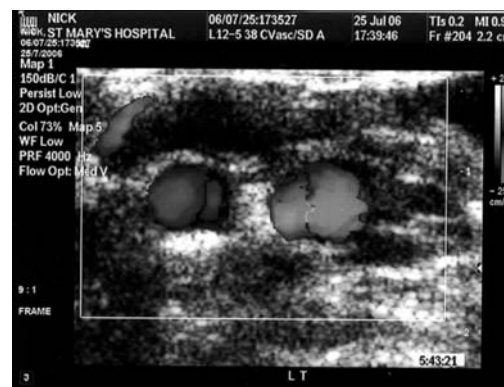
We acquired in-vivo measurements in the internal and external carotids (ICA & ECA) and common femoral (CFA) arteries of a 23 year old healthy male volunteer.

The patterns of the transverse Doppler scan suggest the presence of swirling flow in both the ICA and ECA. Swirling flows were not visible in the common carotid or the carotid bulb. In the left ICA and ECA, the scan showed the flow to swirl in opposite

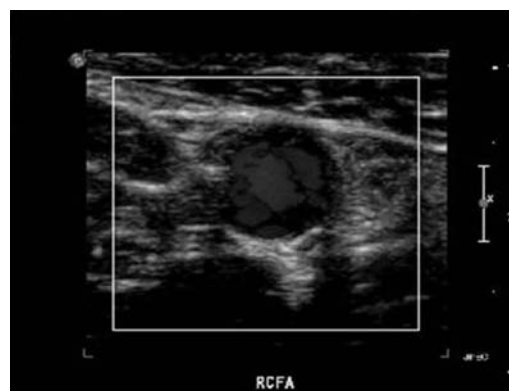
directions. Looking downstream, the rotation was clockwise in the ICA and anticlockwise in the ECA. Mirror image patterns were found in the right carotids.

In the CFA, Dean vortex patterns were visible during systolic flow. Comparison of the scans at two stations, suggests that the Dean pattern rotates along the CFA.

Ultrasound scanning may be of value in visualising secondary flows and increasing understanding of the correlation between the flow and vascular biology and disease.



Swirling flow pattern in the left (LT) carotid arteries of a 23 yr. old healthy male volunteer.



ECG-gated scan in the right common femoral artery (CFA) during systolic flow showing Dean vortices.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC12

Atypical calcium responses evoked by fibrinogen in vascular stem cells

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Recent evidence suggests a role for circulating vascular smooth muscle cell (VSMC) progenitors in atherosclerotic plaque biology [1]. Integrins may play a vital role in the homing of these cells to the lesion site [2]. These extracellular matrix (ECM) protein receptors exert an influence over dihydropyridine receptors (DHPRs) [3], primary mediators of calcium (Ca^{2+}) influx in differentiated VSMCs, thus presenting a link between alterations of the ECM, cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_c$) signalling machinery and subsequent cellular responses. The arginine-glycine-aspartic acid (RGD) tripeptide is a well-characterised ligand for several integrin receptors. It has been proposed that the RGD sequence is exposed in many ECM proteins, such as fibrinogen, a marker of cardiovascular disease [4], following tissue damage [5] and therefore influences cell responses by altering Ca^{2+} levels through integrin-mediated DHPR modulation. Specifically, $\alpha_v\beta_3$ integrin-ligand binding inhibits Ca^{2+} current through the DHPRs, whereas $\alpha_5\beta_1$ integrin counteracts this response by enhancing current [3]. The current study characterised $[\text{Ca}^{2+}]_c$ responses of endothelial progenitor cells (EPCs) to RGD tripeptide and fibrinogen exposure, employing fura-2 videomicroscopy. Exposure of EPCs to fibrinogen or tripeptide elicited an initial elevation of $[\text{Ca}^{2+}]_c$ levels followed by a prolonged decrease relative to resting concentrations, thus implicating both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins in this response. Ca^{2+} responses to fibrinogen were abolished by nifedipine, a DHPR blocker. Dantrolene (10 μM) and ryanodine (10 μM), inhibitors of ryanodine receptors (RyRs), blocked $[\text{Ca}^{2+}]_c$ increases resulting from integrin-ligand binding, leading to a prolonged decrease in $[\text{Ca}^{2+}]_c$ levels without recovery of resting levels. Simultaneous incubation of EPCs with nifedipine and ryanodine blocked Ca^{2+} responses to fibrinogen, implying a coupling of function of DHPRs to that of RyRs. To investigate this hypothesis, EPCs were treated with either cytochalasin D (Cyt-D) (10 μM), which prevents actin polymerisation, or methyl- β -cyclodextrin (M β CD) (1 mM), a cholesterol sequestering agent which disrupts caveoli. Fibrinogen evoked a $[\text{Ca}^{2+}]_c$ response, similar to that of untreated cells, in Cyt-D-treated EPCs incubated with both nifedipine and ryanodine. M β CD-treated cells did not produce a $[\text{Ca}^{2+}]_c$ response to fibrinogen. Our laboratories postulate that the biphasic Ca^{2+} response to fibrinogen through integrins in EPCs is a result of RyR modulation to elevate Ca^{2+} levels and the decrease is owed to a functional coupling of RyRs and DHPRs, possibly due to close apposition of the sarcoplasmic reticulum and the plasma membrane.

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PC13

Haemodynamic effects of the endocannabinoid anandamide in conscious rats with acute hypertension

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A triphasic blood pressure response to anandamide has been described, with the third phase of which, a depressor response, reportedly occurs only in anaesthetised, normotensive rats and in conscious, hypertensive rats (Lake *et al.* 1997). In this context, to date, only chronic models of hypertension have been studied. We have now assessed the cardiovascular effects of anandamide (1 and 3 mg/kg, i.v.) in rats made acutely hypertensive by infusion of angiotensin II (AII; 500 ng/kg/h) and arginine vasopressin (AVP; 50 ng/kg/h). Male anaesthetised Wistar rats (350–450 g; Charles River, UK) were implanted with miniaturised pulsed Doppler flow probes and catheters as described previously (Gardiner *et al.* 2002). On the day of experiment, animals received continuous i.v. infusion of either saline or AII plus AVP, followed by bolus injection (0.12 ml, i.v.) of anandamide 105 min later. Data are shown as mean $\pm$ SEM.

Infusion of AII/AVP significantly ($P < 0.05$, Mann-Whitney U test) increased mean arterial blood pressure (BP; 154 ± 3 vs 114 ± 2 mmHg), reduced vascular conductance (VC) in renal (RVC; 47 ± 4 vs 81 ± 4), mesenteric (MVC; 20 ± 3 vs 62 ± 5) and hindquarter beds (HVC; 12 ± 1 vs 39 ± 2 (kHz/mmHg) $\times 10^3$) and reduced heart rate (HR; 217 ± 12 vs 359 ± 11 beats/min). The haemodynamic responses to anandamide are shown in Table 1.

This study, for the first time, demonstrates that anandamide causes dose-dependent depressor and vasodilator effects in hypertensive, conscious rats.

Table 1. Cardiovascular effects of anandamide. *Significant changes vs baseline within group ($P < 0.05$, Friedman's test)

		1 mg/kg		3 mg/kg anandamide	
		40 s	60 s	40 s	60 s
Saline (n=8 or 9)	HR	+5 $\pm$ 12	-6 $\pm$ 15	-52 $\pm$ 21*	-42 $\pm$ 18*
	BP	+6 $\pm$ 3*	+4 $\pm$ 2	-4 $\pm$ 4	-6 $\pm$ 3
	RVC (%)	-6 $\pm$ 3	+1 $\pm$ 2	+5 $\pm$ 6	+12 $\pm$ 4*
	MVC (%)	-8 $\pm$ 11	-3 $\pm$ 11	-11 $\pm$ 11*	-4 $\pm$ 13
	HVC (%)	+10 $\pm$ 7	+10 $\pm$ 8	+39 $\pm$ 13*	+48 $\pm$ 11*
AII/AVP (n=7 or 8)	HR	+25 $\pm$ 8*	+21 $\pm$ 6*	-6 $\pm$ 11	+17 $\pm$ 5*
	BP	-5 $\pm$ 2*	-3 $\pm$ 2	-14 $\pm$ 7*	-16 $\pm$ 5*
	RVC (%)	+6 $\pm$ 2	+2 $\pm$ 2	+24 $\pm$ 6*	+32 $\pm$ 8*
	MVC (%)	+3 $\pm$ 5	+5 $\pm$ 4	+22 $\pm$ 10	+12 $\pm$ 13
	HVC (%)	+66 $\pm$ 8*	+65 $\pm$ 9*	+82 $\pm$ 20*	+140 $\pm$ 37*

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC14

Modelling t-tubule function in cardiac ventricular myocytes

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Cardiac transverse (t-) tubules form a complex network of membrane invaginations in mammalian ventricular myocytes, also known as the transverse-axial tubular system (TATS). Biophysically realistic computer models have been used to investigate experimental ambiguities and aspects of TATS function not currently amenable to experimental investigation:

1. *Fraction of cell membrane within the TATS:* Detubulation of rat ventricular myocytes causes a ~32% decrease of membrane capacitance (Despa et al., 2003) whereas optical measurements suggest ~56% of the cell membrane within the TATS (Soeller & Cannell, 1999). Analysis of factors that may account for this discrepancy, and calculation of the combinations of t-tubule radius, length and density that produce t-tubular membrane fractions of 32% or 56%, suggest that the true fraction is at the upper end of this range.

2. *Electrical coupling between the surface and TATS membranes:* It has long been speculated that voltage control within the TATS may be inadequate, allowing voltage escape. Analysis based on cable theory, and simulations using models in voltage and current clamp mode, show that voltage spread across both membranes and its equilibration due to their tight electrical coupling is very fast (within 20 µs); thus membrane voltage is almost homogeneous over the whole cell membrane during activity.

3. *Ion diffusion within TATS:* The complexity of the TATS appears to restrict ion diffusion in the TATS lumen. Simulations performed using a model of a single tubule suggest that variable diameter along the length of the tubule and ion buffers in the tubule lumen play a major role in the restricted diffusion and thus in the slowed ion exchange between the TATS lumen and extracellular space.

4. *Ion concentration changes in TATS and their consequences:* Recent detubulation experiments suggest that many trans-membrane ion flux pathways (for I_{Ca} , I_{NaCa} , I_{NaK} and others) are located predominantly within the TATS (Brette & Orchard, 2003). Incorporation of this distribution, and restricted ion diffusion between the tubular and extracellular space, into rat and guinea pig models results in changes of tubular Ca^{2+} and K^+ concentrations during activity, which depend on stimulation rate. These changes (particularly Ca^{2+} depletion) cause a significant decrease of intracellular Ca^{2+} load and hence reduction of intracellular Ca^{2+} transients. These data suggest that the TATS may play an important role in modulating cardiac cell function.

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Soeller C & Cannell MB (1999) *Circ Res* **84**, 266–275.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC15

The effect of reactive oxygen species on whole blood aggregation and the endothelial cell-platelet interaction in patients with coronary heart disease

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Previous aggregation studies in healthy volunteers have demonstrated contrasting results when platelets suspended in buffer or plasma are exposed to reactive oxygen species (ROS). We investigated the effect of ROS on whole blood aggregation (WBA) and the endothelial cell-platelet interaction in coronary heart disease (CHD) patients with and without diabetes. Data are expressed as mean difference with 95% confidence intervals. 14 patients with CHD (7 with diabetes) were recruited to study the effects of ROS on WBA. ROS generated by xanthine (X) and xanthine oxidase (XO) caused a significant dose dependent inhibition of WBA. 100 µM X and 100 mU/ml XO significantly inhibited WBA in response to 3 µg/ml collagen (28.9%, CI 16.8%–40.9%, $p < 0.01$) and 5 µM ADP (36.0%, CI 13.5%–58.5%, $p < 0.01$). Results were similar for both diabetic and non-diabetic subgroups. An additional 9 patients with CHD were recruited for endothelial cell-platelet interaction studies. Addition of 1×10^5 endothelial cells cultured from porcine pulmonary artery caused a significant reduction in WBA in response to 3 µg/ml collagen (31.2%, CI 11.0%–51.5%, $p < 0.01$) and 5 µM ADP (31.6%, CI 0.5–62.6%, $p < 0.05$). Exposing the endothelial cells to 1 minute of ROS using 100 µM X and 10 mU/ml XO did not attenuate the inhibitory effect. In 5 patients, endothelial cells were pretreated with 100 µM L-NAME and this did not alter the effect of endothelial cells significantly. In conclusion, the acute dose dependent effect of ROS on WBA is inhibitory and may differ from the effects of ROS on platelets in less physiological environments such as buffer or plasma. ECs inhibit WBA in patients with CHD. The anti-aggregatory effect of ECs is not prevented by addition of exogenous ROS or L-NAME pretreatment, probably because it involves a predominant non-nitric oxide mechanism.

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PC16

Potential therapeutic benefit of novel DDAH inhibitors for the treatment of endotoxemia

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Introduction

During the onset of sepsis, the induction of inducible nitric oxide synthase (iNOS) and the subsequent generation of large amounts of nitric oxide (NO) is thought to be partly responsible for the marked hypotension ultimately leading to inadequate organ perfusion and cardiovascular collapse. A potential therapeutic target in sepsis is inhibition of excess NO generation, although to date, direct NOS inhibition has proved unsuccessful.

Asymmetric dimethylarginine (ADMA) is an endogenously occurring competitive inhibitor of NOS. ADMA is subject to hydrolysis catalysed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). We have developed novel DDAH inhibitors to pharmacologically elevate endogenous ADMA levels as a potential therapeutic intervention in the treatment of sepsis.

Methods

Novel inhibitors of DDAH were synthesised and characterised using: in vitro enzyme assays and in vitro and in vivo rat models of acute endotoxemia.

Results

Isolated enzyme studies revealed that the novel DDAH inhibitor L-291 resulted in a concentration dependent inhibition of DDAH activity whilst having no direct effect on NOS activity. In vitro functional studies using isolated rat aorta demonstrated that L-291 reversed the iNOS mediated dilatation produced by LPS treatment by $16\% \pm 4.4$ at 100uM and $25\% \pm 4.1$ at 200uM.

In anaesthetised rats, a bolus dose of LPS induces iNOS mediated vasodilatation resulting in a gradual fall in blood pressure. Administration of L-291 in LPS treated rats elevated circulating ADMA levels when compared with saline control (2.42 ± 0.12 uM vs. 1.17 ± 0.09 uM) and attenuated the fall in blood pressure.

Conclusion

We have generated a novel highly selective DDAH inhibitor, which elevates circulating ADMA levels in vivo. In a rat model of acute endotoxemia DDAH inhibition attenuates iNOS mediated hypotension and stabilises blood pressure.

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nels are hetero-octomers consisting of four inward rectifier family Kir6 subunits (Kir6.1 or Kir6.2) and four sulphonyurea receptor subunits (SUR1, SUR2A or SUR2B). Current pharmacological tools do not allow distinction between the roles of each KATP channel subunit. We have therefore developed a molecular approach to specifically alter protein levels of individual KATP channel subunits and so define their role in cardioprotection. We designed shRNA constructs to selectively down-regulate the pore forming subunit KATP channel, Kir6.2. These constructs were engineered into an adenoviral vector system for efficient delivery into isolated adult rat cardiac myocytes. The down-regulation of KATP currents after 24h was confirmed by comparing the currents evoked by a KATP channel opener (p1075, 10uM) of myocytes infected with control virus (9.4 ± 1.3 pA/pF, $n=11$) versus those infected with adenovirus encoding shRNA Kir6.2 (5.4 ± 0.9 pA/pF, $n=14$). To assess the effect of Kir6.2 down-regulation on cardioprotection, myocytes were placed in a bath, superfused with Tyrode solution and synchronized to contract by field stimulation at 1Hz. Myocytes were challenged with a metabolic insult of 4 min perfusion with metabolic substrate free Tyrode solution containing cyanide (2mM) and iodoacetic acid (1mM) followed by reperfusion for 10 min. The specific down-regulation of Kir6.2 by shRNA resulted in a decrease in contractile recovery (control, $82 \pm 2\%$, $n=147$; shRNA Kir6.2, $39 \pm 1\%$, $n=126$). Calcium homeostasis during metabolic stress is a key factor in myocyte survival. Using the calcium indicator Indo1 we measured intracellular calcium levels in response to metabolic stress and reperfusion. Myocytes expressing shRNA constructs down-regulating Kir6.2 showed a higher calcium level after metabolic stress and reperfusion (Indo1 ratio; control, 1.0 ± 0.01 , $n=43$; shRNA Kir6.2, 1.2 ± 0.02 , $n=43$). The effect of Kir6.2 knockdown on ischaemic preconditioning was assessed with an ischaemic pelleting method in which a pellet of myocytes was layered with mineral oil to prevent gaseous exchange to mimic ischaemia, followed by a return to normal medium to mimic reperfusion injury. We found that ischaemic preconditioning was abolished in myocytes infected with Kir6.2 shRNA compared to those infected with control virus (control cell survival $36 \pm 1\%$, $n=71$ compared to $17 \pm 1\%$, $n=102$ survival of Kir6.2 knockdown cells). These results are consistent with a direct role for KATP channels containing the subunit Kir6.2 in the cardioprotective response to metabolic stress.

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PC17

The role of the ATP-sensitive potassium channel subunit Kir6.2 in cardiac protection and ischaemic preconditioning of adult rat cardiac myocytes

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ATP-sensitive potassium channels (KATP) play a central role in protecting cardiac myocytes from metabolic stress. KATP chan-

PC18

Vascular antioxidant actions of Liver Growth Factor (LGF) on arteries and SMC derived from SHR

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Background. Liver Growth Factor (LGF) is a mitogen for liver cells with regenerative properties in several biological systems,

including the cardiovascular system. We have previously reported that short-term treatment with LGF improves vascular structure and function and lowers blood pressure in spontaneously hypertensive rats (SHR) (1), a rat model of human essential hypertension with high levels of oxidative stress. LGF is an albumin-bilirubin complex, and bilirubin and albumin-bound bilirubins are known to have antioxidant properties (2,3). We hypothesize that the antihypertensive and antifibrotic effects of LGF in SHR might be related to its antioxidant capacity. The aims of the study were: 1) to test if the production of superoxide anion is reduced in arteries and smooth muscle cells (SMC) derived from SHR rats treated with LGF (SHR-LGF); 2) to test the direct antioxidant properties of LGF on nitric oxide (NO)-mediated responses in the presence of a superoxide anion donor. Methods. Superoxide anion production was assessed by dihydroethidium (DHE) and confocal microscopy in mesenteric resistance arteries and in SMC primary cultures derived from adult male WKY, SHR and SHR treated with LGF (4.5 µg LGF/rat, i.p., twice a week during 2 weeks). Isometric tension was studied in intact carotid arteries isolated from adult male Sprague-Dawley rats. Concentration-response curves to Ach were obtained before and after 20 min pre-incubation with the superoxide anion donor pirogallol (10⁻⁶ M). Thereafter, the antioxidant effects of LGF (0.3 nM) were compared with those of the superoxide anion scavengers superoxide dismutase (SOD, 15 U/ml) and tiron (10 mM). Results. Superoxide anion production, measured by intensity of fluorescence emitted by DHE was significantly higher in SHR compared with WKY and it was reduced in SMC and arteries derived from LGF-treated SHR. Pirogallol reduced to 42% Ach-mediated relaxation. In the presence of pirogallol, the addition of LGF, SOD or tiron improved Ach relaxation to 71%, 65% and 89%, respectively. Conclusions. 1) SHR treatment with LGF reduces oxidative stress in vascular tissues; 2) LGF also has antioxidant effects on arteries "in vitro", protecting NO from degradation by superoxide anion. These data suggest that LGF might exert its vascular protective actions in hypertension, at least in part, by reducing oxidative stress.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC19

Vasoconstriction induced by metabotropic calcium channel activation in the coronary artery

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We have described in rat basilar arterial myocytes, that in the absence of extracellular Ca²⁺ influx, activation of L-type Ca²⁺ channels is coupled to Ca²⁺ release from the sarcoplasmic reticulum (del Valle-Rodríguez et al, 2003). This mechanism, denoted

as calcium channel-induced Ca²⁺ release (CCICR), depends on the conformational change of L-type Ca²⁺ channels and the downstream activation of the G protein-phospholipase C (PLC) biochemical cascade, leading to synthesis of InsP₃ and vasoconstriction in the absence of extracellular calcium. We have investigated whether CCICR is present in coronary arterial myocytes. We measured cytosolic Ca²⁺ concentration in isolated myocytes by microfluorimetry and arterial tension was monitored by using a Mulvany-Halpern myograph. In isolated myocytes 70K+ solution induced calcium release in the absence of extracellular calcium. Ca²⁺ channel activation induced by depolarization or by FPL-64176 (FPL), a Ca²⁺ channel agonist, evoked arterial ring constriction in the absence of extracellular calcium that was suppressed by Ca²⁺ channels antagonists as nifedipine, diltiazem or D-600. Using a pharmacological approach we tested whether the metabotropic cascade activated by Ca²⁺ channel activation is involved in the vasoconstriction induced by FPL. Exposure of arterial rings to U73122, a PLC inhibitor, significantly reduced the force induced by the Ca²⁺ channel agonist. The role of sarcoplasmic reticulum was studied using inhibitors of InsP₃ and Ryanodine receptors such as 2-APB and tetracaine respectively. Both agents significantly inhibited the vasoconstriction induced by FPL. These results suggest that in the coronary artery, contraction can be triggered by Ca²⁺ channel activation in the absence of external Ca²⁺ through a metabotropic Ca²⁺-channel activation and Ca²⁺ release from internal stores.

Del Valle-Rodríguez A et al. (2003). *EMBO J.* 22, 4337-4345.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC20

The calcimimetic R-568 inhibits mineral deposition by vascular smooth muscle cells

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Vascular calcification (VC) is highly correlated with increased morbidity and mortality in chronic kidney disease (CKD) patients. Allosteric modulation of the calcium sensing receptor (CaSR) by the calcimimetics decreases serum parathyroid hormone, calcium and phosphorus (1), and can inhibit VC in animal CKD models (2). However, it is unknown whether the anti-VC effect is mediated by direct action on vascular smooth muscle cells (VSMC). Here we have examined VSMC CaSR expression, CaSR function in regulating mineralized matrix deposition, and the effects of R-568 on VSMC mineral deposition.

Human and bovine VSMC were isolated and cultured in 10% FCS-DMEM containing 1.2mM free ionized extracellular calcium concentration ($[Ca^{2+}]$). CaSR expression was determined by Western blotting and immunofluorescence. Mineral deposition was assessed by alizarin red staining and ^{45}Ca incorporation. VSMC CaSR expression was demonstrated by Western blotting of cell lysates and immunofluorescence staining of cultured cells. Incubation of confluent VSMC with increasing extracellular $[Ca^{2+}]$ for 24 hours decreased CaSR expression. Western blotting during osteogenic differentiation demonstrated marked CaSR downregulation when mineralized nodules were formed. Acute (5 min) stimulation of VSMC with 5mM Ca^{2+} or 50 μ M Gd^{3+} , a non-permeable agonist, significantly stimulated ERK phosphorylation, suggesting a functional CaSR. Culturing cells with 1.8 or 2.5mM Ca^{2+} or 10 or 50 μ M Gd^{3+} enhanced ($P < 0.05$) mineral deposition compared with VSMC cultured with 1.2mM Ca^{2+} . R-568 (1nM) reduced nodule formation and significantly inhibited mineralisation by cells cultured with 1.8mM Ca^{2+} ($P < 0.001$).

These results demonstrate that VSMC express a functional CaSR that is down-regulated with mineralized matrix deposition. In line with previously reported data (2), there is an association between calcimimetic use and reductions in VC.

These data suggest that the potential inhibitory effect of calcimimetics on VC is mediated by the CaSR. The nature of this mechanism is currently under investigation.

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Lopez I, Aguilera-Tejero E, Mendoza FJ, Almaden Y, Perez J, Martin D, and Rodriguez M (2006). *J Am Soc Nephrol* 17: 795-804.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC21

Characterisation of the DDAH 1 haploinsufficient mouse: A causal role of elevated ADMA in the pathogenesis of cardiovascular disease

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Introduction:

An association exists between elevated levels of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) and a number of cardiovascular diseases. However, to date, no studies have shown a causal relationship between elevated ADMA and cardiovascular pathology. Dimethylarginine dimethylaminohydrolase (DDAH) catalyses the hydrolysis of ADMA in vivo. In order to test the hypothesis

that elevated ADMA levels are causal in the pathogenesis of cardiovascular disease we generated mice deficient in DDAH1 and studied their systemic and pulmonary circulations both in vivo and ex vivo.

Methods:

Mice were generated by targeted deletion of exon 1 of the DDAH1 gene. Homozygous deletion of DDAH1 was lethal in utero whilst heterozygous DDAH1 haploinsufficient mice were viable. DDAH1 protein, DDAH activity and ADMA levels in tissue and plasma were measured in both DDAH1 +/- mice and their wild type littermates. The vascular reactivity of isolated aortas and pulmonary arteries and the effects of 1mM L-arginine on acetylcholine induced relaxations were also assessed. In addition to histological examination of the pulmonary vasculature. In vivo studies were conducted in minimally anesthetized DDAH1 +/- and wild type littermates. Mean arterial and right ventricular pressures were measured using a fluid filled catheter inserted into the right carotid artery or right ventricle respectively and cardiac output, heart rate and systemic vascular resistance were simultaneously measured using echocardiography.

Results:

Heterozygous deletion of DDAH1 resulted in reduced DDAH1 protein and activity and elevated tissue and plasma ADMA levels. Endothelial dysfunction was observed in both systemic and pulmonary vessels from DDAH1 +/- mice ex vivo an effect that was fully reversible by L-arginine. In vivo, mean arterial blood pressure and systemic vascular resistance were increased whilst cardiac output and heart rate were decreased. Furthermore increased right ventricular pressures together with increased muscularisation of resistance arteries in the pulmonary vasculature was observed.

Conclusion:

This study has demonstrated that in DDAH1 +/- mice, elevated ADMA causes endothelial dysfunction and results in increases in both mean arterial and pulmonary pressures indicating that elevated ADMA may be causal in the pathogenesis of cardiovascular disease.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC22

Cryo-injury is an attractive alternative to left anterior descending (LAD) artery ligation for inducing myocardial infarction and heart failure in miceA. Patel^{1,2}, F. Cagampang¹, M.A. Hanson¹ and S.K. Ohri²¹DOHaD, Southampton, UK and ²Wessex Cardiothoracic Centre, Southampton General Hospital, Southampton, UK

A number of mouse models to study cardiovascular diseases have been developed over the last 10 years. Although the mouse has many favourable characteristics, its small size makes surgical procedures technically difficult. Heart failure surgery is classically achieved by ligation of the left anterior descending artery (LAD), however it is technically demanding and can have mortality rates as high as 70%¹⁻⁴. Also the degree of the injury can vary depending on the degree of collateral blood supply of each animal. We have developed a method of cryo-injury to the heart via a trans diaphragmatic approach. It is a procedure that is quickly mastered, produces reproducible results and has very little in the way of complications.

Adult female C57BL/6 (n=18) (weight 20-30 grams) were used. Briefly, animals were anaesthetised and secured to a heated operating table. Myocardial infarction surgery was performed by applying a steel cryo-probe (2mm diameter) cooled in liquid nitrogen to the inferior surface to the heart through the diaphragm via an upper abdominal incision. Animals were randomised to 3 study groups: 20 second, 40 second and a 60 second cryo-injury group (n=6 per group). Animals were recovered for 6 weeks during which time, weekly weight and blood pressure (tail cuff) were measured. After 6 weeks all hearts were harvested for histology and assessed infarct size.

There was no mortality for the duration of the study. Post operative tail cuff blood pressure and weights were measured in all animals. There were no significant differences between the groups.

Hearts were embedded and sectioned 100µm apart, and stained with haematoxylin and eosin. Infarct size was calculated as a percentage of total ventricular volume.

Infarcts were confirmed in all (100%) of hearts.

Infarct size was 5.01% +/- 0.31%, 9.18% +/- 0.257%, 12.35% +/- 0.295% (mean +/- SEM) for 20, 40, 60 second cryo-injury respectively, p<0.001 (20s v 60s, 20s v 40s, 40s v 60s).

Cryo-injury has been shown to be effective in producing myocardial infarction in mice. It has been shown to produce a reproducible injury whose size can be varied by applying the cryo-probe on the heart surface for different periods of time. The procedure has minimal mortality and complications and could be an alternative to LAD ligation in mice.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC23

Influence of cTnI-R145G - a mutation correlated with Hypertrophic Cardiomyopathy - on ventricle cell contraction depends on β -adrenergic receptor stimulationS. Reis¹, C. Littwitz², A. Mügge¹, L. Pott² and K. Jaquet¹¹St. Josef-Hospital, Ruhr-Universität Bochum, Bochum, Germany and ²Zelluläre Physiologie, Ruhr-Universität Bochum, Bochum, Germany

The amino acid exchange R145G in the inhibitory domain of the inhibitory subunit of the cardiac troponin complex (cTnI) causes Hypertrophic Cardiomyopathy. In previous studies we analysed the extent and rate of sarcomere shortening as well as its relaxation rate in response to β 1/2-, β 1- and β 2-adrenergic receptor (AR) stimulation in ventricle cells where contractions were induced by bipolar external stimuli (0.4 ms, 40 V). The localisation of adenovirus driven human cTnI-wt and cTnI-R145G in the sarcomeres is controlled with anti-human cTnI monoclonal antibodies which do not recognize rat cTnI.

In those studies we could show that neither GFP (green fluorescent protein) nor the human cTnI-wt have any influence on the contraction behaviour of adult rat ventricle cells compared with not infected cells. We were also able to show that the amino acid exchange R145G - compared with cTnI-wt - reduced shortening as well as rate of shortening and relaxation significantly in non-AR stimulated and in β 1/2- and β 2-AR stimulated ventricle cells. However, rates of shortening and relaxation were not reduced in ventricle cells stimulated via β 1-AR (ISHR, Manchester, UK, 2006).

In our recent studies we tested the influence of Forskolin on human cTnI-wt and cTnI-R145G infected ventricle cells to analyse on which level of the β -AR pathway the differences are localised. In Forskolin treated cells we could not detect any difference between both cell types. The observed effects might be evoked by factors far downstream of the β -adrenergic signalling pathway, e.g. by different calcium handling. The underlying mechanism is not known yet, but the results clearly underline that β 1- and β 2-adrenergic signalling act differently on regulation of contraction.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC24

The effects of endogenous and exogenous female hormones on vein responses to sympathoexcitation

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Venoconstriction is one of the compensatory adjustments during orthostasis to counter venous pooling. The female hormones oestrogen and progesterone are known for their relaxant effects on arterial vessels and both can cause venodilation (Nekooiean & Pang, 2000; Herket *et al.* 2000). By contrast, oestrogen has been reported to enhance venoconstriction to noradrenaline (Varbiro *et al.* 2002). It is not clear, therefore, how venoconstriction is

modulated during the normal menstrual cycle or by the action of exogenous hormones (oral contraceptive).

To investigate this, we compared calf volume (strain gauge plethysmography) during sympathoexcitation to induce venoconstriction in 6 female subjects with normal menstrual cycles (N), age 21 ± 2 yrs, body mass index 24 ± 5 (both mean $\pm$ SD), and 7 females taking the combined oral contraceptive (O), age 20 ± 1 yrs, body mass index 21 ± 2 . With subjects supine, venous distension was induced 3 times in the left leg by cuff inflation (50 mmHg for 6 mins), one control (CON) and two with the interventions of either contralateral isometric leg exercise (ILE, 40 % maximum voluntary plantarflexion force) or mental stress test (MST, mental arithmetic) applied between mins 3-5. Changes in calf volume during interventions were compared with those during CON distension, such that a decrease in volume would indicate venoconstriction (Zelis & Mason, 1969). All women were tested on two occasion, once during the menstrual phase (M) of their cycle (days 1-5) and again during the luteal (L) phase (days 21-28). Mean arterial blood pressures and heart rates (Portapres, right hand) did not differ between cycle phases in either group (N M 93 ± 7 mmHg, 60 ± 3 bpm; N L 88 ± 4 mmHg, 70 ± 5 bpm; O M 85 ± 5 mmHg, 64 ± 3 bpm; O L 85 ± 3 mmHg, 65 ± 3 bpm). Sympathoexcitation was confirmed by increases in blood pressure and heart rate which were similar in N women in both cycle phases for the ILE intervention (M 4 ± 1 mmHg, 9 ± 3 bpm; L 5 ± 2 mmHg, 10 ± 5 bpm) and MST (M 9 ± 3 mmHg, 14 ± 8 bpm; L 9 ± 2 mmHg, 7 ± 3 bpm). Likewise, O women showed increases in M of 8 ± 4 mmHg and 18 ± 5 bpm to ILE and 6 ± 3 mmHg and 9 ± 4 bpm to MST. Values in L were 7 ± 4 mmHg, 14 ± 5 bpm for ILE and 7 ± 2 mmHg and 13 ± 4 bpm for MST (NS between phases). In response to ILE, there was no significant change in calf volume in any women in either phase. In response to MST, N women did not venoconstrict in either phase whereas O women tended to dilate in both phases. The lack of venoconstriction to sympathoexcitation by ILE is different from men, in whom a significant decrease in calf volume was observed previously (Brown *et al.* 2007). This could contribute to the orthostatic intolerance of women compared with men.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC25

Site-directed mutagenesis of Serine 2815 on the cardiac ryanodine receptor and resultant effects on channel function

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The cardiac Ryanodine receptor (RyR2) is responsible for calcium efflux from the sarcoplasmic reticulum during excitation

contraction coupling. Work by ourselves and others has provided evidence for phosphorylation of RyR2 by calcium/calmodulin dependent protein kinase II delta (CaMKII δ)1. To date however, both the site(s) for phosphorylation and the functional effect of CaMKII-mediated RyR2 phosphorylation remains controversial. To explore this in more detail, we have generated mutations of one putative CaMKII δ site on RyR2, Ser2815. Both a dominant negative (S2815A) mutation and a constitutively active (S2815D) mutation were generated via site directed mutagenesis and sequencing was used as confirmation of a positive result. Full length clones of the mutant and wild type receptors were then expressed in HEK293 cells following transfection with RyR2 cDNA using the calcium phosphate precipitation method. Since GFP was used as a tag, we were able to monitor RyR2 expression in HEK cells by fluorescence intensity. We have successfully expressed both dominant negative and constitutively active mutants as well as wild-type RyR2 in HEK cells. Membrane fractions from these cells have been used for ryanodine binding assays to assess channel open probability (Po) under various conditions. Initial experiments are being conducted to (i) compare mutant and wild-type ryanodine binding in the presence of different free [Ca²⁺] and (ii) examine the capacity of CaMKII for modulation of mutant and wild-type RyR2 Po, reflecting the extent to which Ser 2815 is a key modulatory site for CaMKII on RyR2.

Currie S, Loughrey CM, Craig MA, Smith GL.

Calcium/calmodulin-dependent protein kinase IIdelta associates with the ryanodine receptor complex and regulates channel function in rabbit heart.

Biochem J. 2004 Jan 15;377(Pt 2):357-66.

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PC26

Effects of Angiotensin 1-7 on angiotensin subtype I (AT₁) receptor in the renal vasculature of Spontaneously Hypertensive Rats

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Angiotensin peptides play an important role in the physiological processes regulating blood pressure. Angiotensin 1-7 (Ang 1-7), the heptapeptide formed from Ang I or Ang II, has been reported to have opposing actions to angiotensin II (Le Tran & Forster, 1997). The actions of Ang 1-7 have been shown to be mediated by its own receptor (Le Tran & Forster, 1997) and the AT₁ receptor (Ferrario *et al.*, 1997). Earlier work conducted in our laboratory demonstrated that Ang 1-7 (10⁻⁷M) attenuated the vasopressor responses to Ang II in the renal vasculature of Spontaneously Hypertensive Rats (SHR). The vasopressor action of the heptapeptide is mediated by Ang 1-7 receptor that is coupled to a cyclo-oxygenase dependent pathway and nitric oxide

release (Dharmani *et al.*, 2007). The present study investigated the involvement of AT₁ receptor on the vasomodulatory action of Ang 1-7 in the isolated perfused kidneys of SHR using receptor binding assay, semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting. Ninety min perfusion with Ang 1-7 (10⁻⁷M) lowered the renal AT₁ receptor density compared to the vehicle control (534 ± 22.05 fmole/mg v.s. 739.6 ± 44 fmole/mg protein, P < 0.05). No significant changes in the mRNA and protein expression of the AT₁ receptor in the SHR kidney were observed following perfusion of the angiotensin peptide.

In summary, the vasomodulatory action of Ang 1-7 in the isolated SHR kidney is partly mediated via the AT₁ receptor. Furthermore, the result suggests that Ang 1-7 induces AT₁ receptor internalisation to compensate the ongoing vascular changes in the hypertensive state.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC27

Complex distributions of Na_v1.5, KChIP2, and Kir2.1 transcripts in the heart as revealed by *in situ* hybridization

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We have investigated the distribution of three ion channel transcripts, Na_v1.5, KChIP2 and Kir2.1, in the heart. Na_v1.5 is responsible for the Na⁺ current and thereby determines the upstroke of the action potential, KChIP2 increases the cell surface expression of K_v4 channels and thereby increases the transient outward K⁺ current, and Kir2.1 is responsible for the background inward rectifier K⁺ current, I_{K,1}, and thereby generates the resting potential. Rabbits (~1.5 kg) were humanely killed, the hearts rapidly excised, the sinoatrial node, atrioventricular node and ventricles dissected and frozen in liquid nitrogen, and the preparations cryosectioned. To investigate the distribution of the transcripts, *in situ* hybridization was carried out using ~400 nucleotide riboprobes. If present in a cell, transcripts were present in a ring around the nucleus (presumably corresponding to the rough endoplasmic reticulum). Na_v1.5 was: absent from the centre of the sinoatrial node, but present in the periphery of the sinoatrial node; present throughout the atria; absent from the inferior nodal extension, but present in the penetrating bundle (atrioventricular node); and present throughout the ventricles. KChIP2 was: present in the centre of the sinoatrial node, but curiously absent from the periphery of the sinoatrial node;

present throughout the atria; absent from throughout the atrioventricular node; more abundant in the midmyocardium of the interventricular septum than in the periphery of the septum; and more abundant in the epicardium than in the endocardium of the left and right ventricular free walls. Kir2.1 was: curiously equally abundant in the sinoatrial node and surrounding atrial muscle; more abundant in the ventricles than in the atria; more abundant in the midmyocardium of the interventricular septum than in the periphery of the septum; and more abundant in the epicardium than in the endocardium of the left and right ventricular free walls. In conclusion, *in situ* hybridization reveals a highly complex distribution of ion channel transcripts in the heart; transcript abundance can vary both within a tissue (e.g. centre versus periphery of the sinoatrial node) as well as between tissues (e.g. sinoatrial node versus ventricle).

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PC28

Spatially inhomogeneous Ca²⁺ release in myocytes isolated from the rabbit atrio-ventricular node

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In the heart, Ca²⁺ release from the sarcoplasmic reticulum (SR) may play an important role in pacemaking in the sino-atrial node (SAN): it has been suggested that a rise of sub-sarcolemmal [Ca²⁺] due to spontaneous Ca²⁺ release from the SR activates inward Na⁺/Ca²⁺ exchange current, thus triggering the action potential (Hüser *et al.*, 2000; Bogdanov *et al.*, 2001). However, comparatively little is known about intracellular Ca²⁺ in the atrio-ventricular node (AVN; Hancox *et al.*, 1994), which also exhibits pacemaker activity. We have therefore investigated the spatio-temporal characteristics of intracellular Ca²⁺ in myocytes isolated from the AVN. Myocytes were loaded with the Ca²⁺-sensitive fluorescent indicator fluo-3 AM (10 µM for 30 min) and imaged at room temperature using a confocal laser scanning microscope. The myocytes showed spontaneous contractions (0.5 ± 0.1 Hz; mean ± sem; n = 13). Transverse and longitudinal line scan images obtained from 1 µm sections through the centre of the cells revealed that the systolic Ca²⁺ transient was spatially inhomogeneous, with some areas showing little rise of intracellular Ca²⁺. The regions that showed an increase, or no increase, of [Ca²⁺] were consistent from beat-to-beat. When plotted against time, mean fluorescence for each line scan revealed a rapid rise of intracellular Ca²⁺ during the systolic Ca²⁺ transient (time to peak: 86.8 ± 7.8 ms; n = 13) followed by a slower decline (time constant: 182.7 ± 11.0 ms; n = 13); the transient was frequently (77.9 ± 7.1 %) preceded by a slow ramp increase of [Ca²⁺] lasting 234.4 ± 32.4 ms (n = 13). Immunohistochemistry showed the ryanodine receptors (RyR) located in regular transverse bands, as reported previously in other cardiac cell types. These data suggest that Ca²⁺ handling in AVN cells may be similar to

that reported previously in SAN cells; the spatial inhomogeneity does not appear to be due to inhomogeneous RyR distribution but may be due to local differences in Ca^{2+} entry or RyR sensitivity.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC29

Is there metabolite sensitisation of stretch-sensitive muscle mechanoreceptive afferents in man?

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Stretch-sensitive muscle mechanoreceptive afferents are known to decrease resting vagal tone and increase heart rate (HR) (Gladwell *et al.*, 2005). This HR rise can be seen even when opposed by baroreflex activation, induced by neck pressure change, in resting subjects. Sustained isometric exercise and subsequent post-exercise circulatory occlusion (PECO) causes an exercise pressor reflex and hence increases baroreceptor activation. We have shown previously that during baroreflex activation by PECO, passive stretch of the human calf muscle does increase HR by a similar magnitude to that seen at rest (Drew *et al.*, 2006). However, it remains unclear whether this effect is dependent upon metabolite sensitisation of the mechanoreceptive afferents as a constant inhibitory mechanoreflex input from the muscle acting against an increased excitatory input from the baroreflex would not be expected to result in the same HR rise. By activating muscle mechanoreceptive afferents during PECO in either the limb that generated the exercise pressor reflex or the contralateral previously inactive limb, a sensitising effect of trapped metabolites on the reflex could be revealed. With local ethical committee approval, subjects rested for 5mins and then on separate occasions performed each of the following trials. They either rested for a further 1.5mins with occlusion of the circulation to the right leg (R-CON) or left leg (L-CON), or performed 1.5mins of one-legged isometric plantarflexor exercise at 50% of their maximal voluntary contraction with either the right calf (R-EX) or left calf (L-EX). This activated the muscle metaboreflex and increased mean arterial pressure (MAP) by 8 ± 1 and 6 ± 1 mmHg for the right and left legs, respectively, during 3.5 min of PECO. This was followed by a further 3mins of concurrent occlusion and sustained passive stretch of the right calf muscle in all trials. During PECO in the R-EX trial (metaboreflex activation in the right calf), concurrent passive stretch of the right calf increased HR by a similar magnitude compared to during the R-CON trial (3 ± 2 v 4 ± 2 b.min⁻¹, respectively). However, in the L-EX trial (metaboreflex activation in the left calf), concurrent passive stretch of the right calf caused a smaller HR increase compared to both the R-EX (1 ± 1 v 3 ± 2 b.min⁻¹, respectively), and L-CON (1 ± 1 v 2 ± 2 b.min⁻¹, respectively) trials. This data suggests that trapped metabolites sensitise the muscle

mechanoreceptive afferents, and is compatible with the idea that this facilitates their response to standardised mechanoreflex activation. This increased inhibitory reflex input is then more effectively able to oppose baroreflex-mediated excitation of cardiac vagal neurones.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC30

Dimethylarginine Dimethylaminohydrolase-1 and the developing embryo

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Nitric oxide (NO) has been linked to a number of embryonic processes, yet the role of nitric oxide signalling in development remains largely unknown. Dimethylarginine dimethylaminohydrolase 1 (DDAH1) catalyzes the breakdown of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide production, and may also have nitric oxide-independent functions. We generated transgenic mice targeting the DDAH1 locus. The recent characterization of these mice indicated that homozygous deletion of DDAH1 appeared lethal in utero. Moreover, the loss of DDAH1 activity in DDAH1^{+/-} mice leads to a reduction in NO signalling and consequently vascular dysfunction. Here we report that DDAH1 null embryos are fertilised at low frequency: 4 DDAH1^{-/-} blastocysts detected in 79 scored (5.1%). Furthermore, the null embryos fail to undergo uterine implantation and are thus non-viable. In addition, DDAH1^{+/-} females have a high frequency of embryo resorption (21 resorbing embryos of 73 scored (28.2%)). To elucidate an underlying role for DDAH1 which could explain these phenomena we documented its presence in the embryo. DDAH1 RNA is expressed in the left ventricle, cardiac outflow tract, developing vasculature, and developing limb buds. This pattern overlaps with the RNA expression pattern of the nitric oxide synthase (NOS) isoforms, and with NOS activity. Our data suggests that DDAH1 is necessary during several phases of embryonic development, possibly through specific effects on NO pathways. Leiper JM *et al.* (2007). *Nature Medicine* **13**, 198-207.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC31

Effects of STZ-diabetes on CGRP and NPY concentrations along the rat tail artery

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We have previously reported that the noradrenaline concentration in segments of the rat tail artery increases after 10-12 weeks

of STZ diabetes (Morrison et al, 2004). The aim of this study was to investigate the changes in the concentration of the sympathetic postganglionic co-transmitter NPY, and the concentration of CGRP, a marker for the afferent fibres. NPY and CGRP are synthesized in the cell bodies of the sympathetic and dorsal root ganglia and are transported to the periphery of the axon, whereas noradrenaline is synthesized in the varicosities. In diabetic autonomic neuropathy it is believed that axoplasmic transport is impaired, and the experiments were performed to investigate whether transport of NPY and CGRP to the most distant nerve terminals is preferentially affected.

Male rats were injected with STZ (60 mgs/kg) at 10 weeks of age, and tissues were taken from diabetic animals that had been decapitated after 12 and 16 weeks of hyperglycaemia, and were compared with the age-matched controls. The tail artery was removed and divided into three segments – proximal, middle and distal – each approximately 5 cms in length, and the peptides were extracted from these segments and stored before radioimmunoassay. The rat tail artery contains some of the longest afferent and sympathetic postganglionic neurones in the body and we have examined the concentrations of CGRP and NPY in the three segments of the artery of diabetic and control rats.

In the control animals the concentration of NPY decreased from proximal to distal segments ($p < 0.02$), whereas the CGRP concentration increased distally ($p < 0.0002$) at 26 weeks of age. STZ-diabetes caused a fall in the concentrations of NPY and CGRP in the middle and distal segments of the tail after 12 and 16 weeks of hyperglycaemia, relative to the age-matched control rats. The results were significantly reduced in the middle segment of the artery for NPY ($p < 0.001$) and in the distal segment for CGRP ($p < 0.0001$) at 12 weeks; whereas at 16 weeks only the NPY level had fallen significantly ($p < 0.001$).

The results suggest that the transport of CGRP and NPY to the most distant terminals may be impaired in STZ diabetes. These results also contrast with the changes in noradrenaline concentration reported previously (Morrison et al, 2004); the differences may be explained by impaired axonal transport of peptides in STZ-diabetes.

Morrison, JFB, Dhanasekaran, S and Sheen, R. (2004) The effects of age and STZ-induced diabetes on biogenic amines in the rat tail artery. *Molecular and Cellular Biochemistry* 261: 77-82.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC32

Protective action of 9-hydroxypinoresinol against oxidative damage in brain of mice challenged with kainic acid

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Title only.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC33

Monoamine oxidase and reactive oxygen species: a role in neuronal apoptosis

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Reactive oxygen species (ROS) participate in an array of biological processes, including normal cell growth, oxidant signalling and cell death [1]. Monoamine oxidase (MAO) is thought to be an important contributor to increased steady state concentrations of ROS within both the mitochondrial matrix and the cytosol [2]. MAO activity can yield the formation of oxygen radicals, hydroxyl radicals, and hydrogen peroxide [3]. Many protein targets are thought to respond to intra- and extra-cellular signals by altering their primary function as catalysts to become important mediators in biological events such as cell death by utilising their oxidative capacity [4]. Here we investigate the role of MAO in neuronal cell death, which has particular relevance to the loss of dopaminergic neurons in Parkinson's disease.

Apoptosis was induced in human neuroblastoma (SH-SY5Y) cells using the protein kinase inhibitor, staurosporine (STS)[5]. In this model, the mitochondrial (caspase-9) pathway was activated 1 hour post treatment, but not the receptor mediated (caspase-8) pathway. This was followed by activation of the downstream apoptotic executioner caspase-3. Activation of caspase cascades was preceded by a dramatic and increased production of ROS evident within 1 hour of treatment. MAO activity was significantly increased during STS-induced apoptosis, peaking after 1 hour treatment, accompanied by an increase in MAO protein, but not MAO mRNA. All these events were associated with activation of the mitogen activated protein kinase (MAPK) enzymes JNK and p38, and depleted levels of the anti-apoptotic protein Bcl-2.

The specific MAO inhibitor, clorgyline, significantly protected cells from STS-induced-apoptosis, and reduced the effects on MAPK signalling and survival proteins. This suggests an involvement of MAO in the apoptotic cascade and signifies the involvement of MAPK signalling in MAO-mediated cell death. The antioxidant N-acetyl cysteine (NAC) also protected cells from apoptosis, to a similar extent to clorgyline, but the antioxidant vitamin C was less effective. In turn clorgyline and NAC dramatically reduced ROS production following STS treatment whilst vitamin C again was less effective. The use of anti-oxidants targeted to mitochondria will allow us to confirm whether most of the ROS produced is derived from mitochondria.

In conclusion, our data provide evidence that MAO, through its production of ROS as a by-product of its catalytic activity, is recruited by the cell to enhance apoptotic signaling.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC34

The effect of atorvastatin on the age-induced alterations in the profile of rat brain lipids

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Lipids play a crucial role in the structure and physiological function of neuronal cell membranes. Brain contains large concentrations of lipids and any modification of this normal profile can result in brain dysfunction. Aging has been associated with alterations in the profile of polyunsaturated fatty acids, phospholipids and cholesterol in brain tissue. Statins are cholesterol-lowering agents that have been shown to possess neuroprotective effects (1). The molecular mechanism of this action is therefore of interest.

The aim of this study was to profile lipids from young and aged rat brain tissue, and study the effect of atorvastatin with the view to understand the molecular mechanism of the latter.

Young (3-4 months) and aged (22-25 months) male Wistar rats were treated with atorvastatin (5 mg/kg body weight). Lipids were extracted from brain tissue samples and analysed using high field proton nuclear magnetic resonance spectroscopy (1H-NMR) (2). Atorvastatin in brain tissue was analysed by liquid chromatography-electrospray mass spectrometry (LC-ESI-MS/MS) (3).

1H-NMR analysis indicated that there was a significant decrease in the levels of the polyunsaturated fatty acid docosahexaenoic acid, as well as choline and ethanolamine glycerophospholipids in the lipid extracts of brain tissue from aged animals as compared to the corresponding controls. Conversely, age increased the levels of cholesterol and sphingolipids in the brain of the aged animals. The atorvastatin treatment did not affect the levels of cholesterol in the brain of the aged animals. However, LC-ESI-MS/MS analysis of the atorvastatin-treated brain tissue indicated that small concentrations (0.059 ng/mg protein $\pm$ 0.018) of atorvastatin were present.

Our results suggest that the age-induced alterations such as increased cholesterol in the brain lipid profiles were not reversed by the atorvastatin treatment although small amounts of atorvastatin were detected in the brain. Therefore the molecular mechanism of statin neuroprotection may be attributed to other effects and not modulation of central nervous system cholesterol homeostasis.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC35

RNAi-mediated inhibition of glutathione biosynthesis using a novel tissue-specific and temporally regulated genetic tool

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Parkinson's disease (PD) is characterized by an extensive loss of dopaminergic neurons (DN) in the substantia nigra pars compacta (SNc) and their terminals in the striatum. Lowered glutathione content has been found in the SNc of patients with pre-symptomatic PD. We recently reported that inhibition of glutamate cysteine ligase (GCL), i.e. the heterodimeric enzyme that catalyzes the first rate-limiting step in glutathione biosynthesis, leads to neurodegeneration in vitro either in the presence or in the absence of astroglial cells in co-culture (1). To assess whether this effect occurs in vivo, we have now designed a temporally regulated DNA construct to inhibit GCL catalytic subunit in a tissue-specific manner. The 9 kb tyrosine hydroxylase rat promoter (2) has been bound to a tamoxifen-inducible form of Cre recombinase (3). In the presence of tamoxifen, Cre promotes the recombination of a Lox-flanked region that contains the green fluorescent protein (GFP), an H1 promoter and a short-hairpin RNA (shRNA) targeted against GCL. This temporally regulated recombination, which can be detected by GFP fluorescence, leads to the expression of a GCL shRNA specifically confined to DN. The implementation of this system for the generation of transgenic mice thus represents a novel tool to investigate the mechanism of DN death by oxidative stress in a PD-like model, as well as for the search of novel therapeutic strategies against this devastating disease

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC36

3-nitrotyrosine in not increased in rat brain 7 days after status epilepticus

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Purpose

Many of the short and long term consequences of epilepsy may be due to excitotoxicity, of which protein nitrosylation is a central feature. Levels of 3-nitrotyrosine (3NT) are raised in other neurological diseases, and known to have key functional consequences. We have already shown that 3NT is raised in rat brain following seizures (novel finding). We set out to establish the time course of this change.

Methods

Self-sustaining SE (perforant path model) was induced for 3 hours, then terminated with diazepam. 7 days later, the rats were decapitated under anaesthesia, the hippocampal subregions dissected on ice, and snap frozen. Sham controls underwent identical procedures, with the exception of electrical stimulation. 3NT was quantified using high performance liquid chromatography with electrochemical detection.

Results

Mean hippocampal 3NT levels (expressed as a ratio to L-tyrosine) were not different in status animals ($3.27 \times 10^{-5} \pm 3.96 \times 10^{-6}$ SEM) compared with controls ($3.75 \times 10^{-5} \pm 1.02 \times 10^{-5}$ SEM). Subregional analysis confirmed this finding in the dentate gyrus/hilar (DG/H) region ($SE\ 3.48 \times 10^{-5} \pm 5.23 \times 10^{-6}$, sham $4.29 \times 10^{-5} \pm 1.86 \times 10^{-5}$), CA1 ($SE\ 2.98 \times 10^{-5} \pm 1.06 \times 10^{-6}$, sham $2.03 \times 10^{-5} \pm 5.36 \times 10^{-6}$) and CA3 ($SE\ 3.36 \times 10^{-5} \pm 4.56 \times 10^{-6}$, sham $4.92 \times 10^{-5} \pm 2.46 \times 10^{-5}$) regions.

Conclusions

This study is the first to describe the time course of changes in 3NT expression following seizures in a rat model of epilepsy, and has implications for our understanding of the morbidity of seizures. Further studies are required to understanding the functional localization and consequences of protein nitrosylation following SE.

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PC37

Linking cell cycle and neurodegeneration: ubiquitin ligase APC-Cdh1 protects neurons against excitotoxic damage

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Cell division at the end of mitosis and G1 is controlled by Cdh1, an activator of the E3-ubiquitin ligase anaphase-promoting complex (APC) that promotes the ubiquitylation and degradation of mitotic cyclins and other substrates (1). The finding that Cdh1-APC is active in post-mitotic neurons (2) has raised the interest in the role of cell cycle proteins in neurodegenerative diseases. Recently, we reported that Cdh1 silencing, using a small hairpin RNA (shRNA) strategy, triggered cyclin B1 accumulation that preceded apoptotic death in post-mitotic neurons (3). Though these results pointed out the essential role of Cdh1 for neuronal survival, it remained elusive whether Cdh1 would directly promote cellular protection in relevant neurodegenerative paradigms. Thus, here we aimed to investigate the role of Cdh1 at

dictating neuronal vulnerability in excitotoxic damage, i.e. a common hallmark in several neurodegenerative disorders. To achieve this, rat cortical neurons in primary culture were incubated with glutamate (100 μ M plus 10 μ M glycine) for 5 min, and Cdh1 expression was analyzed by Western blotting. We found that Cdh1 protein levels significantly increased in neurons as from 1 hour after glutamate treatment, while no signs of apoptotic death were observed, as assessed by annexin V staining using flow cytometry. However, Cdh1 silencing using the shRNA strategy increased neuronal vulnerability to glutamate treatment, whereas Cdh1 over-expression prevented neurons from excitotoxic damage. Thus, besides its role at regulating cyclins stability in post-mitotic neurons, Cdh1 plays a key neuroprotective role in excitotoxicity. These results may be relevant for understanding the molecular mechanisms involved in certain neurodegenerative diseases.

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PC38

Slow endocytosis in cerebellar granule cells

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In order to maintain communication at the synapse, synaptic vesicles must be retrieved and recycled by endocytosis.

At least two distinct mechanisms of vesicle retrieval exist; fast endocytosis and slow endocytosis. The mechanism of slow endocytosis is currently poorly understood, but it is known that strong stimulation is needed to trigger this pathway. Slow synaptic vesicle endocytosis is controlled by cyclin-dependent kinase 5 in an activity dependent manner. We have characterised this slow endocytic pathway in cerebellar granule neurons in order to determine the molecular mechanisms of this pathway. To do this we have determined the strength of stimulus required to activate slow endocytosis. We have investigated how these vesicles replenish the readily releaseable pool and the reserve pool. We have also looked at the essential molecular players in this process by over-expression and use of dominant negative mutants. These studies have highlighted the importance of the slow endocytic pathway in normal physiological function at the nerve terminal.

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PC39

Presynaptic interaction partners of the neuronal splice variants of SRCT.M. Abdelhameed¹, G. Evans² and M. Cousin¹¹*Center of Integrated Physiology, University of Edinburgh, Edinburgh, UK and* ²*Cell and Molecular Biology, University of York, York, UK*

SRC splice variants are multi-domain nonreceptor tyrosine kinases. C-src – which is found almost in all types of tissue - has been a subject of investigation for years. Alternatively, the neuronal splice variants N1 SRC and N2 SRC, also called neuronal SRCs, are not as well characterized in terms of their neuronal function. N1 src has six extra amino acids in its SH3 domain, while N2 SRC has 18 extra amino acids. We have embarked on a project to identify the interaction partners of the neuronal splice variants of SRC, N1 and N2. This has involved generating both SH3 domain and the full length SRC splice variants and determining their binding partners in lysates from presynaptic nerve terminals. We have identified a number of selective interactions of N1 and N2 by MALDI-tof mass spectroscopy. A number of these proteins have essential roles in synaptic vesicle recycling. The next step will be to determine whether their interactions occur in vivo, and which of the binding proteins are also SRC substrates. The results suggest that the SRC neuronal splice variants might have specific roles in the synaptic terminal and implicates them in a putative role in the synaptic vesicle life cycle.

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PC40

Melatonin has no modulatory effect on cholecystokinin-induced noradrenaline release in hypothalamic paraventricular nucleus of virgin female ratM. Aydin¹, S. Kutlu¹, M. Ozcan², E. Alcin¹, A. Ayar¹ and B. Yilmaz¹¹*Physiology, Firat University Medical School, Elazig, Turkey and* ²*Biophysics, Firat University Medical School, Elazig, Turkey*

It is known that noradrenergic projections from brainstem has a stimulatory effect on oxytocin secretion, and intravenous administration of cholecystokinin (CCK) causes an increase of noradrenaline (NA) concentration in supraoptic and paraventricular nucleus (PVN), and plasma oxytocin levels. It is suggested that melatonin inhibits basal and induced oxytocin secretion but the precise mechanism of this effect is yet to be discovered. The aim of this study was to investigate the effect of melatonin on the CCK-induced NA release in PVN. Virgin female rats (250-300 g) were anaesthetized with choral hydrate (400mg/kg) and placed on a stereotaxic frame. A microdialysis probe was set into PVN with the guidance of the rat stereotaxic atlas, and artificial cerebrospinal fluid was run through a micropump throughout the experimental period. One hour later, microdialysis samples were collected with 20 minutes intervals

for 80 min. After collection of the first sample (control), 50µg/kg CCK was intravenously administered to the CCK group (n=8). In addition to CCK, 1µg melatonin was intracerebroventricularly infused to the melatonin group (MLT, n=8). The control (n=10) and CCK groups were received intracerebroventricularly vehicle infusion. NA content of samples were analysed by a high performance liquid chromatography-electrochemical detector system. Data were evaluated and expressed as percentages of the baseline levels. NA levels were normalized by nominating the control level as 100%, and its levels in 20-mins samples were expressed as percentage of these values.

Data were statistically analysed by One-way Analysis of Variance (ANOVA) followed by a pairwise comparison between control and each CCK and MLT groups using a Tukey's t-test for multiple comparisons. P<0.05 was considered statistically significant. In the second interval (20-40 min), NA content of PVN was significantly elevated in the CCK group (p<0.001) compared to control values. But melatonin did not alter this CCK-induced rise in NA content compared to CCK administered group for this interval. Melatonin group also had significantly higher NA levels than both the control group (p<0.005) and its initial control values (p<0.02) in this second interval. There was no significant differences between the values of any group for the subsequent interval (p>0.05).

The results of the present study indicate that CCK has stimulatory effect on NA content in PVN which is not subject to modulation by melatonin. Our results does not rule out the possibility that, the reported inhibitory effect of melatonin on oxytocin secretion may involve mechansim(s) other than NA mediation.

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PC41

Effect of zinc chloride on aluminum-induced learning and memory impairment in rats

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Uptake of aluminum may disturb the learning and memory of humans or animals. But zinc has been shown to exert beneficial effects on memory deficits.

Objective: The aim of the present study is for evaluating the effect of zinc chloride on aluminum-induced learning and memory impairment in rats.

In this research, we examined coadministration of zinc chloride (30mg/kg/day) and aluminum chloride (600mg/kg/day) in drinking water for two weeks. The shuttle box was used for this text. Also we used gavage method for oral administration. Two criteria were considered to behavioral test, including latency in entering dark chamber and time spent in the dark chamber. This experiment showed that after 2 weeks coadministration of Zncl2 (30mg/kg/day) and Alcl3 (600mg/kg/day) doses, passive avoidance memory has been improved (P<0.001).

In conclusion: zinc chloride consumption with (30mg/kg/day) dose, could have a significant effects on memory and could improved the negative effect of AlCl₃ on learning and memory. It seems zinc modulated NMDA receptor, which participate in memory formation, and could enhance LTP in the CA1 region of the hippocampus in aluminum-induced learning and memory impairment in rats. Also, zinc adequate doesn't permit the entry in the brain of toxic metals such as aluminum, from blood-brain-barrier (BBB).

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PC42

Hypoxic modulation of glutamate transporter expression in cortical astrocytes

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Glutamate uptake by astrocytes is vital in the prevention of excitotoxicity, especially in circumstances such as stroke, when oxygen levels are severely compromised. However, the effects of hypoxia on glutamate transporter expression have not been studied in detail. Here, we report the effects of 24h hypoxia (1% O₂) on glutamate transporter levels in rat cortical astrocytes.

Using subtype-specific antibodies, we identified the presence of transporters EAAT1, EAAT2 and EAAT3 in astrocyte homogenates (isolated as previously described; Smith et al., 2004) by western blotting. Exposure of astrocytes to hypoxia reduced protein EAAT1 levels by 39.4 ± 10.9% (n=5) and EAAT2 levels by 39.7 ± 6.0% (n=5). EAAT3 levels were unaffected. RT-PCR results show that the observed reduction in protein expression caused by hypoxia is mirrored by a reduction in mRNA levels of EAAT1 and EAAT2. The effects of hypoxia on protein expression levels of both EAAT1 and EAAT2 were prevented by the addition of the NF-κB inhibitor, SN50 (5μM), indicating a role for the transcription factor NF-κB in modulating the transcription and subsequent expression of EAAT1 and EAAT2. Immunocytochemistry also revealed that hypoxia caused translocation of NF-κB to the nucleus of the cell, and that this was prevented by SN50.

NF-κB is known to activate, and be activated by, TNFα. As TNFα has been reported to regulate EAAT2 expression (Sitcheran et al., 2005) and as TNFα is produced by astrocytes following ischemic injury (Yu & Lau, 2000), we measured TNFα levels in astrocyte media following 24h hypoxia. Hypoxia caused a 7-fold increase in TNFα production, and application of exogenous TNFα (10ng/ml.) mimicked the effects of hypoxia on EAAT expression, indicating a possible role for TNFα in hypoxic regulation of the EAAT expression.

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PC43

The CRF-like peptide urocortin restores striatal DA release in a neuro-inflammatory paradigm of Parkinson's disease

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Parkinson's disease (PD) is an incurable neurodegenerative disorder which can only be treated pallitively. We have recently observed that the CRF-like peptide urocortin (UCN) reverses key features of nigrostriatal degeneration in the lipopolysaccharide (LPS) neuro-inflammatory paradigm of PD (Abuirmeileh et al. 2007). While these findings suggest a restoration of dopaminergic transmission, we are unsure if they reflect a restoration of striatal extracellular DA levels. To address this we have measured basal and evoked striatal DA release using microdialysis in conscious rats.

Male Wistar rats (250-280g; n = 4-6 per group) were anaesthetised with isoflurane and stereotactically injected with LPS or UCN (T0) as previously described (Abuirmeileh et al. 2007). In some experiments UCN was given 7 days after LPS (T7). 14 days after LPS, rats were given 0.5 mg kg⁻¹ of apomorphine to evaluate ipsilateral circling, implanted with dialysis probes (under isoflurane anaesthesia) as previously described (Whitton et al. 1992), and 24h later perfused with artificial cerebrospinal fluid (Whitton et al. 1992) at 1.0μl/min. After 1 h, 4 x 30 min samples were collected prior to infusion of 100mM K⁺ (exchanged for equimolar Na⁺) for 30 min, followed by collection of two further 30 min samples. DA was estimated as previously described (Biggs et al., 2006). Data were subjected to two way ANOVA and Dunnett's post hoc test.

Compared to rats given LPS alone, apomorphine induced circling behaviour was reduced by injection of UCN (18.4 ± 4.01 vs 5.11 ± 1.4 and 8.77 ± 3.06 turns/120 s for LPS, LPS + UCN (T0) and LPS + UCN (T7) respectively, $p < 0.05$). Striatal dialysis of shams showed basal extracellular DA at 25 ± 3.2 fmol/10ul (mean $\pm$ s.e.mean, $n = 10$) which increased to around 1000 fmol/10ul by K⁺ infusion. LPS reduced both basal and stimulated DA levels by 70–80 %. However, when UCN was given either at T0, or 7 days after LPS (T7) basal and evoked extracellular DA was comparable to sham treated rats.

These data provide further evidence for a potential neuroprotective role for UCN using a neuro-inflammatory model of PD. This may have particular significance, since neuro-inflammation is currently receiving considerable attention in the aetiology of PD (e.g. see Whitton, 2007). Since UCN can restore striatal extracellular DA concentrations, presumably local DA receptors will be engaged leading to the reversal of motor deficits. The observation that UCN is effective when given at T7 indicates that it is capable of promoting restoration once the lesion has become established (Abuirmeleh et al., 2007). These observations suggest that UCN or similarly acting molecules offer therapeutic promise in PD.

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PC44

A study comparing the pro-epileptogenic actions of a non-selective muscarinic acetylcholine receptor agonist and a specific M₁ receptor agonist *in vitro*

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Selective M₁ muscarinic acetylcholine receptor (mAChR) agonists are being developed for possible therapeutic application in a number of disease states. However, activation of mAChRs using broad-spectrum agonists has also been shown to produce hyper-excitability within the brain which, at the behavioural level, is manifest as seizure activity. The aim of the current study was to assess whether selective M₁ receptor agonists promote pathological patterns of activity within the hippocampus *in vitro*. Extracellular recordings were used to monitor spontaneous field potentials in 450µm coronal rat hippocampal slices [1]. Application of the M1 selective agonist 77LH281 (10µM) resulted in a gamma frequency oscillatory activity ($n=10$, mean dominant frequency = 32 ± 3.6 Hz) that was sensitive to AMPA receptor blockade (2µM NBQX). In contrast, application of the broad spectrum mAChR agonist oxotremorine-M (10µM) induced interictal-like epileptiform bursts which, in many instances ($n=11$ of 22), transformed into more prolonged ictal-like discharges.

We also assessed whether mAChRs modulated established epileptiform activities. Specifically, convulsant regimes including potassium channel blockade (4AP, 20µM), blockade of GABA_A receptor-mediated inhibition (bicuculline, 20µM) or enhanced NMDA receptor activation (0mM Mg²⁺ perfusion medium) were used to induce epileptiform activity [1]. Subsequent co-application of 10µM 77LH281 did not produce any significant changes in epileptiform burst frequency in any of the three models examined (120 ± 30 %, 144 ± 74 % and 114 ± 35 %; all $p > 0.05$; $n=10-17$). Conversely, co-application of 10µM oxotremorine-M significantly increased epileptiform burst frequency in all models investigated (180 ± 34 %, 401 ± 108 % and 148 ± 21 %; all $p < 0.05$; $n = 8-10$). Moreover, in the majority of slices tested, 10µM oxotremorine-M converted the network from inter-ictal-like bursting behaviour to prolonged ictal-like patterns of activity ($n=15$ of 24, 9 of 13 and 11 of 16 slices for 4AP, bicuculline and 0mM Mg²⁺ models respectively).

These results suggest that, in contrast to the broad spectrum mAChR agonist oxotremorine-M, the selective M₁ receptor agonist 77LH281 does not display a propensity to induce, or enhance, epileptiform activity.

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PC45

Effects of ramelteon, a melatonin agonist, on sleep in the rat

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A recent report published by the Institute of Medicine of the National Academy of Sciences concluded that sleep disorders represent a substantial unmet public health problem in the US [1]. Ramelteon ((S)-N-[2-(1,6,7,8 tetrahydro-2H-indeno-[5,4]furan-8-yl)ethyl]propionamide), the first of a new class of insomnia drugs which are melatonin receptor agonists, recently gained FDA approval for the treatment of insomnia characterised by difficulty with sleep onset [2]. Ramelteon was demonstrated to be effective at promoting sleep in cats [3] and monkeys [4]. This study compared the effects of ramelteon with melatonin and zolpidem, a GABA(A) receptor agonist and the dominant prescription hypnotic, on the sleep-wake cycle in the rat using the EEG telemetry model which is routinely used in drug development as a predictor of clinical hypnotic activity.

In radioligand binding assays on recombinant human MT₁ and MT₂ receptors, both ramelteon and melatonin showed high affinity, but little subtype selectivity (ramelteon; pK_i MT₁ 10.05 ± 0.04 ; MT₂ 9.70 ± 0.07 , melatonin; pK_i MT₁ 9.29 ± 0.02 ; MT₂ 9.34 ± 0.04). In a functional model, the pigment aggregation response of *Xenopus* melanophores [5], ramelteon, like melatonin, was a potent full agonist (EC₅₀ 11.48 ± 0.02 ; melatonin EC₅₀ 10.45 ± 0.02).

Adult male Sprague-Dawley rats (~350g, n=6) housed under a 12:12 L:D cycle were implanted (i.p.) with telemetric probes (F40-EET; DSI) under general anaesthesia (75mg/kg i.p. ketamine, 0.5mg/kg i.p. medetomidine) to record cortical EEG and neck muscle EMG. Semi-automated scoring using SleepSign™ (Kissei-Comtec) was used to discriminate 10 s epochs into non-REM (NREM) sleep, rapid eye movement (REM) sleep, or wake (W). Ramelteon, melatonin and zolpidem (all at 10 mg/kg, i.p.) were administered in a randomised protocol at mid-dark. Time to NREM and REM onset, and NREM, REM and W durations were then determined. Data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. Ramelteon, melatonin and zolpidem significantly decreased the latency to NREM sleep onset (vehicle 46.6±5.9 min, ramelteon 26.8±1.4 min, melatonin 25.1±4.4 min; zolpidem 9.7±1.5 min, $p<0.05$). Ramelteon and zolpidem increased NREM duration up to 1 h post-injection compared to vehicle ($p<0.05$). Neither ramelteon nor melatonin had any significant effect on REM latency or REM duration, but zolpidem eliminated REM sleep for >2 h after injection. Power spectra during NREM episodes in the first hour demonstrated no change with melatonin or ramelteon, but an increase in delta power (0-3 Hz) and a decrease in theta power (4-8 Hz) with zolpidem.

All three agents were effective at promoting NREM sleep, but ramelteon and melatonin appear to produce a natural sleep and are relatively short-acting, while zolpidem has a longer duration of action and altered sleep architecture.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC46

Synthetic oligomers of beta-amyloid alter synaptic transmission in hippocampal CA1

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Small soluble oligomers of the beta-amyloid peptide (A β) are believed to play a central role in the pathology of Alzheimer's disease. The low-weight aggregates are capable of disrupting synaptic plasticity in the brain and this is thought to contribute to the memory and learning deficits observed in Alzheimer's Disease. Application of A β peptide oligomers to hippocampal slices

has previously been demonstrated to block the induction of long-term potentiation (LTP), a form of synaptic plasticity and a model for mechanisms of memory and learning. An understanding of the mechanisms behind the effects of A β at hippocampal synapses is crucial if therapeutic intervention in early onset Alzheimer's disease is to become a realistic option.

Aim – to understand how low molecular weight A β oligomers influence hippocampal synaptic transmission.

Hippocampal slices (400 μ m thick) were prepared from adult rats (90-150g) in accordance with Home Office approved procedures, and incubated with a variety of low molecular weight A β oligomers. To provide a measure of CA1 excitability field potential EPSPs were recorded from CA1 stratum radiatum with increasing intensity of Schaffer collateral stimulation to generate input-output curves.

Pre-incubation of slices with low nM concentrations of synthetic A β oligomers increased hippocampal excitability compared with untreated slices. This enhanced excitability in the presence of A β was prevented by application of the GABA-A receptor antagonist, picrotoxin, and occluded by the GABA-A receptor agonists taurine (100 μ M) and muscimol (2 μ M).

These preliminary results show that low concentrations of A β oligomers cause an increase in hippocampal excitability that may arise from interference with fast inhibitory synaptic transmission. Since the concentrations used here are also closer to physiological concentrations than previous studies, our results may indicate an early effect of A β oligomers at hippocampal inhibitory synapses in Alzheimer's disease.

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PC47

GABA and nitric oxide containing neurones are closely associated with target specified presympathetic neurones of the paraventricular nucleus of the hypothalamus

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The central nervous system plays a key role in maintaining cardiovascular homeostasis e.g. plasma volume regulation, through the actions of the sympathetic nervous system. Recently it has emerged that activity present in the sympathetic nerves appears to be elevated in a number of cardiovascular diseases such as heart failure (Esler & Kaye). Functional studies indicate that within the PVN both GABA and nitric oxide (NO) play a role in determining the level of excitability of presympathetic neurones and that an alteration of this action may be in part responsible for the sympatho-excitation manifest in heart failure (Li & Patel, 2003). Anatomically, there is sparse evidence indicating how GABAergic and NO producing neurones might regulate presympathetic neurones. Therefore, the purpose of this study was to identify the location of GABAergic and NO producing neurones in the PVN in relation to a group of presympathetic neurones that play a role in plasma volume regulation.

All procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986. Male Sprague Dawley rats

were anaesthetised with Isoflurothane (4% in 2.5 l/min O₂) and the left adrenal gland isolated via a retroperitoneal approach. This was injected with 10 µl of herpes simplex virus conjugated with green fluorescent protein (HSV-1-GFP; 10⁹ pfu/ml). The animals recovered for 4 days when they were killed humanely (Euthatal), perfused-fixed (4% paraformaldehyde) and the brain and spinal cord removed. Frozen sections (30 µm) containing virally labelled presympathetic neurones were incubated in either anti guinea pig GABA or anti guinea pig nNOS (neuronal NO synthase) for 24 hrs. The tissue was then treated with a biotinylated secondary antibody followed by streptavidin Alexa Fluor 350 or 594. The tissue was examined under epifluorescence. Presympathetic neurones containing HSV-1-GFP were scattered throughout the 5 parvocellular nuclei of the PVN, with the majority occupying the dorsal cap region. GABAergic neurones were located at the lateral boundaries of the dorsal cap subnucleus and also intermingled with the presympathetic HSV-1-GFP neurones in this region. Nitric oxide (nNOS) containing neurones were found scattered through the magnocellular and parvocellular nuclei of the PVN and adjacent to the presympathetic neurones.

We have provided evidence that within the PVN, GABA and NO containing neurones are found in close proximity to presympathetic neurones that project to the adrenal medulla. The close association of these pools of neurones provides an anatomical basis for the involvement of GABA and NO in modulating presympathetic neuronal excitability.

Esler M & Kaye D (1998). *J Auton Nerv Syst* 72, 210-219.

Li Y-F & Patel KP (2003). *Acta Physiol Scand* 177, 17-26.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC48

Temporal coordination between ocular and manual movements

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For a coordinated and consistent goal-directed behaviour, the human brain has to constantly control not only the spatial but also the temporal accuracy of all the movements made by an individual. This is a challenging task when a movement pattern comprises concurrent ocular and manual motor actions. Moreover, the brain is engaged not only in the movement control but also it has to perform the sensory-to-motor translation processes which have to start before the related motor process. Usually, both processes are in sequential order, although they can overlap. What does happen, when two sensory-to-motor translation processes (e.g., a periodic and a discrete) have to be completed in parallel? The temporal accuracy of motor responses under

these highly demanded conditions of a dual task tapping paradigm was subject of this investigation.

A continuation tapping paradigm (1) employed for a periodic task in which, in the continuation phase, subjects tapped at a rate given by an auditory signal in the preceding synchronization phase. In the first experiment, while tapping with the index finger of the dominant hand, the subjects responded as quickly as possible to a discrete auditory stimulus by their non-dominant index fingers. In the second experiment, in response to the discrete stimulus, subjects have to make a fast two-phasic saccade: first, to the right side and then backwards. By means of two force transducers embedded on the tabletop, an accurate recording of right and left index finger taps was possible. In addition, with high resolution laser distance sensors fixed approximately 10 cm above the fingers, the vertical position of both index finger tips was also acquired. For eye movement registration, the IRIS 6500, infra-red light eye movement system was employed. Moreover, active EMG electrodes, MYO 110, fixed on forearms recorded the EMG signals from the extensors and flexors. Data were recorded at a sampling frequency of 1 KHz and stored for offline analysis.

As a result, phase resetting curves (2, 3) showing the temporal relationship between both actions were constructed. It was evident that, in the temporal domain, there is interference between these concurrently performed manual tasks. For the saccades, we found mutual interactions similar to Stuyven et al. (4), but also a novel type of interference: the phase entrainment as well as phase hastening effects (3) describing the mechanism of synchronization between finger tapping and eye movements.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC49

Paraventriculospinal oxytocinergic pathways modulate apomorphine-induced penile erection in the anaesthetised rat

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The non-selective dopaminergic agonist apomorphine (Ki of 101, 32, 26, 2.6, and 10 nM for D1, D2, D3, D4, and D5, respectively) is pro-erectile in the rat and it is thought that the paraventricular nucleus (PVN) mediates these effects (Melis et al, 1987, Succu et al, 2006). The complex neural circuitries which underlie dopamine-induced penile erection remain to be elucidated. However, one potential pathway may involve spinally-projecting oxytocin neurones in the PVN. These neurones may activate the proerectile parasympathetic preganglionic neurones that

innervate the penis by releasing oxytocin at the lumbosacral level of the spinal cord. To test this hypothesis, both intracavernous pressure (ICP, via a 25-gauge needle inserted into one corpus cavernosum) and blood pressure (BP, via the femoral artery) were recorded and analysed in urethane-anaesthetised male rats. Erectile responses were dose-dependently (0.01, 0.05, 0.15, 0.25, 1, 5 mg/kg) increased by apomorphine delivered iv through the femoral vein, which also induced transient decreases in blood pressure. The sub-maximal dose of 0.25 mg/kg was selected for use thereafter. An intrathecal injection (directly into the spinal cord at the L4-L6 segmental region) of either vehicle (3 µl isotonic saline) or the oxytocin receptor antagonist, UK-482743-01 (36, 180, 900 ng, n=6-7 per group) was given 5 min prior to apomorphine injection. Both 180 ng and 900 ng of UK-482743-01 effectively inhibited the mean number of erectile responses, but this effect was only statistically significant for the 180 ng injection ($p < 0.05$). The ability of an oxytocin antagonist delivered locally at the level of the sacral parasympathetic nucleus to decrease the erectile activity induced by systemic apomorphine supports the hypothesis that oxytocin release at the lumbosacral spinal cord has a modulatory role in apomorphine-induced penile erection.

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Succu S et al. (2007). *Neuropharmacology* 52, 1034-1043.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC50

Transition to self-sustaining status epilepticus in rat dentate gyrus

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Status epilepticus (SE) is a medical emergency that can result in permanent neurological damage, particularly to the hippocampus. It is thought that there is a critical time point at which self-terminating seizures develop into self-sustaining SE (SSSE), however endogenous mechanisms of seizure termination are poorly understood. The dentate gyrus (DG) has been proposed as the gatekeeper of hippocampal excitability and is known to play a role in controlling the spread of epileptogenic activity to the seizure susceptible hippocampus.

We have modified an existing model of limbic SE in order to further understand the endogenous anticonvulsive properties of the DG during the transition to SE.

Stimulating and recording electrodes were implanted into the perforant pathway and dentate granule cell layer, respectively, of anaesthetised Sprague Dawley rats. A minimum of 3 days later, rats underwent continuous 3.5mA, 20Hz stimulation to induce SSSE. The perforant pathway stimulation model of SE, induced by 2 hours continuous stimulation has been used to assess seizure severity during and neuronal damage post-SE. Here, groups

underwent stimulation for 30 minutes, 1, 1.5 and 2 hours, to determine the duration of stimulation necessary to induce SE in 50% of animals, thereby allowing SE entry to be increased or reduced through intervention. SE was considered to be induced when animals exhibited epileptic activity for at least 30 minutes after stimulation had ceased. The proportion of animals developing SSSE was assessed alongside seizure severity, observed as behavioural scores and spike amplitude and frequency of EEGs, both during and post-SE.

87.5% animals stimulated for 2 hours (n=8) developed SSSE. 65% of animals stimulated for 1.5 hours (n=8) developed SSSE. 50% animals stimulated for 1 hour (n=8) self-sustained and 25% animals stimulated for 30 minutes (n=8) self-sustained. In all cases seizure severity, as assessed using the Racine score, was greatest after 20-30 minutes of stimulation.

These findings indicate that there is a critical time point at which self-terminating seizures become SSSE.

Further development of this model will enable pharmacological manipulation of the anticonvulsive properties of the DG and so allow a further understanding of the role this region plays in the transition to SSSE.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC51

Glial and neuronal gap junctions contribute to 4-aminopyridine-induced rhythmic network behaviour in the rat dorsal horn *in vitro*

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Network behaviour within the 6-13 Hz frequency range is manifest within spinal dorsal horn (DH) *in vivo* (Sandkuhler & Eblen-Zajjur, 1994). Previous data demonstrated rhythmic network behaviour in rat DH *in vitro* with a dominant frequency of 7-12 Hz (Asghar *et al*, 2002), which could be maintained through gap junction (GJ) interconnectivity, and may have a role to play in somatosensory processing of nociceptive signals. The potassium channel blocker 4-aminopyridine (4-AP) triggers widespread oscillatory activity in spinal DH (Ruscheweyh & Sandkuhler, 2003) *in vitro* but it is not known if GJs contribute to this behaviour. Here, we determined whether GJs localised to glia or neurons may be involved in 4-AP-induced enhanced excitation in DH *in vitro*.

Spinal cords were removed from Wistar rats (aged 12-14 days) terminally anaesthetised with urethane (2g/kg I.P.). Transverse lumbar slices (300µm) were cut and placed in an interface recording chamber perfused with artificial cerebrospinal fluid (aCSF) at 32°C. Extracellular field recordings were then made from substantia gelatinosa (SG) using aCSF-filled glass microelectrodes (1-2MΩ) and oscillations were induced with bath-perfusion of 50µM 4-AP.

Data values for peak amplitude and area of power spectra were quantified before and after application of the non-specific GJ uncouplers, Carbenoxolone (100µM, n=6) and Octanol (1mM,

n=7), and the selective neuronal GJ uncouplers, Quinine (250 μ M, n=10) and Mefloquine (500 nM, n=6). Carbenoxolone and Octanol significantly reduced the peak amplitude (64% and 58% respectively, $P < 0.05$) and area (60% and 57% respectively, $P < 0.05$) with no effect on frequency. Quinine had a small but significant effect only on peak amplitude which was reduced by 33% ($P < 0.05$), whilst Mefloquine significantly reduced both peak amplitude (34%, $P < 0.05$) and area (34%, $P < 0.05$).

These data suggest that both glial, and more specifically, neuronal gap junctions may contribute to 4-AP-induced oscillations in SG. Further studies are now required to reveal the significance of GJ-mediated activation of large populations of DH neurons in the context of spinal nociceptive signalling. All procedures were carried out in accordance with UK Animals (Scientific Procedures) Act 1986. Supported by The Wellcome Trust.

Sandkuhler and Eblen-Zajjur, 1994, *Neurosci.*, **61**:991-1006.

Asghar *et al*, 2002, *J. Physiol.*, **562**:183-198.

Ruscheweyh and Sandkuhler, 2003, *Pain*, **105**: 327-328

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC52

What is the significance for synaptic plasticity of the developmental change in N-methyl-D-aspartate receptor subunit expression?

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Long term potentiation (LTP) and long term depression (LTD) are forms of synaptic plasticity important for learning and memory. In area CA1 of the hippocampus the NMDA receptor is crucial for the induction of LTP and LTD induced by high and low frequency stimulation respectively. The NMDA receptor comprises four subunits, two NR1 and two NR2 from four possible types (NR2A-NR2D). The expression of NR2 subunits is developmentally regulated, with NR2A appearing at P7 while NR2B is already high at birth (Sheng, Cummings *et al.* 1994).

To detect if different subtypes of the NR2 subunit are involved in the induction of LTP and LTD at different stages in development we studied the effect of subunit selective antagonists on the induction of LTD induced by low frequency stimulation (1 Hz, 15 mins) or LTP induced by high frequency stimulation (100 Hz, 1 s) in area CA1 of hippocampal slices from P14 and P56 Wistar rats.

We tested subunit selective antagonists in HEK293 cells expressing NMDAR subunits and found NVP-AAM077 (NVP) to be 14-fold selective for NR2A over NR2B, whereas Ro 25-6981 (Ro) was very selective for NR2B. In P14 hippocampal slices we measured NMDA EPSCs and found NVP and Ro both reduced the EPSC amplitude, but changed the time constant of EPSC decay in a manner consistent with selective actions at NR2A and NR2B receptors respectively. Together this data led us to conclude that 0.1 μ M NVP and 5 μ M Ro are selective doses for antagonising NR2A and NR2B NMDARs respectively.

In P14 hippocampal slices, neither NVP (0.1 μ M), nor Ro (5 μ M) blocked LTD, but higher concentrations of NVP blocked LTD in a linearly dose-dependent manner. This suggests that NR2B NMDARs are not involved in the induction of LTD at this developmental stage under our experimental conditions. In P56 hippocampal slices, LTD could only be induced using the glutamate uptake blocker, threo- β -benzylaspartic acid (TBOA) and this LTD was insensitive to Ro (5 μ M). However, the highest NVP concentration tested (0.4 μ M) completely blocked LTD at this developmental stage. These results suggest that at both developmental stages NR2B NMDARs are not involved, but that extrasynaptic receptors are important at P56.

LTP in P14 slices was reduced 63% and 45% by NVP (0.1 μ M) and Ro (5 μ M) respectively, suggesting that both NR2A and NR2B are involved in the induction of LTP at this developmental stage. However, initial experiments showed that in P42-56 slices LTP is insensitive to NVP (0.4 μ M) and Ro (5 μ M).

Bartlett TE, Bannister NJ, *et al.* Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus. *Neuropharmacology* (2007) 52:60-70.

Sheng, M., J. Cummings, *et al.* (1994). "changing subunit composition of heteromeric NMDA receptors during development of rat cortex." *Nature* 368(6467): 144-7.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC53

Preconditioning interactions in the rat hippocampal slice

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Preconditioning is induced by exposing tissue to sublethal insults resulting in a tolerant state within the tissue protecting against further damage. To investigate the role of adenosine in excitotoxic preconditioning, we evoked responses from rat hippocampal slices exposed to glutamate and ouabain. A significant depression was observed in epsp slope, orthodromic population spike and antidromic population spike amplitudes in response to 10 mM glutamate and 100 μ M ouabain. Antidromic population spikes showed a significantly greater recovery following ouabain exposure ($82.6 \pm 1.6\%$) ($n = 6$) compared with the orthodromic responses (epsp slope $58.6 \pm 7.1\%$, $n = 7$; population spikes $56.6 \pm 8.3\%$, $n = 8$) ($p < 0.05$). The reduction in antidromic spike amplitude was unaffected by 1 mM kynurenic acid, a broad-spectrum glutamate antagonist. We applied an LTP stimulus (100 Hz/1 sec) to orthodromic population spikes one hour following either chemical stimulus; a significant potentiation was induced in slices exposed to ouabain but not those exposed to glutamate. 100 μ M adenosine was applied to orthodromic population spikes to see if preconditioning protection could be induced. A greater recovery was observed following ouabain exposure in preconditioned slices ($79.8 \pm 6.8\%$) ($p < 0.05$) compared with controls ($56.6 \pm 8.3\%$) but no protection was induced against glutamate. Ouabain and glutamate therefore exert different effects on evoked responses from CA1 neurons and adenosine preconditions against ouabain toxicity but not glutamate.

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PC54

Na⁺-independent Cl⁻-HCO₃⁻ exchanger (AE) expression in rat blood-brain barrier

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The blood-brain barrier exerts a strong diffusional restriction on the exchange of solutes between blood and brain [1]. It is also believed to secrete a large proportion of the brain interstitial fluid [2] which requires the regulated transport of ions such as Na⁺, K⁺, H⁺, and HCO₃⁻ across the membranes of the endothelial cells of the brain microvessels. A useful model for the role of acid-base transporters in this secretion has been proposed based on functional data for rat brain endothelial cells in primary culture [3].

This present work investigates functional evidence for the existence of an AE-like transporter and evidence for the molecular expression of AE2 at both the mRNA and protein level. Cells on coverslips were initially exposed to HCO₃⁻ buffered NaCl solution. When Cl⁻ was replaced by gluconate⁻ an alkaline shift followed [3]. This shift was absent in the presence of 250 µM DIDS. If 20 mM trimethylamine was introduced in partial replacement of Na⁺, there was a rapid alkaline shift followed by a recovery towards the original pH (rate $0.042 \pm 0.006 \text{ mM s}^{-1}$, $n = 3$). This recovery was much slower in the absence of HCO₃⁻ ($0.007 \pm 0.001 \text{ mM s}^{-1}$, $n = 3$, $p = 0.02$), in the absence of Cl⁻ but presence of HCO₃⁻ ($0.013 \pm 0.002 \text{ mM s}^{-1}$, $n = 3$, $p = 0.03$) or in the presence of 250 µM DIDS ($0.017 \pm 0.001 \text{ mM s}^{-1}$, $n = 3$, $p < 0.05$). These are the properties expected of an AE-like transporter. Results of real-time PCR showed that AE2 mRNA was expressed at a level comparable to that of NHE1 while AE1 expression was 100 fold lower [3]. Immunocytochemistry in cultured cells using SA6 polyclonal antibody against AE1/AE2 showed a predominantly perinuclear localization reminiscent of Golgi distribution [4]. The presence of the transporter was further supported from the results obtained using western blotting. Immunohistochemistry (SA6 with fluorescent secondary) using frozen rat brain sections revealed prominent fluorescence in brain microvessels after 1% SDS antigen unmasking treatment [5] but not before. AE1 was seen in red blood cells with or without unmasking. It has been shown in other tissues [5] that SA6 detects AE2 only after unmasking. Thus the pattern observed here is evidence for AE2 in the microvessels. These results provide a molecular candidate, AE2, with properties consistent with those required to explain the functional data.

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Polyclonal SA6 was a gift from Dr. Seth Alper, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston.

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PC55

The kinetics of nucleoside uptake across the basolateral side of the sheep choroid plexus epithelium perfused *in situ*

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This study explored the functional activity of nucleoside transporters and kinetics of cellular uptake of nucleosides at the basolateral side (BLS) of the sheep choroids plexus epithelium (CPE) perfused *in situ*. Our previous study has revealed that CPE expresses equilibrative nucleoside transporters (ENT) 1 and 2 and concentrative nucleoside transporter (CNT) 2 at the transcript level (1). In this study we found that the cellular uptake of [³H] adenosine and [³H] uridine across the BLS of the CPE was Na⁺ independent, which suggests that the CNT2, although present in the CPE was not operable at the BLS. This uptake was also insensitive to 1 micromolar nitrobenzylthioinosine (NBTI), which suggests that ENT1, a transporter sensitive to nanomolar NBTI, is also not operable at the BLS of the CPE. However, the uptake of [³H] adenosine was reduced to less than 60% of the uptake in control group when 10 micromolar NBTI was present, which suggest that ENT2, a transporter sensitive to micromolar NBTI mediates the uptake of nucleosides across the BLS of the CPE. Kinetic analysis of uptake revealed that the Michaelis constants K_m for [³H] adenosine, [³H] inosine and [³H] thymidine uptake were 1.733 ± 0.169 , 7.133 ± 1.108 and 1.62 ± 0.146 micromoles, respectively. Sheep plasma and cerebrospinal fluid contained nanomolar adenosine and thymidine and micromolar inosine and nucleobases. Taking in mind these concentrations as well as the estimated K_m s, it appears that the main physiological role of nucleoside transport at the BLS of the CPE is to mediate the transport of nucleoside inosine and nucleobases, which are the waste products of nucleotide metabolism.

Redzic ZB et al (2006) *Cerebrospinal Fluid Res* 29;3:4.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC56

Calcium signalling pathways in the HTB-15 human astrocytoma

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Astrocytes are the most numerous cells in the mammalian brain. Through an intimate relationship with their neuronal neigh-

bours they perform a range of functions including the regulation of synaptic communication. Astrocytes have been implicated in the pathogenesis of numerous diseases including Alzheimer's disease. In cultures of rat astrocytes, the pathogenic A β 1-42 peptide is reported to up-regulate the expression of nicotinic receptors (Xiu et al 2005). To investigate this pathway in cells derived from man we have initiated functional studies of the HTB-15 human astrocytoma. This cell-line is reported to express nicotinic α 7 receptors in a fashion regulated by both cholesterol and the cholesterol lowering drug lovastatin, agents that also modify the processing of the amyloid precursor protein by these cells (Xiu et al 2006).

HTB-15 cells were cultured on glass coverslips in DMEM supplemented with 10% FCS. Cells were loaded with the Ca $^{2+}$ sensitive dye Fura2 by transferring them to a standard HEPES-buffered saline (HBSS) supplemented with 2 μ M Fura2-AM and pluronic F-127 0.02% w/v at 37°C. After 30 mins the cells were washed with, and maintained in, HBSS at room temperature for up to 150 min. before being used in experiments. Ratiometric fluorescence imaging was performed on the stage of an inverted microscope in a constantly perfused low-volume chamber. Ratio images proportional to intracellular Ca $^{2+}$ ([Ca $^{2+}$]_i) were collected every 5 s, using well-established methods. Drugs were applied by addition to the perfusing solution.

Under control conditions a proportion of the HTB-15 cells exhibited large and regular Ca $^{2+}$ oscillations with frequencies between ~0.05 and 0.005 Hz. Removal of extracellular Ca $^{2+}$ caused a rapid drop in basal [Ca $^{2+}$]_i and eliminated oscillations in some, but not all, cells. Re-addition of extracellular Ca $^{2+}$ elicited a rebound [Ca $^{2+}$]_i transient that rose considerably above initial baseline levels. Histamine (50 or 100 μ M) caused a large and reproducible [Ca $^{2+}$]_i mobilization response in >90% of cells. Cell by cell analysis revealed that histamine-induced Ca $^{2+}$ oscillations. Histamine responses were eliminated by the H1 antagonist pyrilamine. Both ATP and ADP produced Ca $^{2+}$ mobilization responses that exhibited profound tachyphalaxis. Muscarinic agonism elicited a pirenzepine-sensitive mobilization response, although this was only seen in around 35 % of cells. Although nicotine (100 μ M) produced clear Ca $^{2+}$ responses these were observed in <15% of cells. These responses were sensitive to 100 nM MLA a selective α 7 antagonist. In addition, a large potentiation of nicotine responses was seen in the presence of the α 7-specific positive allosteric modulator PNU120956 (1 μ M).

Xiu et al (2005) *Neurochem Int.* 47:281-90

Xiu et al. (2006) *Neurochem Int.* 49:459-65

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC57

An investigation of the mechanisms underlying DHPG-LTD in area CA1 of the rat hippocampus

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DHPG-induced LTD (DHPG-LTD) is a chemically-induced, mGluR-dependent, form of synaptic plasticity commonly studied in the hippocampus. Recent data (Hsieh et al. 2006) suggest

the mechanisms engaged by this form of synaptic change may parallel those that contribute to β -amyloid-mediated inhibition of synaptic function. On this basis we have started a new investigation of DHPG-LTD aimed at addressing the involvement of some of the signalling pathways engaged by the pathogenic species present in the Alzheimer's disease brain.

Hippocampal slices were prepared from either 2 or 6 week old male Wistar rats. Extracellular recordings of electrically evoked field EPSPs were made from stratum radiatum of area CA1 of submerged slices. The tissue was constantly perfused with aCSF maintained at 33°C, to which all pharmacological agents were added. In addition standard whole cell patch recordings were made at room temperature from visualised CA1 pyramidal neurones using a Kgluconate-based pipette solution.

In whole-cell current-clamp recordings from CA1 neurones in slices obtained from 2 week old animals the mean resting potential was -67 ± 3 mV. Application of S-DHPG (50 μ M) depolarised the membrane by 13 ± 3 mV ($p < 0.05$). This change in membrane potential was accompanied by a 28% increase in membrane input resistance (control 209 ± 17 M Ω vs. 268 ± 11 M Ω in DHPG $p < 0.05$). Agonist washout resulted in a recovery of the initial membrane potential and resistance.

In extracellular recordings from slices obtained from 6 week old animals 4 minute application of either R,S-DHPG (100 μ M) or S-DHPG (50 μ M) produced a lasting synaptic depression. After 45 minutes of agonist washout this averaged $65 \pm 9.5\%$ ($n=5$, racemate) and $40 \pm 11\%$ ($n=12$, S-isomer). Induction of DHPG-LTD did not require stimulation during agonist application. The DHPG-mediated depression in fEPSP amplitude was accompanied by an increase in paired-pulse facilitation and a change in synaptic latency. The NMDA antagonist D-AP5 (50 μ M) had no effect on the magnitude of DHPG-induced LTD. Similarly the TRPV1 antagonist SB-366791 was without effect. MCPG partially reversed established DHPG-LTD. DHPG-LTD was also observed in slices from 2 week old rats.

β -amyloid mediated inhibition of LTP is reported to involve microglial activation (Wang et al 2004). Consequently we have examined the effect of minocycline (20 μ M), an inhibitor of microglial activation, on DHPG-LTD. This agent produced no consistent effect on baseline transmission. Furthermore, the extent of DHPG-LTD was similar in minocycline-treated slices and interleaved controls ($n=7$ slices per group).

Hsieh et al. (2006) *Neuron* 52:831-43

Wang et al. (2004) *J. Neurosci.* 24:6049-56

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC58

The kinetics of glycine transporter type 1 inhibitors

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Glycine as a cofactor of NMDA receptor has an indispensable role for NMDA receptors in its function. Glycine level is controlled by glycine transporter type 1 (GlyT-1) in the synaptic

clef. GlyT-1 takes place on the membrane of glial cells and controlling the glycine concentration is involved in many CNS functions, as well.

Hypofunction of the NMDA glutamate transmitter system is emerging as a potentially promising hypothesis in the pathology of schizophrenia. The increase in glycine concentration can ameliorate the hypofunction of this system. Glycine concentration elevation in the brain can be achieved experimentally or in the clinics by administration of glycine or by GlyT-1 inhibitors.

Methyl-glycine, NFPS, Org24461 and SSR504735 are the investigated inhibitors in detail.

The GlyT-1 is measured mainly by functional tests, essentially by measuring the amount of tritiated glycine taken up the cells. The uptake is linear with time until the concentration ratio between outside and inside the cells approaches equilibrium. The concentration ratios of glycine and Na⁺/Cl⁻ ions are the driving force of the glycine transport. While the transportable and competitive inhibitors start to inhibit GlyT-1 immediately after their addition, the effect of the non-transportable, non-competitive inhibitors develops slowly, as the compounds bind to the receptor and the binding results in conformation changes, stopping the glycine transport fully or partially.

Our aims are the systematic investigation of some GlyT-1 the inhibitors' kinetics using functional tests. We determined the association and dissociation velocities. The virtual IC₅₀ values depend on the experimental conditions. The slow association and dissociation may have clinical implications.

Harsing LG et al. (2006) *Curr Med Chem* 13, 1017-44

Harsing LG et al. (2003) *Pharmacol Biochem Behav* 74, 811-25

Szabo G et al. (2005) *EWCBR*, p96

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC59

Studies of the expression and localization of novel C-terminal alternatively spliced dystrophin isoforms (delta dystrophins) in the mammalian brain

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Duchenne Muscular Dystrophy (DMD), a muscle degenerative disorder, also affects CNS function and can result in cognitive impairment. In brain full length dystrophin is localized to the post synaptic nerve ending of inhibitory GABA-ergic synapses. Novel alternatively spliced dystrophin isoforms have been identified, these differ from the already identified dystrophin through the substitution of exon 78 by a unique and longer 38 amino acid sequence, named delta exon. We have termed isoforms expressing these isoforms the delta dystrophins. The delta dystrophins have been shown to be expressed in the brain but not in skeletal muscle.

Subcellular fractionation studies show that full length delta dystrophin shows a different pattern of subcellular localization to full length classical dystrophin, Dp427. Dp 427 is highly enriched

in the post synaptic density fraction whereas full length delta dystrophin is most enriched in the microsomal fraction. The two isoforms also show a difference in regional expression levels. Dp427 is abundant in cerebellum and in forebrain PSDs whereas full length dystrophin is expressed relatively weakly in cerebellum compared to forebrain. These observations raised the possibility that delta exon 78 is associated with the alternative trafficking. However, transfection of cultures of dissociated hippocampal neuronal with EGFP-plasmids containing C-terminal fragments of either classical or delta dystrophins shows a similar pattern of localization. The detailed localisation of both classical and delta dystrophins in primary cortical neuronal cultures has been investigated by fluorescence immunocytochemistry using isoform specific antibodies. The results show that delta dystrophins are expressed in a larger number of neurons than the classical dystrophins. Delta dystrophins are localized primarily to the dendritic compartment. Strikingly we found no evidence for the presence of delta dystrophins in dendritic spines or at post synaptic nerve endings. Co-localization studies with GABAergic and glutamatergic markers shows that delta dystrophin is not exclusively associated with GABA-ergic neurons. The present data suggest that classical and delta dystrophins show strikingly different patterns of cellular and subcellular localization in brain and suggests that they are not present within the same dystrophin protein complexes in brain.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC60

The rat dorsomedial hypothalamus activates PVN neurones via a tachykinin pathway

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It has been shown that there is a Substance P (SP) containing neuronal projection from the dorsomedial hypothalamus (DMH) to the paraventricular nucleus (PVN) (Bittencourt *et al.*, 1991), evidence presently suggests that this pathway may be important for integration of the stress response. Previously, however, there have been no electrophysiological studies on the DMH to PVN connection.

To characterise this connection, electrophysiologically, we used cell-attached patch-clamp recording; we measured action current frequency in the PVN upon stimulation of the DMH with glutamate in rat hypothalamic sagittal brain slices. Glutamate (5mM) application to the DMH increased action-current frequency in the PVN in 9/17 neurones by 519±61% (*n*=9) *p*<0.005. Increases in action current frequency in the PVN mediated by glutamate application to the DMH was prevented by the glutamate antagonist kynurenic 200µM acid *p*<0.005 (*n*=7) or synaptic block with a high Mg²⁺/low Ca²⁺ buffer solution *p*>0.05 (*n*=6). Crucially, the selective NK1 antagonist L-703606 (1µM) also inhibited DMH to PVN neurotransmission *p*<0.0005 (*n*=5). This work therefore shows for the first time, an excitatory connection between the DMH and PVN utilising tachykinin NK1

receptors. This pathway may be important for the hypothalamic control of neuroendocrine and/or cardiovascular function.

Bittencourt JC, Benoit R, & Sawchenko PE (1991). *Journal of Chemical Neuroanatomy* **4**, 63-78.

This work was funded by the British Heart Foundation, UK.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC61

Descending inputs to neurones in the central autonomic area of the spinal cord – an immunohistochemical study

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Sympathetic preganglionic neurones (SPNs) are the final common pathway for CNS inputs to influence sympathetic nervous outflow. It is well established that SPN activity is influenced by supraspinal regions. In addition, local interneurons are increasingly being recognised as likely important controllers of SPN behaviour. Recently, the central autonomic area (CAA) was shown to contain GABAergic, presympathetic interneurons (Deuchars et al., 2005). The CAA is innervated by many supraspinal projections (Krukoff et al., 1985). This work aimed to determine if GABAergic neurones in the CAA receive inputs from neurones in supraspinal regions related to sympathetic control. Descending inputs to the spinal cord were identified using immunohistochemistry for 5-hydroxytryptamine (5-HT) or tyrosine hydroxylase (TH). GABAergic neurones in the CAA were visualised using transgenic reporter mice (De Marchis et al., 2004) expressing GFP under control of the promoter for glutamate decarboxylase65 (GAD65), and in rat tissue stained immunohistochemically for GAD67. Animals were anaesthetised with an intraperitoneal injection of Sagatal (60mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Thoracic spinal cord was removed and sectioned at 50µm on a vibratome. Sections were incubated in either sheep anti TH (1:100, Abcam) or rabbit anti-5HT (1:1000, US Biologicals) and mouse anti-GFP (1:1000, Invitrogen) at 4°C for 12-36hrs. Anti-GFP was visualised using a green fluorescing secondary antibody (Alexa⁴⁸⁸-conjugated donkey anti-mouse, Invitrogen) whilst descending inputs with either blue (Coumarin based TSA kit, Perkin Elmer) or red (Cy3-conjugated donkey anti-goat or Alexa⁵⁵⁵-conjugated donkey anti-rabbit, Jackson ImmunoResearch and Invitrogen respectively). Both 5-HT and TH-containing-terminals were highly concentrated in the IML and immunoreactivity extended to the CAA, where terminals were visible in the vicinity of GFP cells. High power examination revealed 5-HT-containing terminals in a dense plexus in the CAA, where some of the terminals were closely apposed to GFP cell bodies and proximal dendrites. Confocal microscopy was used to quantify the fraction of CAA cells receiving close apposition by 5-HT-containing terminals, and the contact density of those cell body appositions. Of 24 cells examined, 23 (95%) received appositions ranging from 0.09 – 2.07 contacts per 100

µm² (1-11 appositions per cell body). Similar analysis is currently underway for TH-containing appositions. Thus we have identified that supraspinal inputs which directly influence SPNs may also influence GABAergic CAA interneurons, providing a possible indirect pathway for affecting sympathetic outflow.

De Marchis S et al. (2004). *Eur J Neurosci* **20**, 1307-17.

Deuchars SA et al. (2005). *J Neurosci* **25**, 1063-1070.

Krukoff TL et al. (1985). *J Comp Neurol* **240**, 103-116.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC62

The role of the planar cell polarity pathway in axonal navigation

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The basic architecture of the cerebral cortex consists of neurons arranged in six layers. This architecture reflects the organisation of projections where pyramidal projection neurons send efferent axons to distant subcortical targets or to other cortical regions. Collaterals of pyramidal cell axons, form stereotyped connections within and between laminae. For instance pyramidal cells in layers 2/3 elaborate axon collaterals in layers 2/3 and 5 but not layers 4 and 6. Similarly, layer 6 cells extend axon collaterals within their layer that ascend and branch in layer 4 but not in layer 5. In addition deep layer neurons of each area send their axons over long distances towards a specific thalamic nucleus. Although it is generally believed that extracellular signals, (attractants or repellents) that act either at a short range or at a long range are detected by the growth cones and are used as guidance cues, little is known about the cellular and molecular mechanisms that lead to the formation of such highly specific connections. Some ideas about the overall nature of those mechanisms can be found in studies of other systems such as the regulation of the pattern of synaptic connections in *Drosophila*. For instance, Dr. L. Zipurski and his colleagues have demonstrated in a series of publications that the axons of the photoreceptors R1-R6 do not select their targets in the optic lobe by recognising a simple "stop" signal that is presented to them. It appears that cross communication between them is also necessary. Screening for genes that might be part of this mechanism they found that a gene called *flamingo* is crucial in regulating target selection of distinct classes of photoreceptors in the developing ommatidia. *Flamingo* encodes for a seven-pass transmembrane cadherin and is part of a signalling pathway that interprets a polarity cue and polarises cells along their planar axis. The acquisition of this lateral polarity is known as Planar Cell Polarity (PCP) or tissue polarity. In vertebrates, the PCP pathway has been shown so far to regulate convergent extension movements (early in embryogenesis) and neural tube closure, as well as the

orientation of stereociliary bundles of sensory hair cells in the inner ear. We are currently investigating if the PCP pathway is similarly involved in the development of cortical axons and their target selection. To do so, we analyze the phenotype of the corticothalamic connections in animals that carry a gene knock out, or in transgenic animals in which specific genes are silenced by a Cre inducible RNAi approach.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC63

How relevant is 8p21 to schizophrenia? A refined association study of a key schizophrenia locus

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Schizophrenia is a multifactorial disease involving the interaction of multiple genes of small effect and their interplay with the environment. It is a complex psychiatric disorder affecting 1% of the population worldwide and is characterised by a range of symptoms. These include delusions and hallucinations (positive symptoms), poverty of thought and affective blunting (negative symptoms) and cognitive deficits.

Current interest in the genes underlying the genetic component of the disorder is intense and a variety of approaches have been attempted, including linkage analysis, study of chromosomal abnormalities, global transcriptome screens and association studies of candidate genes. These have identified numerous schizophrenia-associated loci and genes, several of which have been replicated. Although no causative allele or alleles has been definitively identified, one locus, 8p21, has been repeatedly implicated by linkage analyses and association studies. In order to augment the existing reports, we have performed a case control association study spanning this locus in a large Caucasian population. We have employed polymorphic SNPs in three genes previously shown to be dysfunctional in schizophrenia, NRG1, DPYSL2 and PPP3CC, as well as several genes with functions relevant to the pathophysiology of schizophrenia. To maximise the information obtained from this study we utilised recently published information on recombination and allele frequency within this locus (HapMap)¹. In addition, redundancy of the selected SNPs was minimised by using tagged SNPs within each gene². Association of schizophrenia with individual SNPs, multiple SNPs within a gene and interactions between genes will be analysed.

The economic and social impact of schizophrenia is considerable. Identifying genes involved in predisposition to schizophrenia and their roles in pathophysiology, clinical heterogeneity and responsiveness to antipsychotic medication, therefore remains paramount. We present a further analysis of a strongly replicated locus that harbours intriguing genes with functions consistent with the pathophysiology of schizophrenia.

Altshuler D, Brooks LD, Chakravarti A, Collins FS, Daly MJ, Donnelly P;

International HapMap Consortium. The international HapMap consortium. A

haplotype map of the human genome. *Nature*. 2005 Oct 27;437(7063):1299-320.

Applied Biosystems SNPbrowser software: <http://www.allsnps.com/snpbrowser>

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PC64

Regulation of potassium channel genes in response to neuronal activity

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Epilepsy is the second most common neurological disease and 1 in 20 individuals will suffer an epileptic event sometime during their lifetime. Once a seizure has occurred an individual becomes more susceptible to further seizures. Although it is known that this effect requires changes in gene expression, the exact nature of those changes is not understood. It is likely that changes in expression of genes that regulate neuronal excitability would be important for this response to seizure activity. To investigate how such genes are regulated we have isolated the genes, KCNQ2 and KCNQ3, which encode two potassium channel subunits of the M-channel and function to regulate neuronal excitability. Mutations in either KCNQ2 or KCNQ3, resulting in diminished function in humans are responsible for a form of epilepsy indicating the important role of these subunits. We have used bioinformatic analysis, DNase I sensitivity, gel retardation assays and reporter gene assays to identify functional promoter elements within the human KCNQ2 gene. We show that KCNQ2 and KCNQ3 are regulated by at least some shared transcription factors that respond to seizure activity and that expression levels of these genes in cultured neurons are responsive to neuronal activity.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC65

Apolipoprotein E glycosylation and Aβ pathology in an animal model for Niemann-Pick Type C disease

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Neuronal dysfunction is a common feature and final cause of death in Niemann-Pick Type C (NPC) disease, a rare storage disorder characterized by the accumulation of cholesterol and other

lipids in the endosomal-lysosomal systems. Accumulating evidence indicates that NPC may share some common pathological mechanisms with Alzheimer's disease (AD), and this includes the link between cholesterol and amyloid- β (A β). However, the mechanisms underlying neurodegeneration in NPC is unclear. Apolipoprotein E (apoE) plays pivotal role in cholesterol and lipid metabolism, and its polymorphism was found to modulate the susceptibility to develop AD. Like other cellular proteins, apoE undergoes post-translational modifications including glycosylation. Earlier studies have shown that apoE is differentially sialylated, but the functional significance of this chemical alteration and its association with A β pathology is unclear. In this study, we used an animal model of NPC to examine the glycan profile of apoE and its association with A β deposition and cholesterol metabolism at different stages of disease development.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC66

Varied educational backgrounds of pre-registration Nursing students—implications for teaching

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In recent years there has been a move towards widening access to Nursing education. As a consequence we now have a situation where students come from highly diverse educational backgrounds. Typically Nursing students find Biomedical Science difficult. Previous education or experience in Biology, unsurprisingly, has been shown to significantly influence results (McKee, 2002).

The aim of this study was to investigate the educational background of today's Nursing students and to assess their need for additional support.

An anonymous questionnaire was completed by 38 full-time Adult Nursing Diploma students. The average age of respondents was 25 years, ranging from 19-52 years. Mature students (n=21) were defined as being over 21 years of age on starting their Diploma.

Student Profile: Of the 38 respondents only 8 stated they were not in some form of employment before embarking on their studies. All other respondents thought that their previous work experience had/would aid them in becoming a qualified Nurse. Seven students had previous experience of working as Healthcare Assistants or as Medical Technicians and felt had 'started (their) degree with a very good knowledge of basic Nursing care'. Entry qualifications were varied. Five respondents had degree qualifications ranging from Chemistry to Modern and Contemporary History. One respondent held a Master of Arts (Social Science). Ten students had completed a recognised Access course for Nursing, of these 1 had sat their A levels and 6 had O level

or GCSE qualifications. These individuals were all mature students over the age of 23 years. Eighteen students had A Level qualifications only and 4 entered with Diplomas in Science and Health Studies. 55% of students felt neither A level Biology nor Access courses sufficiently prepared them for studying Biomedical Science. When asked what additional assistance would improve their basic Biomedical knowledge, the students' most popular reply was a foundation course in basic scientific principles, with 29% of respondents preferring this option. More tutorials and an instructional CD/DVD were joint second in popularity with 23% each. Students were asked to rate the difficulty of the Biomedical Science component of the Nursing Diploma. Sixteen students found it very difficult, 20 difficult and 2 just right. Areas where additional help was requested were essay writing skills and examination techniques (45% each).

One of the primary reasons why student Nurses discontinue education programmes is failure to meet course standards (Fullbrook *et al.* 2000). The results of this study demonstrate that Nursing students enter with very varied educational backgrounds, which should be taken into account when offering additional support to these students.

McKee, G. (2002). Why is biological science difficult for first-year nursing students? *Nurse Education Today* **22**(3), 251-257.

Fullbrook, P. *et al.* (2000). 'Fit for Practice: Project 2000' Student Nurses' Views on how well the Curriculum prepares them for Clinical Practice'. *Nurse Education Today* **20**(5), 350-357.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC67

Study of the teaching of biodiversity as a general course in a university of Taiwan

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The development of biodiversity brings huge impact to recent human society and economical lives. It becomes an important issue in the 21st century. Cooperating with biotechnology, they have become the strategy of national long term development. However, in Taiwan there are not many studies concerning the biodiversity curriculum, and most of the recent studies involved are the teachers of primary and secondary students for subject evaluation. It is important to promote the concept of biodiversity among the non-biological undergraduate students and provide enough information for them to understand the basic principle of biodiversity. It is also a challenge to develop the program for biodiversity course in our university, and necessary to study the conceptual analysis and curriculum development in biodiversity course for the undergraduate students. In this study, biodiversity conceptual analysis for the reference of curriculum development and the tool of course evaluation in our university were carried out in order to putting the course into practice, designing the teaching course and preparation of teaching strategy. Six different international and Taiwan domestic issues

of biodiversity were used for the test of 280 undergraduate students after 16 weeks of general biodiversity teaching. Statistical results were integrated into three different conceptual levels, practical, scientific and cultural level respectively. Results between the preference of biodiversity and the three different levels are shown in Table 1. These results indicate that undergraduate students in National Cheng Kung University are more preferable to biodiversity view in practical and cultural issues but not in scientific issues. All these results suggest that in spite of our university being a scientific and technological base institute, students are changing their biodiversity views in many general subjects after suitable teaching of biodiversity, and further study is necessary for the study of biodiversity related to scientific issues. Table 1: Preference of biodiversity related to three different levels

	Not preferable to biodiversity view	Preferable to biodiversity view
Practical level	35%	49%
Scientific level	51%	39%
Cultural level	24%	53%

This work was supported by National Science Council, Taiwan, Republic of China (Grant NSC-94-2511-S-006-003).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC68

A new, low-cost approach to teaching integrative techniques to undergraduate students

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The use of integrative techniques has seriously declined over the last 25 years (Alabaster et al., 2002). Financial constraints and the decreasing number of staff with the relevant experience means that few UK universities offer undergraduates this vital education and those that do are heavily dependent on external funding (Collis 2006; Leggett 2005). Thus, our aim was to develop a new, low cost approach to providing training for undergraduates in this area.

Whilst a module which provided training in specific techniques could have been developed, the diverse background of our students, coupled with staff and financial constraints would severely limit what the students would learn. Instead, the focus was on education rather than training, the objectives being to introduce students to the potential of *in-vivo* studies; to educate them in the diverse range of techniques and experimental disciplines where they are utilised; increase awareness of the issues surrounding their use and to provide students with "hands-on" practical experience. Given HO policies on personal licences, students do not attain costly HO licences that they may not subsequently utilise. Instead, students receive lectures based on HO modules 1-3 followed by lectures covering integrative techniques utilised in specific fields. Given current public perception of such studies, students are required to debate the ethics of animal use in scientific research, ensuring they will be better able

to justify such work should the need arise in the future (IDEA CETL). Experimentally, students receive training in animal handling and undertake a series of behavioural studies to investigate anxiety responses in rodents, participating via delegated authority. These animals are reused as cadavers to provide training in injection techniques, cannulations and surgical procedures culminating in setting up of a complex neuroscience/gastrointestinal preparation; this provides significant ethical and cost benefits. In a subsequent session, they observe the setting up of this complex preparation in an anaesthetised animal, participating again via delegated authority. They also visit our Industrial collaborator, Covance, observing a diverse range of integrative studies, particularly in larger species. Finally, they have to integrate all they have learnt into an end of module essay in which they discuss the role of integrative techniques in modern biomedical research.

Feedback on this module is excellent. More importantly, significant numbers of students go on to undertake *in-vivo* PhD studentships or utilise such techniques in industry

Alabaster V et al. (2002) Trends Pharmacol. Sci. 23: 13–18

Collis MG (2006). Nat Rev Drug Discov. 5: 377–9.

Interdisciplinary Ethics Applied Centre of Excellence in Teaching & Learning <http://www.idea.leeds.ac.uk>

Leggett M (2005) Physiol. News 60: 39–40.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC69

Opportunities for, and obstacles to, the development of bioscience practicals in the A-level curriculum

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It is generally accepted that practical-based teaching forms an important part of the A and AS-level curriculum in science-based subjects. Changes to the curriculum will give teachers greater opportunities to develop their own content and resources. We explored educators' attitudes to the practical components of biology, human biology and physical education in the A and AS level curriculum to identify opportunities for, and barriers to, the development of resources to support this teaching.

Educators, registered with the Science Learning Centre, West Midlands (SLCWM) were invited to complete an online questionnaire. Separately, 15 educators were invited to the SLCWM to participate in structured discussions facilitated by the authors. Respondents (n=29) to the questionnaire rated their agreement with specific statements (Strongly Agree: SA; Agree: A; Unsure: U; Disagree: D; Strongly Disagree: SD). Practical activities were universally considered to be an essential part of student learning (SA: 90%, A: 10%); respondents considered these activities to be valued both by students (SA: 48%, A: 45%) and by universities and employers (SA: 48%; A: 41%). Respondents also

indicated a desire to include more (SA: 62%, A: 31%) or new/revised (SA: 76%, A: 21%) practicals in their courses. Whilst the majority felt they had adequate resources (SA: 14%, A: 55%) to deliver existing practical activities, a significant minority disagreed (D: 21%; SD: 3%); a majority of respondents were either unsure (21%) or disagreed with the statement that they had sufficient resources to develop new or revised activities (D: 41%; SD: 3%). A large majority (SA: 34%, A: 48%) wished training and support to be provided through Science Learning Centers. There was unanimous support (SA: 90%, A: 10%) for practical teaching resources to be available online. Statements from participants in the workshop were consistent with, and substantiated the responses to, the questionnaire. Significant barriers to developing and introducing new, or reintroducing old, practical activities were identified including a lack of knowledge and experience, limited resources, perceived problems with health and safety and an uncertainty that exam boards would endorse particular activities.

In conclusion, our surveyed teachers universally regard practical activities as essential to A and AS level bioscience subjects and wish to develop such resources further. The consistency of the responses expressed suggests that these views may be generalized to the wider bioscience teaching community. The barriers to developing and delivering such new activities must be addressed.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC70

A novel approach to teaching behavioural pharmacological techniques to undergraduate students

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Financial, logistical and ethical factors all currently place a great strain on the provision of suitable training in integrative techniques to undergraduate students. In particular training and experience of *in vivo* drug screening techniques such as functional assays to evaluate the efficacy of potential compounds are particularly difficult to provide at an undergraduate level. Exposure to integrative techniques is very important and should be extended and supported whenever possible, however conventional animal models used in such relevant areas like behavioural pharmacology research (typically involving rodents) require considerable investment of time and resources and for large numbers of undergraduates, it is far from ideal from both an animal welfare and financial perspective. To overcome this, there are alternative models available which may be utilised, replicating drug screening in mammals, but based on research on invertebrates or lower order vertebrates e.g. Zebra Fish (Gerlai, 2003). The use of lower order vertebrates however only refines animal usage and is still costly and logistically hard to manage e.g. Home

Office licence requirements, thus it is only the invertebrate models that offer a far more cost effective, easier to manage and possibly more ethical alternative; the latter also representing a significant contribution to replacement, refinement and reduction. In addition, the small size, short life cycle, and ease of experimental manipulation of invertebrate species provide a rapid, cost-effective means for teaching many of the standard drug screening techniques used in behavioural pharmacology.

The justification for such an approach comes from early work utilising invertebrates in drug discovery centred on neuroethology (Hou et al. 2004). Stereotyped behavioural responses in invertebrates, particularly insects, have been successfully exploited to examine the effects and efficacy of drugs across a variety of different therapeutic areas (Manev & Dimitrijevic, 2005). One such potential non-mammalian model is the blowfly, *Calliphora vomitoria*. In preliminary studies, we have shown that their larvae actively seek dark locations if placed in natural light conditions; a condition in rodents ubiquitously applied for screening for anxiolytic drugs (Brotz & Borst, 1996). Groups of students were easily able to replicate those preliminary light/dark responses in the larvae and further extend the study to show similar responses to anxiolytic compounds as studies utilising rodents. Thus, by mimicking rodent anxiety-like responses, this assay using the blowfly larvae, *C. vomitoria*, has both the potential use as a novel, low cost method of teaching undergraduate students integrative techniques and possibly offers an alternative functional and toxicological screen for novel psychotropic agents.

Gerlai, R. (2003) Behavior Genetics 33, 461-68

Hou J et al (2004) J Neurobiol. 60, 249-261

Manev, H. & Dimitrijevic, N. (2005) Life Sci. 76, 2403-07

Brotz TM & Borst A (1996) J Neurophysiol 76, 1786-1799

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC71

Making the best of laboratory time: Using Perception to deliver pre-laboratory quizzes

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Laboratory classes are an important component of most programmes in the Faculty of Medical and Veterinary Sciences in Bristol. Student learning is broad and includes exposure to the issues of experimental design and data gathering as well as an opportunity to develop practical laboratory skills. Laboratory classes tend to develop in scope and complexity as students progress from year to year and culminate for many with the final year laboratory research project. Most departments value the practical dimension to their programmes very highly.

Within UK HE, there are significant pressures in terms of funding, student numbers and the apparent preparedness of first year students in terms of experience of practical work (at secondary

level) and their confidence with relevant (GCSE) mathematics. Many life science degree programmes have reduced the number of contact hours spent in laboratory classes (Hughes, 2001). Compounding this, a common consequence of the increase in student numbers has been the gradual reduction in the scope and arguably, the quality of written feedback on student practical reports. It is common that students are entering university with few laboratory skills, experiencing reduced opportunity to build on these skills and receiving less feedback to guide their laboratory-based learning. It is hard to escape the conclusion that many students derive less satisfaction and less value from laboratory classes than students in the 1980's and 90's.

To improve student preparation before the laboratory class and their engagement during the practical work, we have developed a series of quizzes that we intend to require all first year physiology students to complete to a satisfactory level BEFORE each laboratory practical class. These are being piloted this academic session and will be mandatory in the 2006_7 academic session. The quizzes are presently hosted by the web-application, Perception (see <http://www.qmp.ac.uk>).

These quizzes focus on four broad areas:

1. Health and Safety – posing questions that are answered in the text of the laboratory handbook.
2. Basic numeracy and literacy in the context of the practical work
3. Experimental design and data acquisition
4. Data handling, plotting, description, testing and contextual comprehension

At present the quizzes are popular, although we have not had opportunity test the hypothesis (by cohort analysis) that student learning is improved by satisfactory completion of the pre-laboratory tests.

Hughes IE.. Do computer simulations of laboratory practicals meet learning needs? (2001). Trends Pharmacol Sci. (2): 71-4.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC72

Effect of acute tensile loading on gender-specific tendon mechanical properties

K.E. Burgess, P. Graham-Smith and S.J. Pearson

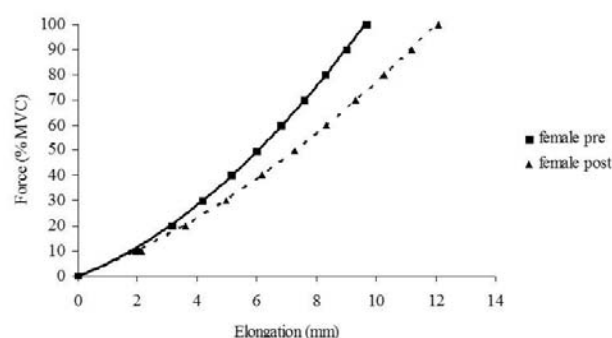
University of Salford, Manchester, UK

Gastrocnemius tendon stiffness in males has been shown to decrease significantly following tensile loading (Kubo et al. 2001). This decrease in tendon stiffness has been proposed as a mechanism for diminished performance following stretch (Cornwell et al. 2002), and may also lead to increased injury risk by increasing the strain experienced by the tendon at any given load (Reeves, 2006). No previous study has examined the relationship between tensile loading and subsequent mechanical changes in female tendon. Therefore, the aim of the present study was to examine and compare the effect of acute tensile loading on the mechanical properties of both female and male gastrocnemius tendon.

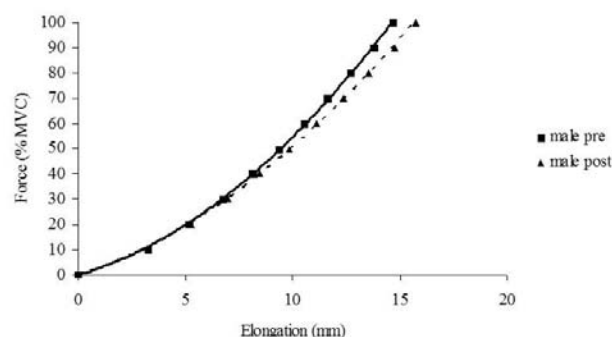
Twenty one subjects (Female n = 8; Male n = 13) performed a five minute passive dorsiflexion stretch. Prior to and post tensile loading medial gastrocnemius tendon stiffness (k), length (l) and cross-sectional area (csa) was measured using ultrasonography and dynamometry. The investigation was approved by the institutional ethics committee and conformed with the principles of the World Medical Association's Declaration of Helsinki. Student's t tests were used to analyse data, alpha values were set to $p < 0.05$.

Stiffness was significantly reduced with tensile loading. Females showed significantly ($p < 0.05$) greater pre to post tensile loading decreases in k (24.4% vs. 11.8%) in comparison to males (see figures 1 and 2). Prior to tensile loading k was significantly less for females than males ($p < 0.05$). Percentage change in maximal strain was significantly greater in females (Female 30.4 vs. Male 10.0%) ($p < 0.05$). There were no significant changes in l or csa for either gender following tensile loading.

The present results show that acute tensile loading reduces stiffness of the gastrocnemius tendon in both females and males, with females showing significantly greater change. The observed disparity between genders may be due in part to variations in initial stiffness and intrinsic differences in tendon composition. These differential changes in tendon mechanical properties have functional and motor control implications.



Female force elongation pre and post stretch. Decreased gradient post stretching illustrates reduction in k



Male force elongation pre and post stretch.

Cornwell A et al. (2002). Eur. J. Appl. Physiol. 86(5): 428-434.

Kubo K et al. (2001). J. Appl. Physiol. 90(2): 520-527.

Reeves ND. (2006). J. Musculoskelet Neuronal Interact. 6(2):174-80.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC73

Skeletal muscle IGF-IEa gene expression in healthy women increases after resistance exercise but is not influenced by age

C.A. Greig, V. Mann, A. Young and B. Noble

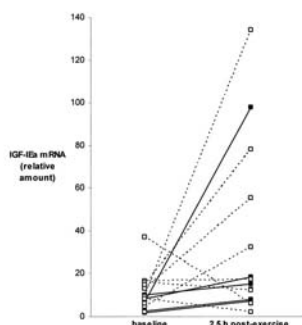
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The locally expressed isoform of the Insulin-like Growth Factor-I (IGF-I) gene has been implicated in the response of skeletal muscle to high intensity resistance exercise. The interpretation of recent reports on the influence of age, of which we know little, is problematic due to the utilisation of different exercise protocols and sampling times (1,2,3). Using an isometric exercise protocol, we have previously shown that the upregulation of this growth factor (IGF-IEa) occurs in younger women (4). Here we aimed to apply our protocol in a study comparing the responses of groups of young and old women in their eighth decade and older.

Sixteen healthy women (n=6 old, median age 81 y, range 76-82 y; n=10 young, median age 26 y, range 21-30 y) undertook 120 maximal voluntary isometric contractions of the knee extensors of one leg as 20 sets of 6 contractions over 90 minutes. Muscle samples were obtained from the lateral mass of the quadriceps at baseline and at 2.5h post-exercise using the needle biopsy technique. Quantification of mRNA for IGF-IEa was by real-time reverse-transcriptase polymerase chain reaction (qRT-PCR). Statistical analysis was by repeated measures ANOVA.

Individual responses of IGF-IEa mRNA to exercise are shown in Fig1. Exercise elevated (geometric) mean (95% CI) mRNA values (relative amounts) for the IGF-IEa transcript in both the young (from 9.91 (5.64 to 17.4) to 21.7 (6.73 to 69.9)) and old (from 4.77 (1.84 to 12.4) to 16.5 (6.13 to 44.3)) women and this was statistically significant when the groups were analysed together (ANOVA $F=5.24$, $p=0.043$). Although the magnitude of the IGF-IEa mRNA response was greater in the old women, (3.5-fold vs 2.2-fold), the effect of age was not statistically significant (ANOVA $F=1.98$, $p=0.19$).

This is the first study to report muscle specific IGF-IEa gene expression after voluntary isometric resistance exercise in young and old women. The IGF-IEa response to exercise appears to be maintained in old age. Furthermore our results suggest that it is not age per se which drives the variability of the growth factor response to resistance exercise.



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Kim J-s, Kosek DJ, Petrella JK, Cross JM, Bamman MM. 2005. *J Appl Physiol* 99: 2149-2158.

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Greig CA, Hameed M, Young A, Goldspink G, Noble B 2006. *Growth Horm. IGF Res.* 16: 373-376.

We are grateful to Research into Ageing for funding this work, to our participants, to Dr Susan Lewis for statistical assistance and the staff of the Clinical Research Facility, Royal Infirmary Edinburgh.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC74

Recovery of bone loss after 90 days of bed rest in humansJ. Rittweger¹ and D. Felsenberg²

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Bone loss as a result of immobilisation is well established. The available literature suggests that recovery of bone losses either after clinical immobilisation (LeBlanc & Schneider, 1991) or space flight (Vico et al., 2000) is rather incomplete. For the first time, we ventured to find out whether bone losses acquired during bed rest can recover.

Twenty-five male healthy participants (23 to 41 years old) underwent -6 deg head down tilt bed rest for 90 days. Bone mineral content (BMC) of the tibia was measured during bed rest and during 360 days follow up by peripheral Quantitative Computed Tomography (XCT2000, Stratec, Germany).

On the 89th day of bed rest, changes in BMC ranged between -15.6% and +0.3% (mean -3.5%, SD 3.6%) at the tibia epiphysis and between -3.5% and +0.7% (mean -0.8%, SD 1.1%) at the diaphysis. After 360 days of recovery, the diaphysis had fully recovered. At the epiphysis, there was still a loss by 0.6% in BMC (SD 1.3%, $p=0.03$). These residual losses were independent of the participants' age and of initial losses at the end of BR ($p>0.45$). However, a repeated measures ANOVA yielded gains by 0.7% (SD 0.9%) in BMC between 180 and 360 days of recovery ($p<0.001$), suggesting that recovery was still ongoing after 360 days. Comparing the individual losses in BMC at the end of bed rest with the gains during 360 follow-up by regression analysis, close correlations were found, with R^2 -values of 0.88 for both tibia sites. These results show for the first time that bed rest induced bone losses are (1) fully recovered within one year at the diaphysis and (2) largely recovered (>80%) in the epiphysis, where recovery seemed to be still ongoing after one year. Moreover, those individuals who lost larger amounts during bed rest depicted larger gains in the recovery period, implying that more or less the same amounts were recovered that had previously been lost. In an extreme case, 17.1% in epiphyseal BMC were gained during recovery, demonstrating the adult skeleton's ability to accrue bone mass at a rate exceeding that during the pubertal growth spurt. On the other hand, the largest individual change from baseline amounted to 1.9%.

These findings clearly demonstrate that the adult skeleton retains a considerable capability of gaining bone mass, which is in contrast to the inefficiency of exercise to increase bone mass normally seen in adults. On the other hand, we found the 'peak bone mass' to be tightly regulated in our study. We would therefore argue that bone strength, including its age-related changes, are regulated by factors outside the bone.

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The Long Term Bed Rest Study was organized by ESA and NASDA. Our participation in the study was supported by DLR (50 WB 0156).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC75

Molecular mechanism responsible for dietary fat mediated inhibition of PDC activation in human skeletal muscle, which is not reversed by acute contraction

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Increasing lipid availability and oxidation suppresses skeletal muscle CHO oxidation by inhibiting pyruvate dehydrogenase complex (PDC) activity; via enhanced expression of pyruvate dehydrogenase kinase (PDK) isoform 4 protein (1, 2), that phosphorylates (inactivates) PDC. Signalling involving the peroxisome-proliferator-activated receptor (PPAR α , δ , γ) and the subfamily of forkhead transcription factors (FOXO) is suggested to increase PDK4 protein (1, 3). We aimed to determine (i) the relevance of this signalling in vivo in humans by using high fat (HF) feeding to reduce muscle PDC activation, and (ii) whether calcium mediated activation of PDC (via PDC phosphatase) during exercise reversed any dietary mediated inhibition of PDC, and impacted on the above signalling pathways.

On 2 randomised occasions, 6 healthy men (22.0 ± 0.6 yr, 24.9 ± 1.4 kg/m²) consumed either a "normal" (55% CHO, 30% fat, 15% protein; CON) or a HF (10% CHO, 75% fat, 15% protein) isoennergetic diet for 3 days. A resting quadriceps biopsy sample was obtained the morning after (fasted), and subjects then cycling at 75% VO₂max for 60 min. Further biopsies were taken after 10 and 60 min exercise. Subjects repeated the same procedures 7 days later, but consumed the alternative diet. Muscle was used to determine PPARs (α , δ , γ), PDK4, FOXO1 mRNAs and PDC activation status (Table 1). Statistical differences were located using repeated measures ANOVA. All values are mean $\pm$ SE.

HF feeding increased resting PDK4 mRNA 3-fold, and is likely to have resulted from increases in PPAR α and γ (1.5-fold) and FOXO1 (3-fold) transcription. Exercise activated PDC in CON and HF, but the magnitude of increase was blunted in HF, and may have been caused by PDK4 and FOXO1 mRNA expression being sustained throughout exercise.

Table 1. Muscle PDK4, PPARs (α , δ , γ), FOXO1 mRNAs and PDC activation at rest (0) and after 10 and 60 min of exercise following 3 days normal and HF dietary intake.

Time	PDK4	PPAR α	PPAR δ	PPAR γ	FOXO1	*PDC
CON 0	1	1	1	1	1	0.60 $\pm$ 0.05
CON 10	1.57 $\pm$ 0.32 ^{***}	1.07 $\pm$ 0.12	1.01 $\pm$ 0.11	1.04 $\pm$ 0.11	1.16 $\pm$ 0.19	1.81 $\pm$ 0.09 ^{***}
CON 60	1.67 $\pm$ 0.16 ^{***}	1.18 $\pm$ 0.15	1.15 $\pm$ 0.18	1.38 $\pm$ 0.32	1.44 $\pm$ 0.35	2.03 $\pm$ 0.09 ^{***}
DCA 0	3.03 $\pm$ 0.68 ^{***†}	1.60 $\pm$ 0.13 ^{††}	1.14 $\pm$ 0.14	1.59 $\pm$ 0.27 [†]	2.66 $\pm$ 0.51 ^{††}	0.50 $\pm$ 0.06
DCA 10	2.97 $\pm$ 0.59 ^{***†}	1.12 $\pm$ 0.22	1.23 $\pm$ 0.04	1.28 $\pm$ 0.17	2.88 $\pm$ 0.51 ^{††}	1.56 $\pm$ 0.09 ^{***†}
DCA 60	2.59 $\pm$ 0.25 ^{***†}	1.27 $\pm$ 0.16	1.07 $\pm$ 0.10	1.25 $\pm$ 0.10	2.14 $\pm$ 0.08 ^{††}	1.58 $\pm$ 0.05 ^{***††}

*mmol/min/kg wet muscle at 37°C. **Different from rest (P<0.01). ***Different from corresponding CON value (P<0.05 and P<0.01, respectively).

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Furuyama T et al. (2003) *Biochem J* 375, 363-371.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC76

Pyruvate dehydrogenase complex activation does not limit mitochondrial ATP production in human skeletal muscle during low intensity exercise

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School of Biomedical Sciences, University of Nottingham, Nottingham, UK

Muscle phosphocreatine (PCr) hydrolysis and lactate accumulation at the onset of intense exercise is partly attributable to inertia in mitochondrial ATP production due to a lag in pyruvate dehydrogenase complex (PDC) activation and flux (the acetyl group deficit; 1). Accordingly, pharmacological activation of the PDC, using dichloroacetate (DCA), results in a stockpiling of acetyl groups in resting muscle and overcomes inertia in mitochondrial ATP production during subsequent intense exercise. During low intensity exercise however the magnitude of metabolic inertia will be less, as the rate of ATP demand will be lower, and will be better matched by the rate of PDC activation and flux at the onset of exercise. One aim of the present study was to investigate whether metabolic inertia at the level of PDC was discernable at the onset of exercise at 60% VO₂max in human skeletal muscle. Secondly, to determine whether the reported effect of DCA on PDC activation persists over more than one bout of exercise.

Nine healthy men (age 27 ± 2 y, body mass 77.4 ± 3.6 kg, VO₂max 43.0 ± 1.9 ml/min/kg) volunteered for a study. On two randomised occasions, separated by 14 days, subjects underwent a 1 h i.v. infusion of DCA (50 mg/kg/h) or 0.9% saline (CON). Thereafter, subjects performed two 6 min bouts of cycling exercise at 60% VO₂max, separated by 15 min rest. Muscle biopsies were obtained from the vastus lateralis immediately before and after each bout of exercise and analysed for glycogen, lactate, PCr, and acetylcarnitine content and muscle PDCA. Statistical analysis was performed using repeated-measures two-way ANOVA and data are expressed as means $\pm$ SEM. DCA increased resting muscle PDCA 4-fold, and was associated with a 5.5-fold increase in muscle acetylcarnitine content, compared to CON (Table 1). Following 15 min of resting recovery,

PDCa had returned to basal in CON, but remained elevated in DCA. However, there was no difference in muscle lactate, PCr, or glycogen content between CON and DCA before or after either exercise bout (Table 1).

In conclusion, although PDC was activated at rest, DCA had no effect on PCr hydrolysis or lactate accumulation during exercise, supporting our suggestion that the magnitude of metabolic inertia at the level of PDC is minimal at the onset of exercise at 60% VO₂max. Also, the effects of DCA on PDCa can persist during recovery between two bouts of exercise.

Table 1. Muscle acetylcamitine, PCr, lactate, and glycogen content (mmol/kg dry muscle) and muscle PDCa (mmol acetyl-CoA/min/kg wet muscle) before and after two 6 min bouts of exercise at 60% VO₂max following i.v. infusion of DCA or 0.9% saline (CON).

	Exercise bout 1				Exercise bout 2			
	CON		DCA		CON		DCA	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
PDCa	0.5 ± 0.1	1.5 ± 0.1	1.9 ± 0.3**	2.1 ± 0.3**	0.9 ± 0.1	1.7 ± 0.3	2.0 ± 0.1**	1.9 ± 0.1**
Acetylcam	1.5 ± 0.4	9.5 ± 1.2	8.3 ± 0.8**	11.0 ± 1.1**	4.6 ± 0.8	10.8 ± 1.2	6.7 ± 1.0**	8.9 ± 1.1**
PCr	71.0 ± 2.1	63.0 ± 4.0	74.4 ± 3.1	57.0 ± 4.0	71.5 ± 4.1	65.6 ± 3.0	73.3 ± 7.0	63.7 ± 4.6
Lactate	5.3 ± 0.9	35.7 ± 6.7	3.7 ± 0.8	33.8 ± 7.2	11.7 ± 2.0	31.9 ± 5.7	12.0 ± 3.0	30.0 ± 6.9
Glycogen	321 ± 33	266 ± 35	320 ± 40	222 ± 30	273 ± 33	185 ± 33	216 ± 22	197 ± 22

** P < 0.01, significantly different from CON pre exercise bout 1 (Treatment effect). Time effect not presented.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC77

Exercise, pulse wave velocity and postprandial oxidative stress in healthy men

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Oxidative stress is postulated to be responsible for the postprandial impairments in vascular function. The purpose of this study was to measure Pulse Wave Velocity (PWV) and markers of postprandial oxidative stress before and after an acute bout of moderate exercise. Ten trained male subjects (age 21.5 ± 2.5 yrs, VO₂ max 58.5 ± 7.1 ml.kg⁻¹.min⁻¹) participated in a randomised crossover design: (1) high-fat meal alone (2) high-fat meal followed 2 h later by a bout of 1 h moderate (60% max HR) exercise. PWV was examined at baseline, 1 h, 2 h, 3 h, and 4 h postprandially. Blood Lipid hydroperoxides (LOOHs), Superoxide dismutase (SOD) and other biochemical markers were measured. PWV increased at 1 h (6.49 ± 2.1 m/sec), 2 h (6.94 ± 2.4 m/sec), 3 h (7.25 ± 2.1 m/sec) and 4 h (7.41 ± 2.5 m/sec) respectively, in the control trial (P < 0.05). There was no change in PWV at 3 h (5.36 ± 1.1 m/sec) or 4 h (5.95 ± 2.3 m/sec) post ingestion

in the exercise trial (P > 0.05). LOOH levels decreased at 3 h post ingestion in the exercise trial compared to levels at 3 h (P < 0.05) in the control trial. SOD levels were lower at 3 h post ingestion in the control trial compared to 3 h in the exercise trial (0.52 units/μl ± 0.05 vs. 0.41 units/μl ± 0.1; P < 0.05). These findings suggest that a single session of aerobic exercise can ameliorate the postprandial impairments in arterial function by possibly reducing oxidative stress levels.

Comparison of PWV following ingestion of high-fat meal

Time interval	Mean PWV (m/sec)	
	Control	Exercise
Baseline	5.2 ± 0.94	4.84 ± 0.6
One hour post ingestion	6.49 ± 2.1*	6.99 ± 2.4†
Two hours post ingestion	6.94 ± 2.4*	7.16 ± 1.2†
Three hours post ingestion	7.25 ± 2.1*	5.36 ± 1.1*
Four hours post ingestion	7.41 ± 2.5*	5.95 ± 2.3*

* P = < 0.05 vs. baseline control trial

P = < 0.05 vs. baseline exercise trial

≠ P = < 0.05 vs. two hours post ingestion exercise trial

Age and physiological characteristics (n = 10 subjects)

Dependent Variable	Mean ± SD
Age (years)	21.5 ± 2.5
Height (cm)	180.3 ± 5.6
Body Mass (kg)	77.0 ± 8.4
BMI (kg/m ²)	23.6 ± 1.6

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Kinlay S et al.(2001) *Hypertension* 38,1049-1053

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC78

Suppression of the neurodegeneration PINK1 locus in Type 2 diabetes

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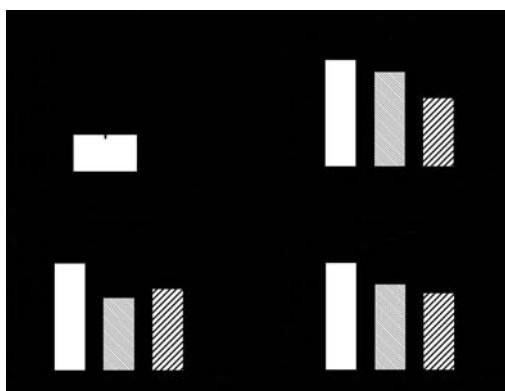
¹SLS, Heriot Watt University, Edinburgh, UK, ²Centre of Inflammation and Metabolism, Rigshospitalet University Hospital, University of Copenhagen, Copenhagen, Denmark, ³The Wenner-Gren Institute, The Arrhenius Laboratories, Stockholm University, Stockholm, Sweden and ⁴Centre for Genomics and Bioinformatics, Karolinska Institutet, Stockholm, Sweden

Mutations in PINK1 have been linked to Parkinson's disease and Drosophila pink1 mutants obtained severe mitochondrial defects. In this study we profiled the gene expression from the PINK1 locus in metabolic syndrome. The PINK1 locus includes PINK1, DDOST (encoding the AGE-R1 receptor) and naPINK1 (a cis-encoded natural antisense to PINK1). We utilized quantitative real-time PCR to examine the gene expression in skeletal muscle and adipose tissue from a human cohort consisting of four groups matched for age, gender and smoking status: non-obese (n=12) or obese (n=14), normal

glucose tolerant (n=13) or type 2 diabetes subjects (n=14). Considering the confounding effect that exercise-inducible genes may have in metabolic disease, we included two control studies, measuring the PINK1 locus genes before and after inactivity or activity in healthy volunteers. In parallel, we examined the expression of PGC-1 α being an established diabetes related gene.

We demonstrate that following 5 weeks of inactivity (n=6, healthy volunteers), PINK1 was decreased in skeletal muscle while naPINK1 was increased. Consistently, the opposite regulation pattern was observed following 6 weeks of activity. The expression of DDOST remained unaltered in both studies. In contrast, all PINK1 locus genes were down-regulated in skeletal muscle of type 2 diabetes. Importantly, skeletal muscle PINK1 expression correlated with diabetes status (fasting glucose and HbA1c) and siRNA knockdown of PINK1 did impair basal glucose metabolism. Surprisingly, the gene expression of the diabetes candidate gene, PGC-1 α , was neither consistently reduced nor related to measures of metabolic syndromes. In adipose tissue, PINK1 expression co-varied with mtDNA encoded mRNA suggesting the operation of the Pink1-Parkin-OXPHOS pathway found previously in *Drosophila*.

In conclusion, PINK1 expression is regulated by physical activity in muscle, while expression of the whole genomic locus is uniquely down regulated in metabolic disease. Whether down-regulation of PINK1 is part of a type 2 diabetes causative pathway or is a secondary effect of hyperglycemia remains to be investigated.



Swedish Diabetes Association, Leverhulme Foundation and Heriot Watt University

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC79

Effects of moderate exercise with and without energy deficit on postprandial metabolism

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Moderate exercise attenuates postprandial lipaemia and insulinaemia, and increases fat oxidation for at least 24 h post-exercise. The magnitude of this effect is related to exercise energy expenditure, but it is unclear whether these changes are the consequence of exercise per se or the associated energy deficit. This study aimed to 1) determine the effects of exercise, with and without energy deficit, on postprandial metabolism, and 2) determine whether exercise-induced changes in postprandial triglyceride (TG) responses were related to changes in whole-body and hepatic fat oxidation. Thirteen men (age 40 $\pm$ 8 y, BMI 31.1 $\pm$ 3.0 kg/m²) [mean $\pm$ SD] completed three 2-day trials in random order. On Day 1: subjects rested (C), walked briskly to expend 27 kJ/kg body mass (E), or completed the same walk with all energy expended replaced (ER). On Day 2: subjects arrived after a 12 h fast and rested for 8.5 h. During this time breakfast and lunch were provided (each with 97 g carbohydrate, 33 g fat, 28 g protein, 3.3 MJ energy). Regular blood samples were collected for measurement of TG, insulin, and 3-hydroxybutyrate (3-OHB; a marker of hepatic fatty acid oxidation). Indirect calorimetry with a ventilated hood was used to determine whole-body substrate oxidation. For 3 d preceding Day 2, subjects avoided alcohol, planned exercise (other than test walks) and had all food provided by the experimenter. Time-averaged postprandial TG concentrations were C: 2.0 $\pm$ 0.3 mmol/l, E: 1.7 $\pm$ 0.3 mmol/l and ER: 1.9 $\pm$ 0.2 mmol/l (p <0.02; ANOVA, for C vs E, E vs ER) [mean $\pm$ SEM]. Postprandial insulin concentrations were C: 44.1 $\pm$ 5.0 mU/l, E: 36.0 $\pm$ 3.8 mU/l and ER: 39.6 $\pm$ 4.5 mU/l (p <0.03 for C vs E, C vs ER, E vs ER). Postprandial 3-OHB concentrations were C: 20 $\pm$ 2 μ mol/l, E: 28 $\pm$ 2 μ mol/l and ER: 23 $\pm$ 2 μ mol/l (p <0.05 for C vs E, E vs ER). Postprandial fat oxidation over the 8.5 h was C: 37.0 $\pm$ 2.0 g, E: 44.6 $\pm$ 2.3 g and ER: 40.6 $\pm$ 2.1 g (p <0.05 for C vs E, C vs ER, E vs ER). Significant correlations were observed between the exercise-induced changes in postprandial TG and postprandial 3-OHB concentrations (r =0.48, p =0.01; Pearson Correlation) and the exercise-induced changes in postprandial TG and postprandial fat oxidation (r =0.74, p <0.0005). In conclusion, prior moderate exercise without energy deficit attenuated postprandial insulinaemia and increased fat oxidation although an energy deficit augmented these effects. An energy deficit was needed to elicit an exercise-induced reduction in postprandial TG concentrations. Furthermore, exercise-induced changes in postprandial TG concentrations were related to changes in whole body and hepatic fat oxidation.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC80

Identification of novel mimics of insulin's phosphorylation of the transcription factor FOXO1a and repression of the gluconeogenic gene PEPCK

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*Neurosciences Institute, Dundee University, Dundee, UK***ABSTRACT**

The compound epigallocatechin gallate (EGCG) is one of several that have been reported to have insulin-like glucose-lowering properties in mammals. EGCG acts at least in part by repression of gluconeogenic genes including phosphoenolpyruvate carboxykinase (PEPCK) but the transcription factors that are targeted by EGCG were unclear until recently. We have found that EGCG mimics insulin-induced phosphorylation of the transcription factor FOXO1a. Like insulin, EGCG induced FOXO phosphorylation is abolished by PtdIns 3-kinase inhibition but not by PD98059 (an inhibitor of mitogen-activated protein kinase cascade) or by rapamycin (an inhibitor of signalling to p70 S6 kinase). In contrast to the effects of insulin and IGF-1 however, EGCG-mediated induction of FOXO phosphorylation is sensitive to scavengers of reactive oxygen species (ROS). The mechanism of action of EGCG and other insulin mimics is discussed. Our results suggest that agents that act on the same target(s) as EGCG may be useful antidiabetic agents.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC81

Changes of immunological and endocrine parameters induced by a prolonged dive in two human healthy subjectsL. Bonanni¹, G. Chioetto¹, F.A. Piazza¹, C. Gurrieri¹, G. Bonanni² and C. Agostini¹

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INTRODUCTION: Space flight conditions may influence a variety of physiologic functions of mammals, including muscle, bone, neurovestibular function and the immune response, that is depressed in more than 50% of the astronauts during and after space flights (1). The human studies in microgravity are limited, although some modelling studies have been done. The most common model is chronic bed-rest with a head-down tilt, but many studies have also used water immersion as experimental model that reproduces more closely effects of acute adaptation to simulated weightlessness (2). Stress hormones, specifically growth hormone, prolactin and cortisol, are widely recognized for their ability to alter circulating leukocyte number and function (3). These results suggest that stresses that occur during space flight could combine with microgravity conditions in inducing the changes seen in immune responses after space flight.

OBJECTIVE: The aim of this study was to investigate the effects of microgravity and stress responses on immunity, taking advantage on the prolonged diving of two healthy volunteer that stayed

for 25 hours in a pool at the depths of 2 meters at room temperature, establishing the new world record of prolonged dive. **STUDY POPULATION** Two male healthy volunteers (DC, 22 years old, and MB, 26 years old) continuously stayed for 25 hours in a pool with air respiratory in a hydrostatic equilibrium. The average depths was 2 m. Thirty milliliter of peripheral blood were obtained the day before the prolonged dive and immediately after its conclusion (within 15 minutes).

RESULTS: After the dive with respect to the basal determinations we found in both the subjects: an overall increase in WBC and in particular in neutrophil numbers; a significant increase in plasma cortisol, growth hormone and prolactin; a decrease in number of NK cell (CD56+ cells) as determined by flow cytometry analysis; a significant decrease of the T-cell proliferative activity in presence of polyclonal stimulation; a significant decrease of the intracytoplasmic expression of IFN-gamma and a parallel increase of IL-4 levels, suggesting a Th1 versus Th2 switch. There were no significant differences in the cytotoxic activity of NK cells

CONCLUSIONS: Our subjects were the first in the world continuously diving for 25 hours. Taken together our data show that the prolonged dive alters the levels of stress hormones and impairs immune system, in particular down-modulating T-cell and the NK-cell subsets, explaining the depression of the immunosurveillance observed in subjects that are maintained in microgravity conditions.

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PC82

The effect of prior exercise on pulmonary VO₂ and efficiency during heavy exercise at different contraction frequencies in humans

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The effect of prior exercise on efficiency and VO₂ kinetics is well established at pedal rates between 60-95 revs.min⁻¹ (Burnley et al, 2002; Sahlin et al, 2005). When considering such responses the contraction frequency at which exercise is performed is crucial as this may alter the position of the working muscle on the efficiency-velocity relationship. Indeed, passive increases in muscle temperature (T_m) have different effects on efficiency depending on contraction frequency (Ferguson et al, 2002). The effect of prior exercise has not been studied at distinct pedal rates. The aim of this study was to investigate the effect of prior exercise on mechanical efficiency and VO₂ kinetics at 60 and 120 revs.min⁻¹.

With ethics committee approval, 6 male subjects (age 25 ± 5 yrs, height 1.82 ± 0.09 m, body mass 79 ± 16 kg) performed 6 min

cycling exercise (power output halfway between LT and $\text{VO}_{2\text{peak}}$) at 60 and 120 revs.min^{-1} , under control conditions (C60 and C120) and after prior exercise (P60 and P120). Prior exercise consisted of two 2 min cycling bouts, separated by 2 min rest, with a third bout to exhaustion, performed at 100% $\text{VO}_{2\text{peak}}$ at 90 revs.min^{-1} . T_m was measured prior to the 6 min exercise. $\text{VO}_{2\text{peak}}$ (breath-by-breath) was measured throughout and modelled using non-linear regression. Net mechanical efficiency was calculated from the $\text{VO}_{2\text{peak}}$, RER and measurement of post exercise blood lactate concentration. Statistical analyses were performed using repeated measures ANOVA and Bonferroni corrected paired t-tests where appropriate. Data is presented as means $\pm$ SD.

Prior exercise increased ($P < 0.05$) T_m by 3.4 ± 0.8 °C. The absolute primary amplitude of VO_2 was higher ($P < 0.05$) after prior exercise at both pedal rates, and to a greater extent at 120 revs.min^{-1} (Table 1). The decrease in the VO_2 slow component as a result of prior exercise was greater ($P < 0.05$) at 120 compared to 60 revs.min^{-1} (Table 1). Consequently, prior exercise reduced mechanical efficiency at 60 and 120 revs.min^{-1} (by 2.4 ± 2.3 and 2.3 ± 1.4 %, respectively).

The present data demonstrate that compared to when T_m is passively increased the effect of prior exercise on efficiency is not necessarily dependent on contraction frequency.

Table 1. Oxygen uptake and efficiency response to exercise with and without prior exercise, at 60 and 120 revs.min^{-1} .

Parameter	C60	P60	C120	P120
Primary time constant (s)	22.2 ± 6.2	20.9 ± 5.6	21.2 ± 8.4	28.9 ± 10.5
Absolute primary amplitude (l.min^{-1})	3.12 ± 0.42	$3.43 \pm 0.35^*$	$3.25 \pm 0.31^\dagger$	$3.71 \pm 0.19^{**\dagger}$
Slow component amplitude (l.min^{-1})	0.31 ± 0.19	$0.14 \pm 0.10^*$	0.39 ± 0.23	$0.04 \pm 0.15^{**\dagger}$
Mechanical Efficiency (%)	24.0 ± 2.3	$21.6 \pm 1.8^*$	24.9 ± 1.2	$22.6 \pm 0.9^*$

* denotes a difference ($P < 0.05$) from the control value at the same pedal rate. † denotes a difference ($P < 0.05$) from 60 revs.min^{-1} under the same condition

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PC83

Investigation of changes in oxygenation of human quadriceps femoris during ischaemia and fatiguing muscle contractions

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Near infrared spectroscopy (NIRS) provides a non invasive method of analysing muscle metabolism and response to stress in human muscle, normally under ischaemic conditions (1,2). The aim of this study was to examine the feasibility of using NIRS during muscle fatigue in non-ischaemic state as a basis for future exploration of tissue oxygenation and muscle performance in humans with metabolic problems. A convenient sample of 10

healthy adults (5M, 5F) mean age 36 (SD 7) years with no known metabolic, neurological or musculoskeletal diseases participated in this ethically approved study. Participants were seated in isometric torque chair and NIRS optodes (NIRO-200, Hamamatsu Photonics Deutschland GMBH) were placed over both quadriceps femoris muscles. Responses of the right limb to (A) proximal limb occlusion with a thigh cuff pressure of 100 mmHg greater than systolic pressure for 15 minutes, (B) percutaneous stimulation at 40Hz for 250 ms every second for 3 minutes (modified Burke fatigue protocol) and (C) proximal limb occlusion for 5 minutes applied immediately after muscle fatigue were recorded. Anthropometric details including skinfold thickness were also recorded. Changes in oxyhaemoglobin ($\Delta\text{O}_2\text{Hb}$) and deoxyhaemoglobin (ΔHHb) during the first 3 minutes of each condition was measured. Repeated measures ANOVA was performed to determine possible significant differences between conditions A, B, and C and post-hoc t-tests were conducted as appropriate. The relationship with anthropometric characteristics was examined using Pearson's correlation coefficients. As expected, measures $\Delta\text{O}_2\text{Hb}$ and ΔHHb were related to skin fold thickness ($r = -0.677$ to -0.783 , $p < 0.05$). For conditions A, B and C, absolute values (mean $\pm$ SEM) for $\Delta\text{O}_2\text{Hb}$ were 133 ± 50 μM , 194 ± 67 μM and 214 ± 60 μM respectively and for ΔHHb were 158 ± 40 μM , 260 ± 39 μM and 154 ± 65 μM respectively. Significant differences were observed in $\Delta\text{O}_2\text{Hb}$ (ANOVA: $F_2 = 8.975$, $p < 0.01$) and ΔHHb (ANOVA: $F_2 = 7.448$, $p < 0.05$). Post hoc analysis confirmed that change in $\Delta\text{O}_2\text{Hb}$ levels was significantly less in condition A ($p < 0.05$) whereas change in ΔHHb levels was significantly greater during condition B ($p < 0.05$). Differences in the response of $\Delta\text{O}_2\text{Hb}$ and ΔHHb during ischaemia in a fresh and fatigued state and during fatiguing muscle contraction support the feasibility of the procedure but unresolved issues include the effectiveness of lower limb occlusion and potential localised volume changes in the lower limb distal to the cuff. The negative correlation between skinfold thickness and measures of $\Delta\text{O}_2\text{Hb}$ and ΔHHb suggests that skinfold thickness may account for the large observed variability.

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PC84

Assessment of 3-nitrotyrosine formation in response to moderate intensity exercise in healthy young adults

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Free radicals, or reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive molecules which are naturally occurring by-products of normal cellular oxidation processes. In some circumstances these species may ultimately overwhelm antioxidant defenses causing damage to proteins, lipids and DNA resulting in oxidative stress (1). There are number of disease pathologies where oxidative and nitrative stress have been implicated (2).

Physical activity or exercise can alter the balance of oxidative and anti-oxidative species within the human body. Although it was first assumed that due to an increased oxygen metabolism, exercise would induce an increased release of damaging reactive species, more recent research has indicated that exercise induced radical species are key to adaptive processes (3). However it remains unclear what role exercise intensity and duration play in the production of radical species and potency of free radical damage, in addition to the role of training and health status of the individual undertaking exercise. It has been proposed that damage may only occur under exhaustive exercise conditions in untrained individuals (4). In this study 3-nitrotyrosine (3-NT) in plasma was assessed as a stable marker of oxidative and nitritative stress. It is formed from the reaction between peroxynitrite (ONOO⁻) and tyrosine. Healthy young adults (n=15) undertook a moderate bout of acute exercise (40 min at 70% VO₂ max on a cycle ergometer). The results showed a significant increase in the level of 3-NT in response to exercise ($1.07\mu\text{M} \pm 0.49$ pre- compared to $3.8\mu\text{M} \pm 0.93$ post-exercise; $p=0.003$) across all subjects, regardless of training status or gender. Training status did not have a significant effect on 3-NT levels post exercise, although levels in trained men (n=5) were higher than those in untrained men (n=5) ($8.3\mu\text{M} \pm 2.88$ trained vs. $2.04\mu\text{M} \pm 0.49$) ($p=0.08$). Increased 3-NT may indicate increased presence of nitric oxide in tandem with oxidative species. In this study exercise resulted in an increase in 3-NT which was greater in trained males than untrained males. In some circumstances an increase in plasma 3-NT may cause a modification of protein function. Further investigations are required to assess the implications of raised 3-NT following exercise.

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PC85

The hormone response to three different exercise bouts in young men

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Exercise is a potent physiological stimulus for the release of growth hormone (GH), cortisol, testosterone and prolactin, and also inhibits insulin release. These hormones may have roles in substrate metabolism during and after exercise, but different

types of exercise may produce varying hormone responses. The aim of this study was to investigate the hormone response to different types of exercise in young men.

Eight men aged 18-25 years were familiarised before completing three trials, at least 7 days apart, in a random order: 30-s cycle-ergometer sprint (Sprint); 30-min resistance exercise bout (Resistance); 30-min cycle at 70% VO₂max (Endurance). All trials took place in the morning following an overnight fast. Blood samples were taken pre- and immediately post-exercise, and 20, 40 and 60 min post-exercise. Area under the curve (AUC) was calculated using the trapezoid method. Differences between means were investigated using ANOVA and Bonferroni adjusted t-tests. Statistical significance was accepted at $P < 0.05$.

In the endurance trial there was a significant increase from pre-exercise to peak concentrations for GH, cortisol, prolactin and testosterone, and a significant decrease in insulin with no change in glucose concentrations. In the sprint trial there was a significant increase in GH, cortisol, testosterone and glucose concentrations and in the resistance trial there was a significant increase in testosterone and glucose concentrations. Endurance exercise elicited the greatest AUC for GH and testosterone and the greatest suppression of insulin concentrations. Sprint exercise caused the greatest AUC for cortisol, prolactin and glucose concentrations (Table 1).

The nature and magnitude of the hormone response was influenced by exercise type, perhaps reflecting the roles of these hormones in regulating substrate metabolism in the face of differing demands during and after resistance, sprint and endurance exercise.

Table 1: AUC (mean (SD)) for hormones in response to exercise. ^a Significantly less than endurance trial. ^b Significantly less than sprint trial. ^c Significantly less than both the resistance and sprint trial.

	Resistance	Sprint	Endurance
Cortisol (nmol/L/min)	392 (128) ^a	530 (157)	497 (104)
Prolactin (mU/L/min)	221 (83) ^b	372 (161)	249 (73)
Testosterone (nmol/L/min)	15.9 (3.3) ^a	25.0 (4.4)	25.4 (4.2)
GH (μg/L/min)	3.2 (4.5) ^a	6.3 (4.1)	9.3 (6.4)
Insulin (mU/L/min)	8.0 (5.3)	8.3 (5.0)	3.7 (2.4) ^c
Glucose (mmol/L/min)	3.8 (0.6)	4.0 (0.4)	4.0 (0.2)

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PC86

Induced alkalosis affects mitochondrial but not buffer capacity adaptations to exercise

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The main purpose of this study was to investigate the role of differing H⁺ accumulation on changes in muscle buffer capacity (β_m). In study 1, 16 human females performed an incremental

cycle test to determine maximal oxygen uptake ($\text{VO}_{2\text{max}}$) and the lactate threshold before and after training. They were then assigned to placebo (NaCl) or alkalosis groups (NaHCO_3). In study 2, 24 male Wistar rats performed an incremental treadmill test to exhaustion and assigned to one of three groups, baseline, placebo or alkalosis. Endurance performance for studies 1 and 2 was determined before and after training. Training for studies 1 and 2 consisted of 12 high-intensity, 2-min intervals performed 3 times a wk for 5–8 wks. The alkalosis groups for studies 1 and 2 ingested NaHCO_3 or a placebo prior to each training session. Muscle biopsies from the vastus lateralis (VL) of humans and the soleus (SOL) and extensor digitorum longus (EDL) muscles of the rats were sampled to determine βm . Mitochondrial respiration was determined on the SOL and EDL.

Following the training period, there were significant ($P < 0.05$) increases in βm (10–20%) in all muscles (VL, SOL and EDL) in the human and rat studies for both the alkalosis and placebo groups, but there were no differences ($P > 0.05$) between the groups. Both the human (placebo 1.2-fold v alkalosis 1.6-fold) and rat studies (placebo 8.2-fold v alkalosis 12.5-fold) also resulted in significant ($P < 0.05$) improvements in endurance for both the alkalosis and placebo groups, but with greater improvements for the alkalosis group than the placebo group. In the human study, the lactate threshold improved significantly ($P < 0.05$) more for the alkalosis group than placebo (placebo 15% v alkalosis 26%). Compared to baseline, there was a significant ($P < 0.05$) increase in SOL mitochondrial respiration with training (placebo 30% v alkalosis 67%), with a significantly greater increase for the alkalosis group. As there were similar changes in βm for all groups, our results suggest training intensity may be the key stimulus for increasing βm . Alternatively, the intense nature of our training regimen despite induced alkalosis may have resulted in a sufficient accumulation of H^+ and stimulated changes in βm . In both studies, the group ingesting NaHCO_3 during training had greater improvements in endurance performance. The greater increase in the lactate threshold and mitochondrial respiration of the alkalosis groups indicates that the improved endurance performance may be related to differences in muscle oxidative capacity.

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PC87

Exercise, heat stress and the interleukin-6 response: support for neuroendocrine regulatory mechanisms

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Whilst it is widely recognized that systemic interleukin-6 (IL-6) concentrations increase as a consequence of prolonged exercise, confusion exists over the plasma IL-6 response to exercise with additional heat stress, since the heat may (Brenner *et al.* 1999) or may not (Niess *et al.* 2003) augment circulating levels. Recently Rhind and colleagues concluded that the exercise-

induced elevation in core temperature “appears to be an important physiological mechanism triggering the hormone–cytokine axis” (Rhind *et al.* 2004). Therefore, the purpose of the present investigation was to clarify a) if the addition of heat stress augments the IL-6 response to exercise, and b) whether there is an associated neuroendocrine response during exercise in the heat. Following Local Ethics Committee approval and having obtained informed consent, eight non-heat acclimated males (26 ± 8 years, $\text{VO}_{2\text{max}}$: 60 ± 7 ml kg^{-1} min^{-1} , Wmax : 339 ± 34 W) each performed two 60-min bouts of cycling at $70 \pm 2\%$ $\text{VO}_{2\text{max}}$ at either 20°C (CON) or 35°C (HOT), in a balanced cross-over design. Data were analysed using analysis of variance with repeated measures for time, Tukey’s honestly significant difference *post hoc* procedure and paired samples *t*-tests where appropriate. All eight subjects reported exercise as more difficult during HOT than CON, as evidenced by significantly higher ratings of perceived exertion (13.8 ± 0.5 versus 12.6 ± 0.5 ; $p < 0.01$). The rise in oesophageal temperature was greater during HOT than CON ($p < 0.05$), increasing by 1.8°C and 1.2°C , respectively. Plasma levels of IL-6 increased approximately twice as much in the HOT condition than CON (3.7 ± 0.6 versus 1.7 ± 0.2 pg ml^{-1} ; $p < 0.05$). Concentrations of growth hormone were significantly higher during HOT than CON (91 ± 9 versus 58 ± 8 mU l^{-1} ; $p < 0.05$) as were concentrations of prolactin (728 ± 145 versus 316 ± 50 mU l^{-1} ; $p < 0.01$). Estimated total carbohydrate (HOT: 119 ± 22 g, CON: 112 ± 20 g) and fat oxidation (HOT: 57 ± 10 g, CON: 60 ± 11 g) were similar between trials (both $p > 0.05$). The present results confirm the additive effect of heat stress to the IL-6 response during exercise (Brenner *et al.* 1999; Rhind *et al.* 2004). It is unlikely that this response is related to fuel selection, as whole-body carbohydrate and fat oxidation were similar between conditions. Rather, the greater rise in core temperature during HOT was accompanied by significantly higher levels of growth hormone and prolactin, supporting a role for neuroendocrine regulation of exercise-induced IL-6 (Rhind *et al.* 2004).

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PC88

Protein ingestion following acute eccentric exercise speeds force production restoration

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Food components and the timing of supplements have been shown to affect many aspects of skeletal muscle metabolism, such as glycogen depletion and protein synthesis (Kuo *et al.* 1999; Rasmussen *et al.* 2000; Tipton *et al.* 2001). The aim of the study was to examine whether ingestion of protein following eccentric exercise (EE) could affect recovery and alleviate symptoms associated with delayed onset of muscle soreness (DOMS) in the 72h post exercise period. Methods: Eight male subjects (Age 21 ± 1 yrs; Mass 76 ± 3 kg; Height 180 ± 1 cm Mean $\pm$ SEM) volunteered to take part in the study. Each subject visited the lab on two sep-

arate occasions separated by 14 days. A 10ml venous blood sample was taken following an overnight fast and analysed for serum Creatine Kinase (CK) and Protein Carbonyl (PC) content by ELISA. Peak isometric maximum voluntary contraction (MVC) and peak power output (PPO) were determined using a custom built isometric rig and SRM fitted cycle ergometer, respectively. Subjects completed a 30-minute downhill run (-10°) at a target heart rate of 150 ± 5 bpm (Braun and Dutto, 2003). On completion, the subjects ingested either a protein meal containing 40g EAA (PRO) or placebo (PLA) solution in a randomised, blind procedure. Venous blood was taken; MVC and PPO were recorded in the subsequent 24, 48 and 72h. DOMS was assessed by algometry. Each subject repeated the experiment a minimum of two weeks later taking the different meal to their first visit. Differences between treatments was analysed for statistical significance using stats. Ethical approval was granted by the University of Brighton Ethical Committee. Results: MVC significantly declined ($p < 0.05$) at 24h post EE in PLA group (-7%) and remained depressed at 48h in the placebo condition (-10%), whereas MVC stayed within pre EE values in the PRO trial at all time points. PPO also declined, reaching a significant ($p < 0.05$) nadir at 24h in the PRO group (-7.9%) and at 48 h in the PLA group (-6%) when PPO had returned to pre EE values in the PRO group. CK, PC and muscle soreness significantly increased ($p < 0.05$) post EE in both groups, which peaked at 24h and endured to 72h, with PRO demonstrating no difference from PLA. Conclusion: Eccentric exercise resulted in a decline in MVC and PPO and an associated increase in CK, PC and DOMS in the 72 h post exercise. Protein ingestion attenuated the performance depressive effect of DOMS on MVC, with no significant effect on PPO recovery. PRO did not affect the increase in muscle damage markers following EE. Protein supplementation may therefore improve MVC recovery following muscle damaging exercise.

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PC89

The effects of prolonged strenuous exercise on salivary secretion of IgA subclasses in man

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Low levels of secretory IgA (sIgA) have been associated with recurrent upper respiratory tract infection (URTI) (Gleeson et al., 1999). Saliva contains approximately 60% sIgA1 and 40% sIgA2 in normal adults (Tappuni & Challacombe, 1994). Because sIgA1 is more susceptible to proteases degradation produced by mucosal pathogens, the higher the proportion of sIgA1 and the lower the sIgA2 concentration, may increase the risk of bacterial mucosal infection (Kilian et al., 1996). The aim of the pres-

ent study is to investigate the effects of acute prolonged intensive exercise on saliva flow rate, sIgA level, and sIgA1/sIgA ratio. Following approval by the local Ethics Committee, eight male volunteers (age 21.3 ± 1.0 years, body mass 66.8 ± 2.0 kg, VO_{2max} 52.5 ± 2.1 mL.kg $^{-1}$.min $^{-1}$, peak power 259 ± 6 watt; means $\pm$ SEM) performed either a single bout of cycling at 55% peak power for 2 h (started at 9:00) or a separate resting trial separated by at least a week in a counterbalanced design. Timed, unstimulated saliva samples and venous blood samples were obtained at 10 min before exercise (Pre-EX), the last 2 min of exercise (Post-EX), and 1 h (P-EX-1h) and 2 h (P-EX-2h, saliva only) after exercise. Water ingestion was allowed ad libitum during the trials except for the 5 min preceding each saliva sample collection. The concentrations of sIgA and sIgA1 were determined by ELISA. Results were analyzed using a two-factor (trial $\times$ time) repeated measures ANOVA with post hoc Tukey tests and paired t tests applied where appropriate. The results of this study showed that a 2-h cycling at 55% peak power significantly decreased blood glucose concentration ($P < 0.01$) and increased plasma concentrations of cortisol and adrenaline (both $P < 0.01$) as shown in Table 1. However, there were no significant effects on saliva flow rate, sIgA level, and sIgA1/sIgA ratio. These findings suggested that a single bout of cycling at 55% peak power for 2 h does not appear to acutely affect oral immunity.

Table 1. The changes in plasma levels of glucose, adrenaline & cortisol and salivary levels of saliva flow rate, sIgA & sIgA1/sIgA2 ratio.

		Pre-EX	Post-EX	P-EX-1h	P-EX-2h
Glucose (mM)	EX	5.17 $\pm$ 0.06	4.42 $\pm$ 0.27	4.13 $\pm$ 0.21**	
	RE	4.80 $\pm$ 0.18	4.78 $\pm$ 0.15	4.64 $\pm$ 0.19	
Adrenaline (nM)	EX	1.33 $\pm$ 0.08	3.21 $\pm$ 0.31**	1.41 $\pm$ 0.15	
	RE	1.53 $\pm$ 0.16	1.59 $\pm$ 0.11	1.06 $\pm$ 0.26	
Cortisol (nM)	EX	347 $\pm$ 29	571 $\pm$ 49	517 $\pm$ 49	
	RE	329 $\pm$ 38	195 $\pm$ 14	187 $\pm$ 14	
Saliva flow rate (μ L/min)	EX	490 $\pm$ 99	281 $\pm$ 42	384 $\pm$ 109	538 $\pm$ 139
	RE	428 $\pm$ 51	493 $\pm$ 98	621 $\pm$ 152	476 $\pm$ 116
sIgA level (mg/L)	EX	142 $\pm$ 12	151 $\pm$ 11	163 $\pm$ 11	149 $\pm$ 13
	RE	151 $\pm$ 10	150 $\pm$ 8	153 $\pm$ 9	134 $\pm$ 12
sIgA1/sIgA2 ratio (%)	EX	56 $\pm$ 5	74 $\pm$ 6	69 $\pm$ 6	63 $\pm$ 5
	RE	62 $\pm$ 4	70 $\pm$ 4	65 $\pm$ 5	61 $\pm$ 6

Values are mean $\pm$ SEM (n=8). Significantly different from pre-EX (** $P < 0.01$); significantly different from resting control (RE) trial ($^{\#}P < 0.01$)

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC90

Increased daily activity improves glycaemic control and cardiovascular function in obese, type 2 diabetes individuals

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Physical activity and diet-induced weight management have been shown to reduce progression from impaired glucose tolerance to type 2 diabetes by 58% (Pan et al., 1997; Tuomilehto et al.,

2001). Recent recommendations are for a minimum of 150 min/week of moderate to vigorous physical activity, with no more than 2 consecutive days without activity (Sigal et al., 2006). Walking has been advocated as a low impact exercise mode for sedentary individuals, with 10,000 steps/day suggested to conform with daily activity recommendations (Tudor-Locke et al., 2004).

Seven physically inactive non-diabetes individuals (2 male, 5 female; age 51 ± 12 yrs, weight 77.4 ± 11.3 kg, height 1.64 ± 0.05 m; Mean $\pm$ SD) and seven physically inactive, diet treated individuals with Type 2 diabetes (4 male, 3 female, age 54 ± 7 years, weight 107.2 ± 18.9 kg, height 1.66 ± 0.12 m) took part in this study. The walking intervention consisted of accumulating 10,000 steps/day, for 6 weeks, as monitored using a pedometer. HbA1c % and total cholesterol were measured from venous blood samples at rest, whilst glucose concentrations were measured for 2 hr during an oral glucose tolerance test (OGTT). Paired sample t-tests (SPSS version 14) were used to determine differences pre and post training in each group ($P < 0.05$).

Subjects increased steps/day from 4414 ± 1796 and 5267 ± 931 steps/day to 9517 ± 2059 and 9873 ± 1001 steps/day in non-diabetes and Type 2 diabetes individuals respectively. Neither group demonstrated significant differences in fasting glucose (4.5 ± 0.8 vs. 4.1 ± 0.3 mmol.L⁻¹ and 6.8 ± 1.2 vs. 6.7 ± 1.2 mmol.L⁻¹), peak glucose (5.9 ± 0.9 vs. 5.7 ± 1.0 mmol.L⁻¹ and 11.8 ± 1.0 vs. 11.8 ± 0.9 mmol.L⁻¹), and 2 hr glucose (4.3 ± 0.3 vs. 3.9 ± 1.0 mmol.L⁻¹ and 11.8 ± 1.0 vs. 11.8 ± 0.9 mmol.L⁻¹) pre and post intervention. A decrease was observed in HbA1c % in the Type 2 diabetes group following the 6 wk intervention (7.6 ± 1.2 to 6.5 ± 0.6 ; $P = 0.05$). Glucose AUC_{response} decreased significantly in the type 2 diabetes group from 634 ± 78.4 to 541 ± 76.1 (mM.min⁻¹ $P = 0.05$). Resting heart rate (73 ± 18 to 64 ± 14 beats.min⁻¹ $P = 0.018$), mean arterial pressure (103.4 ± 13.1 to 98.8 ± 12.6 mm.mg $P = 0.05$) and total cholesterol (209.5 ± 11.5 to 174.9 ± 1.9 mg.dL⁻¹ $P = 0.02$) were lower post training in the type 2 diabetes group.

The present study demonstrates that six weeks of accumulated exercise is effective in improving glycaemic control, mean arterial pressure and resting total cholesterol in individuals with type 2 diabetes. Our data suggests that daily walking should be recommended to glucose intolerant and type 2 diabetes individuals for improving health and maintaining glycaemic control.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC91

The glycogen binding domain of the β -subunit of AMPK functions as a sensor of glycogen quality

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The activity of the AMP-activated protein kinase (AMPK) is central to the maintenance of both cellular and whole body energy homeostasis. AMPK functions as heterotrimer with the catalytic activity residing in the α -subunit and the γ and β -subunits performing regulatory functions. While the role of the γ -subunit in the allosteric binding of AMP has been established in recent years, the regulatory role of the β -subunit has remained elusive.

The β -subunit has a C-terminal ASC domain required for interactions with both the α and γ -subunits. In addition to the ASC domain the N-terminus of the β -subunit has a 120 amino acid segment with strong homology to "N-isoamylase-like domain". The latter are non-catalytic domains found in enzymes that metabolise the α 1-6 glycosidic linkages of polysaccharides such as glycogen and starch.

Previous work by this laboratory (1) and others (2) revealed that this domain is capable of binding to glycogen both in-vitro and in-vivo. This domain has subsequently been re-named the glycogen-binding domain (GBD).

Glycogen is the main storage polysaccharide found in mammalian cells. It represents a plentiful source of glucose that is readily available for ATP generation. Glycogen molecules are large, heterogeneous, tree-like structures nucleated by a single glycogenin protein. Within the glycogen particle glucose molecules are linked by α 1-4 glycosidic linkages with branches appearing every 10-12 glucose units via a α 1-6 glycosidic link.

In the study reported here we have found that glycogen can inhibit AMPK activity. Using the recombinant AMPK complex we have shown that the inhibitory effect of glycogen is blocked by mutation of two key tryptophan residues within the GBD. We have additionally shown that mutation of these two tryptophan residues within the isolated domain abolishes glycogen binding to the GBD.

Attempts to define fragments of glycogen that bind to the GBD resulted in the discovery that AMPK interacts with and is inhibited by isomaltose. Isomaltose is a α 1-6 linked disaccharide of glucose that mimics the branch-points of the glycogen particle. It is additionally reported that degradation of glycogen by phosphorylase to expose the branch-points increases the potency of AMPK inhibition, while chemically synthesised branched oligopolysaccharides have a similar effect. This work suggests the GBD of AMPK specifically recognises the branch-points of the glycogen particle with consequences for the regulation of the AMPK system. In this sense AMPK may therefore monitor the quality as well as the quantity of cellular glycogen.

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PC92

Spot the Difference! Differential expression analysis of the aging rat skeletal muscle proteome

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Sarcopenia can be defined as the age-related loss of muscle mass, strength and function. Although there is no specific level of lean body mass or muscle mass at which one can say sarcopenia is present, any loss of muscle mass is of importance because there is a strong relationship between muscle mass and strength. The introduction of new techniques such as proteomics will be necessary to understand the clinical and biochemical pathophysiology in the field of muscle aging. The concept of the proteome was first introduced in 1994 and is defined as Protein expressed by a given genome. Two-dimensional electrophoresis (2DGE) is the first, important step in this proteomics strategy, followed by MALDI-ToF mass spectrometry. Using both technologies it is possible to be able to take a 'snapshot' of a cell so that many proteins can be separated, quantified and identified. We optimized 2D-gel electrophoresis to obtain high resolution 2D maps of 3 month and 30 month gastrocnemius muscle. The gels were stained, and the differentially expressed proteins were identified using 2D image analysis software. These proteins were then identified using peptide mass fingerprinting. The identified proteins were involved in energy metabolism, myofilament, cytoskeleton and detoxification.

This work was supported by a principal investigator grant from Science Foundation Ireland (SFI-04/IN3/B614), as well as equipment grants from the Irish Health Research Board (HRB-EQ/2003/3, HRB-EQ/2004/2).

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to reveal phosphorylation levels using a fluorescent phospho-sensor dye (Pro-Q Diamond dye) and subsequent MALDI-ToF mass spectrometry analyses to identify differentially expressed proteins.

This work was supported by a principal investigator grant from Science Foundation Ireland (SFI-04/IN3/B614), as well as equipment grants from the Irish Health Research Board (HRB-EQ/2003/3, HRB-EQ/2004/2).

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PC94

Decay and re-induction of dry-heat acclimation following 12 and 26 days without exposure to heat stressA.S. Weller¹, D.M. Linnane¹, A.G. Jonkman² and H.A. Daanen²

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Compared with the induction of heat acclimation (HA), studies investigating the decay and re-induction of HA (RA) are relatively sparse and have yielded conflicting results (Pandolf, 1998). Consequently, the aim of this study was to quantify HA decay and the number of days required to attain RA following two periods without exposure to heat stress. Following local ethics approval, 16 semi-nude men (mean $\pm$ 1 S.D.: age, 28.1 ± 7.2 y; body mass, 74.2 ± 10.6 kg; peak oxygen uptake ($\text{VO}_{2\text{peak}}$), 51.9 ± 5.2 ml min⁻¹ kg⁻¹) were acclimated to dry-heat by undertaking an exercise protocol in a hot chamber (dry-bulb temperature, 46.1 ± 0.1 °C; relative humidity, 17.9 ± 0.1 %) on 10 consecutive days (HA1–10) in winter UK. Thereafter, the subjects were divided into two groups and re-acclimated to heat for up to 7 days (RA1–7) after 12 (Group 1) and 26 days (Group 2) without exposure to heat stress. The exercise protocol consisted of 60 minutes of treadmill walking at a fixed speed/incline (45 ± 4 % $\text{VO}_{2\text{peak}}$), followed by a 10-minute rest period and 40 minutes of treadmill walking/running, the intensity of which was increased across HA to maintain rectal temperature (T_{re}) at ~ 38.5 °C. T_{re} , mean skin temperature (T_{sk}), heart rate (f_c) and rate of water loss (m_{wl}) were recorded. At 60 minutes, T_{re} , T_{sk} , f_c and m_{wl} did not change after HA7, and HA was taken as the mean of HA8–10. 60-minute heat strain data at HA8–10 and RA1, and the day at which RA was attained are given in Table 1. For both groups there was no decay in T_{re} , and for all measured variables, RA was attained by RA2 and RA4 in Groups 1 and 2, respectively. These results are of practical significance to all individuals working/competing in hot environments, and suggest that once adaptation to heat has been attained, the time that individuals may spend in cooler conditions before returning to a hot environment could be as long as one month, without the need for extensive re-adaptation to heat.

Table 1. Heat strain variables at 60 minutes

PC93

Sarcopenia: mapping of the phosphoproteome

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The progressive muscle wasting that occurs with age is called Sarcopenia. Ageing is synonymous with frailty, caused by the loss of strength and movement. This deterioration usually starts in adults aged approximately forty and up to 40% loss in muscle mass can occur. There are many purported reasons for Sarcopenia such as the reduction in exercise, changes in hormone levels, denervation of muscle, muscle remodeling, decrease in mitochondrial regeneration and decreased rates of protein synthesis. The mapping of the Aged muscle Phosphoproteome will play a substantial part in identifying novel disease markers that occur during ageing. To identify these Biomarkers, total differential proteomic analysis of Wistar rats was carried out. The separation of full cell protein lysate was carried out by two-dimensional gel electrophoresis. Then the gels were fluorescently stained and imaged

	Group 1		Group 2	
	HA8-10	RA1	HA8-10	RA1
T _{re} (°C)	38.12 ± 0.30	38.11 ± 0.44 (1)	38.16 ± 0.17	38.16 ± 0.21 (1)
T _{sk} (°C)	36.32 ± 0.93	36.52 ± 0.96 (1)	36.40 ± 0.80	37.19 ± 0.72 * (3)
f _c (b min ⁻¹)	137.0 ± 9.4	144.3 ± 8.2 * (2)	134.1 ± 13.8	141.0 ± 16.5 * (2)
m _{wl} (kg h ⁻¹)	1.1 ± 0.2	1.0 ± 0.2 * (2)	1.1 ± 0.2	0.9 ± 0.2 * (4)

Differences (Newman-Keuls) between HA8-10 and RA1 are shown by * ($P < 0.05$), and the RA day in which there was no difference with HA8-10 are given in parentheses.

Pandolf KB (1998). *Int. J. Sports Med.* **19**, S157–S160.

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PC96

Systemic oxidative stress response to regional alterations in skeletal muscle and cerebral tissue oxygenation

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Exercise and hypoxia independently stimulate free radical generation by human skeletal muscle (Bailey *et al.*, 2007) subsequent to a decrease in intracellular oxygenation rather than an increase in oxygen (O₂) flux (Bailey *et al.*, 2004). In the current study, we examined if the combination of exercise and inspiratory hypoxia would further compound regional tissue de-oxygenation and thus by consequence, increase oxidative stress. We also determined if inspiratory hyperoxia, by normalising tissue oxygenation, would subsequently attenuate oxidative stress.

Eight healthy male volunteers aged 26 ± 2 (mean $\pm$ SD) were examined at rest in normoxia (21%O₂) and within 10 minutes of passive exposure to 12%O₂ (passive hypoxia) followed by a standardised cycling test to volitional exhaustion (active hypoxia). An additional measurement was performed following 5min passive recovery in 100%O₂ (passive hyperoxia). Absolute changes in oxy-haemoglobin concentration in cerebral (cO₂Hb) and skeletal (mO₂Hb) tissue were measured using continuous near-infrared spectroscopy (OXYMON Mk III). Differential path length factors of 6.0 and 4.0 were employed to correct for brain and muscle calculations. Plasma from a forearm antecubital vein was extracted into a high-sensitivity multi-bore aqueous cell prior to X-band electron paramagnetic resonance spectroscopy for the direct detection of the ascorbate free radical (A[•]) expressed in arbitrary units (AU)/√line width in Gauss (G). All data were normally distributed (Shapiro-Wilk-W tests) and analysed using a one factor repeated measures ANOVA followed by Bonferroni-corrected paired samples *t*-tests.

Compared to the normoxic control, the decrease in mO₂Hb observed during passive hypoxia ($-1.3 \pm 2.8 \mu\text{mol/L}$, $P < 0.05$) was further compounded by exercise ($-12.2 \pm 5.3 \mu\text{mol/L}$, $P < 0.05$) and increased markedly in hyperoxia ($+9.6 \pm 6.6 \mu\text{mol/L}$, $P < 0.05$). Passive hypoxia decreased cO₂Hb ($-4.5 \pm 3.5 \mu\text{mol/L}$, $P < 0.05$) with increases observed during exercise ($+4.1 \pm 10.7$

$\mu\text{mol/L}$, $P < 0.05$) and hyperoxia ($+23.1 \pm 18.4 \mu\text{mol/L}$, $P < 0.05$). Compared to the normoxic control, passive hypoxia increased plasma A[•] by $150 \pm 111 \text{ AU}/\sqrt{\text{G}}$, $P < 0.05$) with further increases observed during exercise ($+371 \pm 194 \text{ AU}/\sqrt{\text{G}}$, $P < 0.05$) and hyperoxia ($+430 \pm 164 \text{ AU}/\sqrt{\text{G}}$, $P < 0.05$).

The present findings confirm the oxidative synergy that exists between hypoxia and exercise that may be related to the magnitude of muscle but not cerebral de-oxygenation. The hyperoxic data suggest that intracellular hyper-oxygenation may be equally as pro-oxidant as hypo-oxygenation.

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Bailey DM *et al.* (2007). *Free Rad Res* **41**, 182-190

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC97

Ca²⁺ spikes in myocytes of small intestine

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Parasympathetic control of gastrointestinal smooth muscles (SM) is mediated via muscarinic (M2/M3) receptors, activation of which causes smooth muscle cell (SMC) depolarization via opening of muscarinic cationic channels (MCCs). Plasma membrane (PM) depolarization increases the action potential (AP) frequency thus facilitating Ca²⁺ entry via voltage-gated Ca²⁺ channels (VGCCs). Activation of M2 receptors gates MCCs via G_{oα}. Activation of M3 receptors stimulates PLC-β, which hydrolyses PIP₂ to DAG and IP₃. MCC opening is synergistically facilitated by intracellular Ca²⁺ and IP₃ [1]. As inhibitors of VGCCs greatly attenuate cholinergic contraction, it was suggested that Ca²⁺ entry via VGCCs is the major mechanism of Ca²⁺ mobilisation. Analysis of the mechanisms of the carbachol (CCh) - induced Ca²⁺ mobilisation brought us to revision of this idea.

The experiments were conducted on SMCs freshly isolated from the longitudinal layer of the guinea-pig ileum. Using fast confocal imaging we analysed [Ca²⁺]_i changes following stimulation of M2/M3 receptors with CCh and related the spatial pattern of CCh-induced [Ca²⁺]_i transients to the intracellular distribution of IP₃ receptors (IP₃Rs) and ryanodine receptors (RyRs). By combining Ca²⁺ imaging with tight-seal recording we related the changes in [Ca²⁺]_i to the changes in the cell membrane potential. Using pharmacological tools we analysed the contribution of VGCCs, IP₃Rs and RyRs to CCh-induced [Ca²⁺]_i transients. The effect of some of these agents on VGCCs, MCCs, CCh-induced depolarization and SM contraction was tested.

CCh-induced APs were associated with abrupt increases of [Ca²⁺]_i (Ca²⁺ spikes) at sub-PM SR elements enriched with IP₃Rs and poor in RyRs. Summation of Ca²⁺ spikes gave rise to an initial [Ca²⁺]_i transient followed by a delayed phase/[Ca²⁺]_i oscillations. Block of RyRs (50-100 μM tetracaine or 100 μM ryanodine) abolished the delayed phase but had little effect on Ca²⁺

spikes/initial $[Ca^{2+}]_i$ transient. Block of VGCCs (5 μ M nifedipine) or IP₃Rs (2 μ M xestospongine C or 30 μ M 2-APB) suppressed both the initial and delayed phases of the $[Ca^{2+}]_i$ transient and abolished Ca^{2+} spikes. Current through VGCCs was partially inhibited by xestospongine C, but was slightly augmented by 2-APB. 2-APB partially inhibited MCCs but not CCh-induced depolarization. Cholinergic contraction was strongly attenuated by 2-APB or tetracaine.

Thus, genesis of Ca^{2+} spikes by VGCCs involves IP₃Rs-mediated Ca^{2+} -release. Both IP₃Rs and RyRs are engaged in the full-scale contractile response to CCh.

Gordienko DV & Zholos AV (2004). *Cell Calcium* **36**, 367-386.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC98

Fluorescent ligand binding in α 1-adrenoceptor knockout mice demonstrates that all three α 1-adrenoceptor subtypes exist in smooth muscle cells in the mouse carotid artery

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The distribution of α 1-adrenoceptors (α 1-ARs) in smooth muscle cells (SMCs) of the carotid artery was examined using QAPB, a fluorescent ligand for α 1-ARs, in the wild type (WT) and α 1-AR knockout (KO) mice.

Methods Fresh carotids were incubated in 100nM QAPB/PSS solution (21°C, 120 minutes). α 1B/D-KO arteries were also co-incubated with 100nM QAPB/100nM RS100329 (α 1A-AR selective antagonist). Arteries were sliced open and laid flat on a microscope slide (Miquel RM et al., 2005). All arteries were visualised using the Bio-Rad Radiance 2100 Confocal Laser Scanning System. An excitation/emission of 488nm/515nm was used with a X40 oil immersion objective (NA 0.75). Integrated intensity was obtained from region statistics. The number of pixels at each intensity was obtained and used to calculate the total fluorescence at each intensity level. Mean data was displayed in histogram plots to quantify the observations made by eye.

Results QAPB binding was evenly distributed throughout the WT carotid, in which SMCs were clearly defined. In the α 1B-KO, SMCs were clearly defined by QAPB and evenly distributed. In both the α 1B/D-KO and α 1D-KO QAPB binding was markedly reduced and varied between individual cells. The leftward displacement of the histograms and the decrease in total intensity (Table 1) demonstrated that fluorescence was reduced for all three KO compared to the WT. RS100 329 abolished QAPB binding (total intensity $2.35E+6 \pm 6.3E+5$).

Conclusions The reduced intensity in the α 1B-KO suggests that the total α 1-AR population was reduced and that the α 1B-AR is present in the WT. In the α 1D-KO fluorescence was reduced more than in the α 1B-KO, consistent with a major α 1D-AR population in the WT. Furthermore, the presence of QAPB binding in the α 1B/D-KO, which was abolished by RS100 329, highlights that an α 1A-AR population may be present in the WT. In conclusion, the total α 1-AR population is decreased in the KOs and suggests that all three α 1-AR subtypes exist in SMCs in the WT carotid artery.

Table 1. Integrated intensity to QAPB in the WT and KO

Mouse Strain	Integrated Intensity	% Reduction	n
WT	1.06E+7 $\pm$ 2.9E+6	-	4
α 1B-KO	5.84E+6 $\pm$ 6.6E+5	45	4
α 1D-KO	3.43E+6 $\pm$ 9.3E+5	68	4
α 1BD-KO	3.68E+6 $\pm$ 6.8E+5	65	4

* $p < 0.05$, ** $p < 0.01$ compared to WT (one-way ANOVA, Bonferroni's post test).

Miquel RM et al., (2005). *Mol Imaging* **4** 1-13.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC99

Automated Neurite Outgrowth image analysis – validation versus manual measurement

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In vitro neurite outgrowth analysis by fluorescence microscopy is an important tool for studies in the fields of neurodegenerative diseases such as Alzheimer's and Parkinson's, neuroregenerative processes such as spinal cord injury repair, and basic neurological research. Manual measurement of outgrowth is a time consuming process and potentially biased process. Using the Neurite Outgrowth application module of the MetaMorph® 7.0 software package we analyzed two dimensional fluorescent images of PC12 cells in vitro, stimulated with NGF, and compared the results with those using manual measurements. Variances and correlations are shown between multiple users, multiple measurements by the same user, and software analysis of replicate experimental samples. These results demonstrate that automated analysis can provide results faster and more reproducibly and statistically similar to traditional manual methods.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC100

Two-photon microscopy for imaging of lymphocytes recruitment in intact mouse carotid artery

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For evaluation of atherosclerotic processes, imaging of 3D structures in intact vascular tissues and functional aspects of the diseased artery are required. Multiphoton imaging has been applied

in atherosclerosis to image murine vascular structures. However, homing of cells to the vessel wall has not been addressed. In our laboratory, we have established multiphoton scanning microscopy to allow 3D imaging of the entire structure of an isolated intact ApoE^{-/-} mouse carotid artery, identifying homed fluorescently tagged adoptively transferred lymphocytes, in auto-fluorescent adventitial connective tissue and medial elastin fibres. Cell suspensions were prepared from peripheral and mesenteric lymph nodes of female C57BL/6 mice and labelled with Cell Tracker™ Red CMTPX (Molecular Probes Inc.). Lymphocytes (2–5 × 10⁶) were injected i.v. into female ApoE^{-/-} or C57BL/6 recipients. Twenty four hours after transfer excised intact mouse carotid arteries were imaged by two-photon microscopy. The use of this system provided us to clearly image lymphocyte infiltration mainly within the adventitia of an intact ApoE^{-/-} mouse carotid artery. Consequently, this system could be a powerful tool to study immune cells behaviour in artery disease.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC101

Confocal imaging of acute injury by suture in human articular cartilage

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Introduction

The scope of this work was to visualize the acute effect of the sutures used in the cartilage repair. The known protocol applied to the viable cartilage tissues was improved to address this problem (1). Even though the repair of mature adult cartilage is limited, the viable chondrocytes are crucial for maintaining cartilage homeostasis. As the chondrocytes remain viable *post-mortem* it is possible to use cartilage explants also for comparing damage caused by other surgical equipment (scalpel, coring instruments, bipolar probes etc.) by this method. We are studying the acute injury produced by the suture passage in human explants which has not been described appropriately yet. Such approach would allow choosing optimal needle size, design, and material to be used in the clinical practice.

Materials and Methods

It is of therapeutic importance to assess the impact of suture on tissue damage indirectly by differential live-dead staining of the tissue explants after artificial injury of cartilage. In this trial series we used cartilage from osteochondral cylinders (6mm in diameter with 10mm of vertical thickness of the subchondral bone) procured from human cadaveric donors of different age and sex. Cylinders were stored in culture medium for 24–48h at 4°C. After inserting the needle we pulled it through the cartilaginous part of the cylinder to simulate chirurgic suture. With a vibrating blade microtome we then sliced the cylinder into 200 µm thick slices perpendicular to the direction of suture. The slices were incubated in Viability assay kit for animal cells (Molecular Probes, Eugene, OR) containing Calcein AM and Ethidium homodimer-1 for approximately 1h. Confocal images of stacks 10 µm apart

were scanned. As the fluorescent signal from both dyes was strong enough to obtain quality images (with pinhole closed to 0.7 Airy Units) through averaging no deconvolution was applied.

Results

The first results of confocal imaging of 200 µm thick slices from human articular cartilage showed in details the volume affected by acute injury produced by varying suturing materials. High quality 3D images helped interpret surface area based results of damaged area. In case of uncertainty check if the increased area of damaged cells actually corresponds to huge amounts of damage or if it is an artifact of nonsuperimposed injury section. From image analysis we can now statistically evaluate and compare the impact of different surgery tools on the extent of damage. Using confocal imaging for damage assessment presents an advantage over classical fluorescent microscopy in the field of volumetric study.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC102

Use of fluorescent imaging methods to assess mitochondrial physiology in cochlear explants and slices

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Mitochondrial dysfunction has been implicated in age related hearing loss and in the response to both noise damage and ototoxins. Documentation of mitochondrial function in the inner ear however is scarce and little is known about their role in cochlear physiology and pathophysiology. Recent advances in techniques available for the study of mitochondria have furthered our understanding of their biochemical and bioenergetic functions in single cell systems. In addition to ATP production, mitochondria participate in numerous other processes within the cell; including Ca²⁺ homeostasis, regulation of intracellular redox potential and cell death.

The cochlea is a complex, multicellular organ known to have a high metabolic demand. It exhibits a tonotopic gradient such that high frequency sounds are encoded at the basal end and low frequency sounds at the apex. There is also a base to apex gradient in the sensitivity of sensory hair cells to noise and ototoxins. Explant cultures were prepared from postnatal day 3 or 4 rat cochleae and used after 1 day *in vitro*. In addition, cochlear slices were prepared from postnatal cochleae and used within a few hours.

The mitochondrial transmembrane potential (ψ_{mt}) is central to oxidative phosphorylation. We assessed ψ_{mt} using the fluorescent lipophilic cation tetramethyl rhodamine methyl ester (TMRM). Analysis of mitochondrial TMRM fluorescence suggests a difference in ψ_{mt} in inner hair cells from the base of the cochlea compared to the apex. To further investigate differences in base-to-apex mitochondrial physiology, we used the endogenous autofluorescence of NAD(P)H and FAD. Measurement of NAD(P)H and FAD autofluorescence provides an assessment

of mitochondrial metabolism. The spectral properties of the two signals enable them to be imaged simultaneously using excitation with 351 nm and 488 nm laser lines. To calibrate the signals, 3 mM NaCN was applied to inhibit mitochondrial respiration and induce a state of maximal reduction. This was followed by 5 μ M FCCP, an uncoupler that collapses ψ_{mt} and establishes a state of maximal oxidation. Calibration of the signals in this way provides an estimation of cell redox state. We are presently continuing this work in order to confirm the presence of base-to-apex differences in mitochondrial function in subtypes of cochlear cells.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC103

Targeting of inositol 1,4,5-trisphosphate receptors to the endoplasmic reticulum

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Inositol 1,4,5-trisphosphate receptors (IP₃Rs) are the intracellular Ca²⁺ channels, whose ability to generate spatially organized cytosolic Ca²⁺ signals depends upon their precise targeting to specific subcellular compartments. IP₃Rs are tetrameric. Each subunit has an N-terminal IP₃-binding site and towards the C-terminal there are six transmembrane domains (TMDs), the last pair of which with their linking luminal loop form the pore of the channel. We previously demonstrated that a fragment of IP₃R1 comprising only TMD1-2 and the intervening luminal loop was targeted to the ER, while TMD1 or TMD2 alone were expressed in mitochondria (Parker et al., 2004). Here we identify the minimal requirements for ER targeting for IP₃R1 using N-terminal fragments of IP₃R1 tagged with enhanced green fluorescent protein (EGFP) and expressed in COS-7 cells. IP₃R1 truncated after the TMD2 was targeted to the ER, whereas after truncation immediately after TMD1 it was cytosolic. Extending the region beyond TMD1 by 8 or 18 amino acid residues allowed only inefficient targeting to the ER. Addition of 26 amino acid residues improved ER targeting and with 36 residues the IP₃R was effectively targeted to the ER. We conclude that the TMD1 includes a signal recognition sequence that is recognized by the signal recognition particle (SRP) only after enough downstream residues are translated, to allow expulsion of this signal from the ribosomal tunnel.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC104

Lysophosphatidic acid and lysophosphatidylcholine with polyunsaturated fatty acyl chains are efficient substrates for mammalian lipoxygenases

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Oxygenation of lysophospholipids with unsaturated fatty acyl chains, 1-linoleoyl, 1-arachidonoyl or 1-docosahexaenoyl group by mammalian lipoxygenase (LOX), reticulocyte or leukocyte LPO, was examined by monitoring the formation of conjugated dienes. In support of the above, the hydroperoxy derivative of each lysophospholipid as oxygenation product was confirmed by LC/MS analyses. Further, the oxygenation products of lysophospholipids were found to contain hydroperoxide group mainly at C-13 and C-15 for linoleoyl and arachidonoyl chain, respectively. The kinetic study indicated that lysophosphatidylcholine and lysophosphatidic acid containing polyunsaturated fatty acyl chains were no less efficient than the corresponding polyunsaturated fatty acids as substrate of reticulocyte LOX. Similarly, these lysophospholipids were also found to be oxygenated effectively by porcine leukocyte LOX. In contrast, these lysophospholipids were not oxygenated efficiently by potato LOX. Thus, polyunsaturated lysophospholipids could be utilized as selective substrates for some mammalian lipoxygenases.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC105

Lycopene inhibits TNF- α -induced monocyte-endothelial cell adhesion and ICAM-1 expression in human endothelial cells

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Inflammatory mediators such as TNF- α , IL-1 β , and IL-8, which can enhance binding of LDL to endothelium and smooth muscle cells and upregulate expression leukocyte adhesion molecules on endothelium during atherogenesis. Lycopene, a natural carotenoid from tomato and other sources, has been shown to inhibit IL-1 β -induced ICAM-1 and VCAM-1 expression in human endothelial cells (Martin et al., 2000). However, its action

mechanism is still largely unknown. In the present study, we studied the effect of lycopene on TNF- α -induced signaling in human umbilical endothelial cells (HUVECs). TNF- α -induced ICAM-1 mRNA and protein expression in HUVECs was inhibited by lycopene, whereas its expression in smooth muscle was not affected. A gel electrophoresis mobility shift assay (EMSA) further demonstrated that lycopene inhibited TNF- α -induced NF- κ B-DNA complex formation. This possibly attributed to that lycopene attenuated TNF- α -induced I κ B phosphorylation and NF- κ B translocation from cytosol to nucleus. In a functional study, lycopene blocked THP-1 monocyte adhesion to endothelial monolayer, while it exhibited no obvious inhibitory effect on THP-1 monocyte adhesion to various matrix proteins such as collagen, laminin and fibronectin. Taken together, our results for the first time demonstrate that lycopene is able to inhibit TNF- α -induced monocyte-endothelial interaction, ICAM-1 expression and NF- κ B translocation, suggesting an anti-inflammatory role of lycopene.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC106

Crucial role for endogenous melanocortin type 3 receptor in mesenteric post-reperfusion injury

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Melanocortin peptides are endowed with anti-inflammatory properties; their actions are mediated by a subgroup of G-protein coupled receptors: out of the five receptors identified so far, MC1 and MC3 have been indicated, in distinct experimental settings, for bringing about the anti-inflammatory effects of melanocortins. Here we have used null mice to determine the counter-regulatory role of MC3 and MC1 in vascular inflammation monitoring leukocyte-endothelial dynamics after mesenteric ischaemia reperfusion (I/R).

C57Bl/6 (WT), MC3-/- or MC1 mutant (recessive yellow e/e colony) (~15-20 g body weight) were anaesthetised and surgically prepared; ischaemia was produced by the occlusion of the superior mesenteric artery (SMA) by a small clamp for 35 min; after clamp removal, reperfusion was allowed for 90 min [1] or 4 h. Leukocyte-endothelial interactions were monitored by intravital microscopy, quantifying by off-line analysis the extent of cell rolling, adhesion and emigration. Sham-operated animals underwent the same surgical procedure without clamping. In some animals, the MC3 agonist dTrp⁸- γ -MSH was given i.v. before SMA clamping, at the dose of 10 μ g [2].

The IR procedure provoked significant and time-dependent increases in the parameters measured, with maximal responses

already at 90 min, when compared to sham operated mice. At 90 min post-reperfusion, MC3-/- mice displayed marked augmentation of cell adhesion and emigration (two-fold increase vs. WT mice, $P < 0.05$, $n = 6$), whereas no changes in cell rolling were evident. By the 4 h time-point, MC3-/- mice still had higher extent of cell adhesion in the inflamed microcirculation ($n = 6$, $P < 0.05$). Of interest, even the modest response observed in sham-operated mice was increased in the absence of MC3. Experiments in the MC1 mutant (inactive) colony yielded different results: no difference from WT was obtained at 90 min post-reperfusion on any of the parameters under analysis ($n = 6$) and the same was true at the 4 h time-point. Using the 35/90 min protocol, we could observe that single treatment of WT mice with dTrp⁸- γ -MSH (10 μ g/mouse) inhibited (>60%) both cell adhesion and emigration ($P < 0.05$; $n = 6$), with no effect on cell rolling.

Collectively these data indicate the existence of an endogenous counter-regulatory circuit centred on MC3 and rapidly activated after vascular injury as obtained with an IR procedure. Therefore, therapeutic targeting of MC3 may be beneficial for the treatment of acute IR-related injured, such as in aorta aneurisma, coronary disease and stroke.

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PC107

Protective effect of GH releasing peptide-2 in LPS-induced liver injury

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Background/Aims: It has been reported that GHRP-2, a potent ghrelin receptor agonist, has anti-inflammatory effect. We investigated whether this GH secretagogue attenuates liver injury in LPS treated rats.

Methods: Wistar rats were divided in control and LPS-treated rats (1 mg/kg), and were injected i.p. with saline or GHRP-2 (100 μ g/kg) at 17:00 h and at 08:00 h the following day. All animals were humanly killed 4 h after the last injection. Serum levels of aspartate and alanine transaminases were measured as an index of liver damage. Circulating nitrites/nitrates and hepatic IGF-I and TNF- α were evaluated as possible mediators of GHRP-2 actions.

Results: LPS increased serum levels of transaminases and nitrites/nitrates. Moreover, LPS increased hepatic TNF- α and decreased hepatic IGF-I mRNAs. GHRP-2 administration attenuated LPS effects on transaminases, nitrites/nitrates, TNF- α and IGF-I "in vivo". To elucidate whether GHRP-2 is able to modulate transaminases production directly on the liver, cocultures of

hepatocytes and nonparenchymal cells were incubated with LPS (100ng/ml) and GHRP-2 (10⁻⁷M). GHRP-2 prevented endotoxin-induced increase in transaminases release as well as in TNF- α and IGF-I mRNAs and decreased nitrite/nitrate release from cocultures of hepatocytes and nonparenchymal cells.

Conclusions: These data suggest that GHRP-2 has a protective effect on the liver in LPS injected rats that seems to be mediated by IGF-I, TNF and NO.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC108

Comparative assessment of anti-inflammatory potential of *Asparagus racemosus* and *Uraria picta*

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OBJECTIVE:

Herbal drugs have an advantage of having ability to modulate more than one cellular event involved in inflammation. A pharmacological study was carried out to evaluate the anti-inflammatory potential of *Asparagus racemosus* (AR) and *Uraria picta* (UP) and investigate their mechanisms of action.

AR was selected for anti-inflammatory evaluation since it contains flavonoides and steroidal saponins. It is reported for anti-ulcer and immunomodulatory activity. UP is an ingredient of traditional Ayurvedic medicine, Dashamoola, used for treating inflammations but till now no scientific evaluation has been carried out.

METHODS:

Aqueous extract of roots of AR (ARAE) and Ethanolic extract of whole plant of UP (EUP) were studied for their *in-vitro* inhibitory activity in NO radical scavenging assay (20-300 μ g/ml) and lipoxygenase assay (50-100 μ g/ml).

In-vivo evaluation was carried out in carrageenan induced rat paw edema at 200-600 mg/kg (p.o.).

The most active dose (600 mg/kg) for both the plants was taken up for the mechanistic studies in mediator (bradykinin, histamine, arachidonic acid) induced rat paw edema. The involvement of nitric oxide (NO) system was studied by injecting L-Arginine (300 mg/kg, i.p.) 2 hours after the injection of carrageenan in the rat hind paw.

RESULTS:

ARAE and EUP showed a maximum of 62.99% and 59.33% (at 300 μ g/ml) scavenging of NO radical respectively. EUP showed 25.48 % inhibition (at 100 μ g/ml) while ARAE failed to inhibit lipoxygenase activity.

ARAE and EUP showed a dose dependent inhibition of carrageenan induced inflammation with ED₅₀ of 366.46 mg/kg and 328.94 mg/kg respectively.

ARAE and EUP (at 600 mg/kg, at 20 min) produced inhibition of 49.3%, 31.48% and 58.88%, 48.27% in bradykinin and histamine induced rat paw edemas respectively. EUP showed 45.10% (at 75 min) inhibition. in arachidonic acid induced rat paw edema while ARAE was inactive. L-Arginine reduced anti-inflammatory activity (at 4 hr) from 63.74% to 32.237% for ARAE and from 68.53% to 46.27% for EUP.

CONCLUSION:

Asparagus racemosus and *Uraria picta* have significant anti-inflammatory activity possibly mediated by inhibition of histamine, bradykinin and suppression of NO pathway. The anti-inflammatory effect of EUP could also be mediated via the inhibition of lipoxygenase activity. The investigation revealed that UP has better anti-inflammatory potential than AR.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC109

Trefoil peptide and mucin changes associated with mucosal barrier failure in neonatal necrotising enterocolitis

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The supramucosal barrier protects the underlying epithelium from luminal aggressors. Mucins and trefoil peptides are important components of the protective mucosal barrier in the intestinal mucosa and play a fundamental role in epithelial repair and restitution (1 – 4).

Necrotising enterocolitis (NEC) is the most common gastrointestinal (GI) emergency in the human neonate (5). The mechanisms leading to severe necrosis of the bowel and multiorgan failure are poorly understood. The role of mucosal protection in the pathogenesis of NEC has not been investigated.

We propose that prematurity is associated with an inefficient mucosal barrier, due in part to aberrant trefoil peptide and mucin synthesis and secretion (1, 2, 4). We have examined the expression of MUC1-5AC and TFF 1-3 mRNA and mapped the gene product distribution in the GI tract of fetuses, neonatal controls, and infants with NEC.

Consent was obtained from the parents of neonates up to 44 weeks gestation having a laparotomy and bowel resection. The bowel samples from resection specimens, and GI tract extractions from products of conception at termination of pregnancy were collected. Trefoil and mucin mRNA expression and protein localisation was determined by real time PCR, in situ hybridisation and immunohistochemistry.

A total of 15 fetal and 44 postnatal (24 with NEC, 16 acute, 8 recovery phase) and 20 control samples were analysed in this study. Fetal mucin and trefoil gene and protein expression in the first trimester showed the same pattern as described in mature bowel

(1, 2, 4). Expression of both membrane bound and secreted mucins was unaffected by NEC. Low levels of TFF 1-2 mRNA and protein were detected in the NEC specimens with no evidence for co-ordinated upregulation. A significant downregulation of TFF3 expression was found in 83% of NEC patients. Immunohistochemistry revealed a decrease in TFF3 protein adjacent to areas of mucosal injury in NEC.

Our results suggest a lack of TFF expression in response to NEC in the premature bowel, while mucosal barrier mucin is preserved. This may lead to weakened barrier function and impaired mucosal restitution, and contribute to the cascade of bowel necrosis and generalised sepsis characteristic of NEC.

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We wish to thank Dr A. Giraud, Univ. Melbourne, Australia for the anti-TFF3 antibody.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC110

The sulphonylurea glibenclamide inhibits fMLP-activated elastase release from human neutrophils

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Inappropriate neutrophil activation and subsequent proteolytic enzyme release has been implicated in the pathogenesis of chronic inflammatory conditions such as chronic obstructive pulmonary disease (Brown *et al.*, 2003). Neutrophil activation is dependent upon an increase in the intracellular concentration of Ca^{2+} , mediated by store operated Ca^{2+} entry (SOCE; Itagaki *et al.*, 2002). Human neutrophils express a range of K^{+} ion channels (Gallin, 1991), inhibition of which would be expected to produce membrane depolarisation, inhibit SOCE and consequently neutrophil activity. We have tested this hypothesis by studying the effects of drugs known to block K^{+} channels on neutrophil elastase (NE) release from fMLP-activated human neutrophils using methods described previously (Brown *et al.*, 2003). Neutrophils were purified from peripheral venous blood collected from healthy human volunteers and resuspended (2.5×10^6 cells ml^{-1}) in Hanks' balanced salt solution. 150ml of this cell suspension was placed in each of a number of microcentrifuge tubes containing cytochalasin B (final concentration; 5 mgml^{-1}) along with different concentrations of the drug under study (or vehicle). Cells were primed by the addition of $\text{TNF-}\alpha$ (10 ngml^{-1} ; 30 min at 37°C) before being activated by fMLP (10^{-7}M ; 45 min). The samples were centrifuged and 25 ml of the supernatant placed into the respective wells of a 96-well microplate. 150 ml of TRIS buffer was added to each well along with 20 ml of NE substrate N-ethoxysuccinyl-ala-ala-pro-val-p-nitroanilide. After 30 min at room temperature the amount of

the coloured elastase product, proportional to the amount of NE present, was quantified by measuring absorbance at 405 nm in a microplate reader. Data were normalized and expressed as percentage inhibition of the NE release obtained from neutrophils in the absence of drug after correction for basal NE release. Data was analyzed using ANOVA and Dunnet's post hoc test with $P < 0.05$ considered significant. All experiments were repeated three times in duplicate.

NE release was inhibited in a concentration dependent manner by both the calcium entry blocker SKF96365 ($1\text{--}100 \mu\text{M}$; IC_{50} 16 μM ; maximum inhibition $96.0 \pm 0.5\%$) and glibenclamide ($1\text{--}100 \mu\text{M}$; IC_{50} 7 μM ; maximum inhibition $93.6 \pm 2.3\%$, Fig 1). Another sulphonylurea, glipizide was also effective but only at the highest concentration tested ($32.5 \pm 5.1\%$ inhibition at $100 \mu\text{M}$). The K^{+} channel blockers tetraethylammonium (TEA; $0.1\text{--}10 \text{ mM}$), 4 aminopyridine (4-AP; $1 \mu\text{M}\text{--}1 \text{ mM}$) and verapamil ($1\text{--}100 \mu\text{M}$) had no significant effect on NE release.

These results suggest ATP-dependent potassium channels play a role in regulating neutrophil activity and that blockers of these channels could offer a novel means of inhibiting neutrophil function.

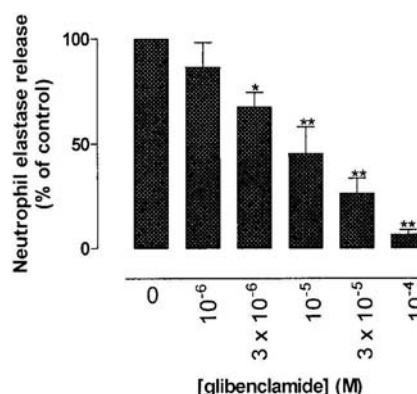


Figure 1: Effects of glibenclamide on NE release from human neutrophils. * $P < 0.05$; ** $P < 0.01$ compared to control.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC111

Rosiglitazone, a PPAR γ agonist, prevents microparticle-induced vascular hyporeactivity through pro-inflammatory protein regulation

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Microparticles (MPs) are plasma membrane vesicles with pro-coagulant and proinflammatory properties. We recently demon-

strated that MPs induce vascular hyporeactivity and evoke upregulation of pro-inflammatory protein expression. This study dissected the effect of either in vitro treatment or short-term oral administration of the peroxysome proliferator activated receptor- γ agonist, rosiglitazone (RZ), on MPs-induced vascular hyporeactivity of mice vessels.

MPs were produced from human lymphoid CEM T cells by actinomycin D treatment. The effects of RZ on mice aortic rings incubated with or without MPs were investigated. Both, aortic rings treated in vitro with RZ or taken from mice treated by oral administration of the same agonist, completely prevented MPs-induced vascular hyporeactivity in response to U46619. These effects of RZ occurred at a concentration at which it did not significantly modify blood parameters and vascular reactivity. The mechanisms involved abrogation of nitric oxide (NO) and prostacyclin overproduction linked to upregulation of inducible NO-synthase and cyclo-oxygenase 2 elicited by MPs. Also, RZ treatment attenuated the ability of MPs to evoke NF- κ B activation.

These results suggest that RZ counteracts vascular dysfunction associated with increase release of pro-inflammatory proteins elicited by MPs. They underscore therapeutic perspective for RZ in vascular diseases involving enhanced participation of MPs.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC112

Endogenous MC3R and inhibition of inflammatory arthritis

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The pharmacological anti-inflammatory properties of melanocortin peptides, including α -melanocyte stimulating hormone and pituitary hormone adrenocorticotrophin₁₋₃₉, has long been established, however what their main molecular target is still remains unclear; out of the 5 melanocortin receptors cloned so far, there is evidence for a predominant role of MC1R and MC3R, as determined in models of acute inflammation and tissue injury. Furthermore, the patho-physiological impact of this important homeostatic anti-inflammatory system has been poorly studied. Here, we focused on inflammatory arthritis.

Arthritis was induced by K/BxN serum transfer into wild type (WT), MC1R mutant (recessive yellow *e/e* colony) and MC3R null mice (*n*=12), by injecting 150 μ l of serum i.p. on day 0. First signs of arthritis developed from day 2 and were monitored both as clinical score and paw volume (using a water plethysmometer). The results obtained indicated a marked disease exacerbation in MC3R null mice, with a peak at day 6 (*P*<0.05; two-way ANOVA), whereas no different profile of disease development was displayed by the MC1R mutant and WT mice. This heightened presence of disease in MC3R null mice was confirmed by

histological analysis of the paw joints. In contrast to WT animals, a substantial cellular infiltrate was present with pannus formation and evidence of cartilage/bone erosion in the absence of endogenous MC3R. Interestingly, resolution of arthritis was substantially delayed in MC3R null mice, and this genotype had a much higher disease penetrance when compared to WT and MC1R null mice. The MC3R null phenotype was striking, for instance, 100% of MC3R null mice had developed clear signs of arthritis by day 5, whereas disease penetrance was only 25% in wild type animals and 40% in MC1R nulls (*P*<0.05, Fisher's exact test).

In conclusion we have demonstrated that when MC3R and not MC1R is void in the mouse system, a host anti-inflammatory response cannot be modulated with consequent exacerbation and prolongation of arthritic joint inflammation. The MC3R could be a novel target for the development of innovative anti-arthritic drugs.

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PC113

Signaling events regulating CD11c and CD11b integrin-mediated monocyte adhesion and cytoskeletal reorganization on fibrinogen

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The beta 2-integrins expressed on leukocytes play a key role in contributing to integrin-mediated adhesion and subsequent leukocyte responses in numerous pathophysiological conditions (1). Plasma protein fibrinogen participates in inflammatory responses by promoting the accumulation of leukocytes (2,3). The differential regulation of the integrin-mediated adhesion and cytoskeletal changes of monocytes populations may have relevance to their distinct roles in inflammation. Peripheral blood monocytes despite expressing CD11c, primarily adhered to fibrinogen in a CD11b-dependent manner: 90 % (*p*<0.001, *n*=3) of monocyte adhesion was blocked using anti-CD11b. However, culture of monocyte in human serum resulted in a 3.4-fold increase in CD11c expression and participation in adhesion to fibrinogen in co-operation with CD11b, suggesting the elevated CD11c expression in the differentiated monocyte population leads to its involvement in adhesion. Simultaneous treatment with anti-CD11b and anti-CD11c resulted in inhibition of adhesion by 85% (*p*<0.001, *n*=3). Although both integrins were involved in adhesion, the cytoskeletal reorganization contributing to an increase in spreading of cultured monocytes on fibrinogen was mediated by CD11b. The adhesion of monocytes to

fibrinogen was abrogated upon treatment with the Src family kinase (SFK) inhibitor, PP2. The distribution of CD11c showed significant clustering on cultured monocytes upon adhesion to fibrinogen. The diminished clustering on inhibiting SFK activity indicates an important role for SFK activity in regulating CD11c avidity. We also examine the role of Rac2 in regulating beta 2-integrin dependent adhesion and cytoskeletal changes. The expression of dominant negative Rac2 in monocytes led to changes in cell spreading and formation of actin foci suggesting its involvement in integrin mediated inflammatory responses.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC114

Mast cells express elements of the endogenous anti-inflammatory circuit centred on melanocortins

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Melanocortins (MCs; e.g. ACTH and α -MSH) are peptides with known anti-inflammatory properties in different tissues and systems. Our group has focused one of its interest on cellular and molecular anti-inflammatory properties of MCs in acute and chronic inflammation. We proposed a major role for MC3 receptor, in the regulation of endogenous anti-inflammation, thereby modulating macrophage activation and cytokine production. However, the possible source of MCs at the site of inflammation remains to be elucidated. Mast cells are leukocytes resident in different tissues and play an important role in triggering and development of inflammation. The aim of this study was to determine if elements of the MC system were present in murine mast cells.

Bone marrow-derived mast cells (BMMC) were generated by culture of bone marrow cells for 4-6 weeks in 30% WEHI conditioned medium. Purity of the cultures was assessed by the expression of IgE receptor and CD117 and toluidine blue staining, measuring always purity $\geq 95\%$. Expression of MC receptors as well as of the pro-opiomelanocortin gene (POMC) was monitored by RT-PCR and western blotting. In some cases, cells were stimulated with LPS (1-100 ng/ml). In addition, naïve peritoneal mast cells were also used following positive isolation by magnetic selection.

Expression data revealed POMC gene expression in resting BMMC and peritoneal mast cells. To highlight potential modulatory pathways, BMMC were stimulated with 100 ng/ml LPS, selected because it produced maximal TNF production: POMC

gene was already up-regulated within 1-h of LPS stimulation, and mRNA content remained up to 24h. Interestingly, stressing BMMC by deprivation of WEHI conditioned medium, triggered a delayed up regulation the POMC gene, maximum at 24h. With respect to MC receptors, BMMC and peritoneal mast cells expressed detectable mRNA level for the MC1, MC3 and MC5 receptors. However, western blotting analysis allowed detection only of MC1 protein, at least in resting BMMC. In conclusion, this investigation proposes the existence of an endogenous anti-inflammatory loop operative in the periphery, possibly in conditions of tissue injury and inflammatory resolution, whereby murine mast cells could act in concert or in parallel with the macrophage to promote regain of homeostasis. Parallel studies in mice nullified for elements of this anti-inflammatory break signal confirm the pivotal role played by this system in acute and chronic inflammation. The role that MC production by the mast cells might have in these conditions remains to be determined.

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PC115

Blockade of carrageenan induced inflammatory hypersensitivity by intraplantar injection of Ginkgo biloba extract EGb-761 in rat

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Recent studies in vivo have shown that oral administration of Ginkgo biloba standardised extract (EGb-761) blocked thermal hyperalgesia in rat models of carrageenan induced inflammatory pain and paw incisional pain (Biddlestone and Dolan, 2007; *Br J Pharmacol.*, in press). The aim of this study was to examine the effects of local administration of EGb-761 on carrageenan induced hypersensitivity and inflammation.

Carrageenan (3%; 50 μ l) was injected intradermally into the left hindpaw of adult male Sprague-Dawley rats (n = 6/group; 250-300 g) followed by intraplantar injection of EGb-761 (100 or 300 μ g) or drug-vehicle in 50 μ l into the ipsilateral hindpaw 3 h post-carrageenan. Mechanical response threshold (in grams) and thermal response latency (in sec) to hindpaw stimulation and paw oedema (cm³) were measured before and 2, 4, 6 and 24 h post-carrageenan. The maximum effect (Emax) was calculated as the maximum change in threshold after carrageenan injection from baseline responses. Data (mean $\pm$ SEM) were analysed using one-way ANOVA with post-hoc Tukey's test. Carrageenan induced a significant reduction in mechanical and thermal response threshold, and paw oedema in the ipsilateral paw. Maximum hypersensitivity and paw oedema were observed 6 h post-carrageenan. Intraplantar injection of 100 and 300 μ g EGb-761 had no effect on carrageenan-induced mechanical hypersensitivity but blocked thermal hypersensitivity at 6 h (Emax: 7.7 $\pm$ 4.4% and 14.9 $\pm$ 3.8%, respectively vs. 50.4 $\pm$ 4.4% and 14.9 $\pm$ 3.8%, respectively).

4.3% in vehicle treated group; $p < 0.001$). Intraplantar injection of 300 µg EGb-761 significantly reduced paw oedema at 6 h ($p < 0.05$).

These results demonstrate that local injection of EGb-761 is effective in inhibiting carrageenan-induced inflammation and thermal hypersensitivity, indicating that the observed analgesia induced by EGb-761 may be peripherally-mediated.

Biddlestone, L. and Dolan, S. (2007) Oral Administration of Ginkgo biloba extract, EGb-761 inhibits thermal hyperalgesia in rodent models of inflammatory and post-surgical pain. *British Journal of Pharmacology* (in press)

Brian Leiper, Veronica Graham, Dr Sharron Dolan, Schwabe Pharmaceuticals

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC116

Pharmacology of AS-16; a novel and selective Adenosine A_{2B} receptor antagonist

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There is considerable evidence to suggest that adenosine, through activation of A_{2B} receptors on mast cells and smooth muscle cells, is involved in the pathophysiology of asthma (Holgate, 2005). Here we describe the pharmacology of a novel selective A_{2B} receptor antagonist AS-16 (2-(4-benzyloxy-phenyl)-N-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl]-acetamide) which may have therapeutic utility in asthma and respiratory disease.

In radioligand binding studies utilizing the selective A_{2B} receptor ligand [3H]MRE-2029, AS-16 has an affinity of 22 nM for the human recombinant A_{2B} receptors expressed in HEK-293 cells. It is selective over the other adenosine receptor subtypes, and monoamine receptors in general, having an affinity greater than 1,000 nM at these targets. In functional assays, using recombinant A_{2B} receptors in CHO cells and endogenous A_{2B} receptors in the human mast cell line HMC-1, AS-16 demonstrates an affinity (K_B) of 75 nM and 87 nM respectively.

The *in vivo* efficacy of AS-16 has been examined in sheep which are naturally sensitive to the allergen *Ascaris suum*. In these animals antigen-induced bronchospasm has two phases similar to what occurs with allergen challenge studies in patients, an immediate increase in airflow resistance (early airway response), followed several hours later by a second increase in airway resistance (late airway response). AS-16 (2 mg/kg) was dosed orally twice-daily for 2 days prior to antigen challenge. This dosing regime gave a consistent plasma concentration (70–100 ng/ml) during the experimental phase of the studies. AS-16 reduced the

early airway response by approximately 20 % and almost completely abolished the late airway response. A further feature of the sheep model, which is also consistent with studies in patients, is that antigen challenge induces airway hyperresponsiveness (AHR). AS-16 (2 mg/kg p.o.) completely prevented the AHR to inhaled carbachol, assessed 24 h after allergen challenge. Finally, AS-16 (2 mg/kg p.o.) was able to completely block adenosine-induced bronchospasm, suggesting that in sheep adenosine effects are mediated via the A_{2B} receptor.

Thus, AS-16 is a novel, selective A_{2B} receptor antagonist which will be a useful tool to explore the pathophysiology of asthma and respiratory disease, and may represent a novel therapy for the treatment of these chronic diseases.

Holgate, S.T. (2005) *Br. J. Pharmacol.*, 145, 1009–1015.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC117

Impaired activation and increased Th1 skewing activity of Annexin-1 deficient dendritic cells

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Introduction

Dendritic cells (DCs) are a large family of leukocytes that unlike other antigen presenting cells (APCs), are thought to have no function other than regulate T and B cell responses. This function of DCs requires prior activation and is controlled by their state of maturation. Bone marrow-derived DCs migrate to the periphery as "immature" cells. Upon stimulation with microbial stimuli, they become "mature" and express high levels of major histocompatibility complex (MHC) class I and II, co-stimulatory and adhesion molecules. In recent study we demonstrated that exogenous Annexin-1 (Anx-A1) modulates T cell activation and differentiation (1). Given the key role of DC in influencing T cell response, in this study we sought to characterise the role of endogenous Anx-A1 in DC activation and as well as their capacity to influence T cell responses.

Results

Anx-A1+/+ and Anx-A1-/- DCs were obtained by culturing bone marrow cells from male mice (Balb/C or C57/BL6; aged 6–8 weeks) with GM-CSF derived from X63-GMCSF cell supernatants for 8 days. Analysis of the total number of cells collected after 8 days of differentiation showed a higher number of non-adherent cells from Anx-A1-/- mice compared to control littermate. Furthermore, analysis by FACS staining of markers of DC maturation in Anx-A1 deficient cells showed higher expression of MHC II, CD54, CD80 and CD86. Stimulation of DCs with lipopolysaccharide (LPS) displayed decreased TNF- α and increased IL-12 production in Anx-A1-/. Interestingly, analysis of NF- κ B activation upon LPS stimulation showed lower DNA-binding activity in Anx-A1-/- DC compared to their control littermates. Finally, to study the effect on T cell activation, Anx-A1+/+ and Anx-A1-/- DCs were co-cultured in graded ratios with allogeneic CD4+ T cells in a mixed leukocyte reaction. Consistent with previous results co-culture of immature and mature

DCs from the Anx-A1^{-/-} mice with wild-type T cells in MLR showed increased proliferation and Th1 cytokine production.

Conclusions

Together these findings suggest that genetic deletion of Anx-A1 induces a previously undescribed phenotype in DCs i.e. increased yield of DC differentiation; increased Th1 skewing capability accompanied by and decrease in acute inflammatory cytokine production and increase in longer-term, immunomodulatory cytokine production.

1. D'Acquisto F et al. (2006) Blood 109, 1095-1102.

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PC118

A role for Annexin-1 (ANXA-1) in the healing of damaged gastric mucosa

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Annexin-1 (ANXA-1) is a 37 kDa protein implicated in the control of phagocytosis, cell differentiation and proliferation, and as a mediator of glucocorticoid action during inflammation. Moreover, ANXA-1 regulates leukocyte migration, ischemic damage, pain, as well as acute and chronic inflammation. These effects of ANXA-1 are mediated via formyl peptide receptors (FPR / FPRL-1), which are also activated by bacterially derived, chemotactic, formylated peptides. As ANXA-1 has recently been implicated as a mediator of gastric mucosal defense, an acceleration of the resolution phase of inflammation by ANXA-1 might prove to have therapeutic benefits. The possible role of ANXA-1 in gastric ulcer healing has not been described; therefore, we hypothesized that: 1) ANXA-1 protein expression along the ulcer margin will be increased during the healing of gastric ulcers; 2) The administration of the ANXA-1 peptide mimetic (Ac2-26) will accelerate gastric ulcer healing; 3) ANXA-1^{-/-} mice will exhibit impaired healing to NSAID-induced gastric damage. Methods: C57Bl6J mice (25-28 g) were anesthetized with halothane, opened with a midline laparotomy, and the stomach isolated. 200 µl of 20 % glacial acetic acid (GAA) was applied to the serosal side of the gastric antrum for 1 minute constrained within the barrel of a modified 1 ml syringe top. Total surface area at the time of induction = 28.2 mm². Endpoints: Initial studies looked at both the changes in ulcer surface area (using planimetry), and the time course of ANXA-1 protein expression (by Western blot analysis) during the healing of gastric ulcers. The next set of experiments examined the effects of Ac2-26, Boc2 (ANXA-1 receptor antagonist, FPRL-1 selective), and dexamethasone, on gastric ulcer healing. Finally, we investigated whether ANXA-1^{-/-} mice were susceptible to NSAID-induced gastric damage. For these studies, indomethacin was administered by oral gavage,

and then gastric damage scores were assessed at 5 and 24 hrs. Results: ANXA-1 protein was increased during the healing phase in the gastric ulcer margin: normal = 70 ± 11.7 INT/mm²; ulcer day 2, 121 ± 9.8; day 3, 115 ± 14; day 5, 109 ± 7.8, day 7, 127.3; day 10, 120 ± 10.4 (P < 0.05). In addition, Ac2-26 treatment significantly increased gastric ulcer healing whilst the ANXA-1 receptor antagonist Boc2, impaired the early phase of ulcer healing (P < 0.05). Finally, the ANXA-1^{-/-} mice were more susceptible to NSAID-induced gastric damage (P < 0.05 at 24 hrs post-indomethacin). Though early in the exploratory phase, these data support ANXA-1 as an endogenous anti-inflammatory mediator involved in the healing of damaged gastric mucosa.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC119

NF-κB inhibition sensitizes human monocytic cells to TNF-mediated expression of Nrf2 regulated genes

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Dysregulation of TNF signalling has been implicated in a wide variety of inflammatory diseases including rheumatoid arthritis, Crohn's disease and psoriasis. In most cells, TNF mediates its effects through activation of NF-κB, JNK, p38, and ERK. Here we show that treatment of human monocytic cells with TNF in conjunction with NF-κB inhibition results in an increased expression of Nrf2-regulated genes. Nrf2 has been implicated in conferring protection against many human diseases, including autoimmune diseases, and inflammation. Cytoprotective and detoxification genes including haem oxygenase-1 (HO-1), NADP(H) quinone oxidoreductase (NQO1) and ferritin contain an antioxidant response element (ARE) in their promoter, which is regulated by the transcription factor Nrf2. In this study we showed that TNF treatment plus NF-κB inhibition increased mRNA expression of HO-1, NQO1 and ferritin within 4h in human monocytic cells. Protein expression was observed within 8h of treatment. Nrf2 translocated to the nucleus and bound to the ARE in response to TNF treatment plus NF-κB inhibition. The binding of this complex was inhibited by the addition of Nrf2 supershift antibody. Furthermore, transfection of Nrf2 siRNA significantly inhibited the expression of these genes in response to TNF treatment plus NF-κB inhibition. These results show that treatment with TNF in conjunction with NF-κB inhibition induces the expression of Nrf2 regulated genes in human monocytic cells. Furthermore, they demonstrate a novel TNF signalling mechanism by which Nrf2 may confer protection against certain human disease.

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PC120

Microglial reactive oxygen species production in response to beta-amyloid involves chloride intracellular channel 1 (CLIC1)R. Milton¹, R. Abeti¹, M.R. Duchen¹ and M. Mazzanti²¹Department of Physiology, University College London, London, UK and ²Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Milan, Italy

β -Amyloid (A β) peptides accumulate in the brain in Alzheimer's Disease (AD) and are implicated in its pathogenesis. Microglia, the immune cells of the CNS, are activated by A β and, in response, produce reactive oxygen species (ROS) through activation of the plasmalemmal NADPH oxidase. Generation of ROS by microglia is thought to contribute to the cell death seen in AD. We have previously shown that A β activates a chloride current mediated by the protein CLIC1 (Novarino et al, 2004). The present work highlights attempts to clarify the mechanisms through which this current might be related to microglial-mediated ROS production. Using primary microglial cultures and BV2 cells, we have measured the effects of A β on microglial membrane potential and calcium signalling, and particularly rates of ROS generation using dihydroethidium fluorescence. While application of A β peptides to microglia increases the rate of dihydroethidium oxidation, such a response is not seen when the reverse peptides are used or when the NADPH oxidase is inhibited. The increased ROS production effected by A β was also dependent on the presence of CLIC1. Suppression of CLIC1 protein expression using siRNA, inhibition of the CLIC1 chloride current using IAA-94 or using an antibody to the channel protein, and replacement of extracellular Cl⁻ with impermeant anions, all significantly reduced the ROS response to A β ($p < 0.01$). Novarino G, Fabrizi C, Tonini R, Denti MA, Malchiodi-Albedi F, Lauro GM, Sacchetti B, Paradisi S, Ferroni A, Curmi PM, Breit SN, Mazzanti M. Involvement of the intracellular ion channel CLIC1 in microglia-mediated beta-amyloid-induced neurotoxicity. *J Neurosci*. 2004 Jun 9;24(23):5322-30.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC121

Cell density regulates nuclear localisation of interleukin-1 α and β in murine microglia

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Interleukin-1 (IL-1) is the prototypical pro-inflammatory cytokine, and is implicated in the pathogenesis of acute and chronic neurodegenerative diseases (Allan et al., 2005). Classical IL-1 signalling does not fully account for the effects of IL-1 observed in the literature (Touzani et al., 2002). The pro-forms of the IL-1 family agonists, IL-1 α and β , contain a confirmed and a putative nuclear localisation sequence respectively, and evidence suggests that IL-1 α has intranuclear actions in IL-1 expressing cells (Wessendorf et al., 1993; Werman et al., 2004). Microglia are an early source of IL-1 in the injured central nervous system (CNS, Pearson et al., 1999). Therefore we tested the

hypothesis that IL-1 isoforms localise to microglial nuclei following inflammatory stimuli. Murine primary microglia and a murine microglial cell line (BV-2) were treated with bacterial lipopolysaccharide (LPS) to induce IL-1 expression, and IL-1 subcellular localisation was characterised by immunocytochemistry. The proportion of cells containing intranuclear IL-1 was quantified by blind counting of IL-1 immunostained cells. LPS induced pro-IL-1 α and β localised to the cytosol and nucleus of both primary microglia and BV-2 cells. Nuclear localisation was more pronounced for IL-1 α than for IL-1 β . IL-1 subcellular localisation in BV-2 cells was cell density dependent, being predominantly cytosolic at high density, and both nuclear and cytosolic at low density (Figure 1). Cytosolic localisation of IL-1 in BV-2 cells cultured at low density was induced by co-culture with a confluent HEK cell monolayer, indicating that IL-1 localisation may be regulated by cell contact. Thus IL-1 α and β localise to microglial nuclei in a regulated fashion, and may therefore have intra-nuclear actions in microglia regulating CNS inflammatory responses.

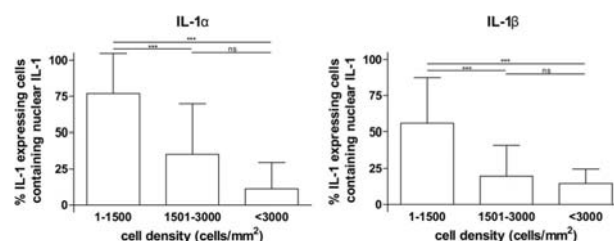


Figure 1: Cell density dependent nuclear localisation of IL-1 α and β in BV-2 cells. BV-2 cells seeded at various densities were treated with 1 μ g/ml LPS for 6h, fixed, immunostained for IL-1 α or β , and nuclei labelled with DAPI. Random fields of view were chosen by focusing on the DAPI stain, and widefield images were captured using a 40x objective. Local cell density was assessed by counting DAPI stained nuclei, and the proportion of IL-1 expressing cells containing nuclear IL-1 was counted in each field of view. The data shown are mean $\pm$ SD from three independent experiments. *** $p < 0.001$, Kruskal Wallis with post hoc Dunn's multiple comparison test.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC122

Interleukin-1 exacerbates excitotoxic brain injury via a neutrophil-dependent mechanism

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Interleukin-1 (IL-1) is a pro-inflammatory cytokine that exacerbates various forms of neurodegeneration, including alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)

induced excitotoxic neuronal death. While injection of AMPA alone into the rat striatum results in localised neuronal loss in the striatum, co-injection of AMPA plus IL-1 (A+IL-1) causes extensive neuronal loss in both the striatum and cortex. This is despite the fact that striatal injection of IL-1 alone produces no perceptible neuronal loss¹. Injection of IL-1 into the brain is known to induce neutrophil recruitment², and several studies suggest that neutrophils contribute to neuronal loss following stroke^{3,4}. The aim of this study was therefore to test the hypothesis that neutrophils contribute to IL-1-mediated exacerbation of AMPA-induced excitotoxic injury.

Neutrophil depletion serum, or normal rabbit serum as a control, was injected into the lateral tail vein of male Sprague-Dawley rats 24h and 1h prior to intra-striatal injection of A+IL-1 (under 3% isoflurane anaesthesia in a mixture of 1.2l/min nitrous oxide and 0.6l/min oxygen; anaesthesia was then maintained by 1.5-2.5% isoflurane in the same nitrous oxide/oxygen mix throughout the procedure). The animals were then sacrificed at 4, 8 or 24h after injection of A+IL-1 and the brain and serum removed. Immunohistochemistry was used to detect neutrophils and c-fos protein expression (to denote neuronal activation) within the parenchyma. Fluoro-jade B was used to detect degenerating neurons at 4 and 8h post-injection. In animals sacrificed at 24h, brain lesion volume was calculated from cresyl violet stained coronal sections. The most noteworthy finding was that neutrophil depletion prior to intra-striatal injection of A+IL-1 significantly reduced the size of the resultant lesion. In the cortex the lesion was reduced by 68.3% and sub-cortical damage was reduced by 40.5% which gave a 60% smaller total lesion volume in neutrophil depleted animals compared to non-neutrophil depleted control animals (Fig 1A). Typically, the cortical neurons salvaged by prior neutrophil depletion were in the deeper cortical layers (Fig 1B). Our findings regarding neutrophil recruitment and neuronal activation and damage at earlier time-points will be presented.

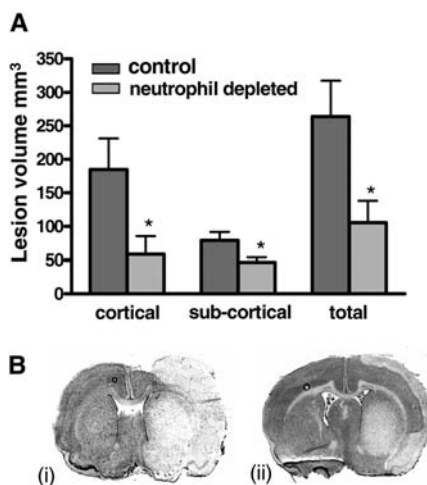


Fig 1. Effect of neutrophil depletion on A+IL-1 induced lesion. (A) A+IL-1 induced lesion volume * $P < 0.05$, Student's *t* test. (B) Cresyl violet stained 20µm coronal sections showing typical A+IL-1 induced lesion (area of pallor) in striatum and cortex of non-neutrophil depleted (i) and neutrophil depleted (ii) animals

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC123

Activation of AMP-activated protein Kinase inhibits Tumour Necrosis Factor alpha-stimulated monocyte adhesion to cultured endothelial cells

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Endothelial cells are key regulators of cardiovascular homeostasis, including the maintenance of blood flow and vascular tone. Endothelium-derived nitric oxide (NO) promotes vasodilatation and inhibits platelet aggregation, leukocyte adherence and VSMC proliferation, which all have a profound influence on blood flow, vascular remodelling and angiogenesis. Endothelial dysfunction, tissue injury and inflammation are associated with activation of the vascular endothelium and increased adhesion of circulating leukocytes. These are critical early events in the development of atherosclerosis.

Endothelial cells recruit leukocytes by selectively expressing adhesion molecules on their surface following stimulation with inflammatory cytokines, such as TNF α and IL-1 β , and certain reactive oxygen species (ROS). Adhesion molecules include vascular cell adhesion molecules (VCAM), intracellular adhesion molecules (ICAM) and endothelial cell selectin (E-selectin).

Our laboratory, and others, has recently demonstrated that the AMP activated protein kinase (AMPK) regulates eNOS phosphorylation and NO synthesis in cultured endothelial cells, and this has been confirmed by the use of constitutively active and dominant negative mutant forms of AMPK. However, the molecular mechanisms and functional effects of AMPK activation in the endothelial remain poorly characterised.

In this study we have utilised the AMPK activator AICAR and mutant AMPK adenoviral constructs to elucidate the effect of AMPK cascade activation on monocyte adhesion and adhesion molecule expression in human aortic endothelial cells (HAECs). Incubation with AICAR and infection with the constitutively active AMPK adenoviral construct was found to reduce U937 pro-monocyte adhesion to HAECs. This response appears to be bi-phasic, comprised of distinct short, NO-dependent (45min) and longer-term NO-independent (4hr) components. The NO-dependent reduction in monocyte adhesion was found to be independent of changes in cell surface expression of VCAM-1, ICAM-1 and E-Selectin adhesion molecules, while the longer term AICAR stimulation produced significant reductions in adhesion molecule expression.

We propose that AMPK negatively regulates leukocyte adhesion in a bi-phasic manner, implying a role for AMPK in atherogenesis and vascular endothelial function.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC124

Whole blood cytokine release assays differentiate PDE4 enzyme inhibitors: implications for safety and toleration

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Inhibitors of phosphodiesterase 4 (PDE4) developed to treat asthma and COPD have so far met with limited clinical success, often due to gastrointestinal side-effects. This may reflect inhibition of the PDE4D isoform (Robichaud et al, 2002). We have investigated whether human whole blood (HWB) assays for release of LTE_4 and $\text{TNF-}\alpha$ can discriminate compounds that are selective for PDE4D from those with a more balanced isozyme inhibition profile that may have a better therapeutic index.

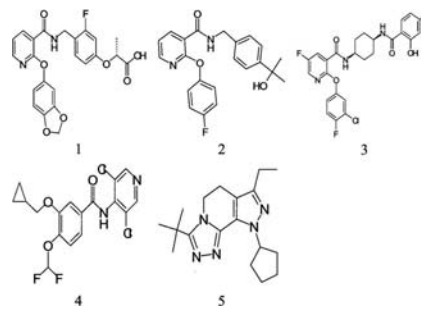
HWB was incubated at 37°C with compound (0.3nM – 100µM) for 30 min prior to addition of LPS (100ng/ml). The assay mixtures were incubated for a further 24 h and $\text{TNF-}\alpha$ then determined by specific ELISA. A similar protocol was used for monitoring LTE_4 release, except that the HWB was treated with sephadex G15 beads (15mg/ml) and PGE1 (1µM). Recombinant human PDE4A, B and D were expressed in Sf9 insect cells. PDE activity was monitored by following the conversion of [^3H]cAMP (0.5µM) to [^3H]5'AMP using a scintillation proximity assay (Amersham UK). IC_{50}s (concentrations reducing stimulated cytokine release or [^3H]5'AMP formation by 50%) were determined using a sigmoidal curve fit algorithm.

The PDE4 inhibitors differed in their relative potency to inhibit LTE_4 release and $\text{TNF-}\alpha$ release in HWB (Table). Overall, the rank order of selectivity for inhibition of LTE_4 release (IC_{50} ratio $\text{TNF-}\alpha$ / LTE_4) mirrored that for inhibition of PDE4D (IC_{50} ratio PDE4A or B / PDE4D); compound 1>2>3>4 and 5. Compounds 1 and 2 showed significantly greater potency against LTE_4 release (>360-fold and >100-fold respectively, $P<0.001$) and this was associated with selective inhibition of PDE4D (≥ 130 -fold and ≥ 11 -fold respectively, $P<0.001$). Compound 3 showed a trend for selective inhibition of LTE_4 release (5-fold, non-significant) and PDE4D (≥ 6 fold; $P<0.001$). In contrast, compound 4 (roflumilast) and compound 5 showed no significant selectivity for LTE_4 release or for PDE4D inhibition.

Our data support the proposal that sephadex-stimulated release of LTE_4 and LPS-stimulated release of $\text{TNF-}\alpha$ in HWB are functional correlates for PDE4D and PDE4B ($\pm 4\text{A}$) inhibition, respectively. Furthermore, when used together, these assays may have utility in clinical trials of PDE4 inhibitors as ex vivo biomarkers for efficacy and toleration. Roflumilast (4), which has shown efficacy in asthma and COPD at tolerated doses (Lipworth, 2005), has a balanced profile for inhibition of PDE4 isozymes and for LTE_4 vs. $\text{TNF-}\alpha$ release in whole blood. In contrast, poor toleration might be anticipated for compounds 1 and 2 which are both highly selective for PDE4D inhibition and LTE_4 release.

Compound	$\text{TNF-}\alpha$	LTE_4	PDE4A	PDE4B	PDE4D
1	>23,200	64 (65)	199 (73)	121 (29)	0.92 (0.1)
2	>38,700	379 (107)	585 (95)	201 (39)	18.3 (1.3)
3	99 (19)	21 (27)	0.57 (0.03)	0.68 (0.03)	0.10 (0.01)
4	15 (2)	15 (1)	1.02 (0.55)	0.98 (0.13)	0.72 (0.04)
5	48 (9)	48 (18)	3.4 (0.28)	4.7 (0.14)	4.0 (0.36)

Table: IC_{50}s for inhibition of $\text{TNF-}\alpha$ and LTE_4 release in HWB, plus PDE4A, B & D. Data are geometric mean with s.e.m in parentheses ($n\geq 3$).



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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC125

Arthritis-induced skeletal muscle atrophy is associated with an increase in atrogen-1 but not in myostatin

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Chronic arthritis is a catabolic state that induces a decrease in body weight gain and skeletal muscle atrophy. The chronic phase of the disease is associated with the inhibition of the insulin like growth factor I (IGF-I) and the activation of the E3 muscle specific ubiquitin ligating enzyme muscle atrophy F-box, also named atrogen 1. Myostatin is a negative regulator of muscle growth that is increased in several situations of muscle wasting such as AIDS and liver cirrhosis-induced cachexia. The aim of this work was to analyze the effect of adjuvant-induced arthritis on muscular myostatin and atrogen-1 gene expression, as well as on IGF-I, from the beginning of the clinical symptoms of the disease. For this purpose, arthritis was induced in male Wistar rats by an intradermal injection of Freund's adjuvant in the sole of the paw.

The clinical symptoms of arthritis began 10 days after the injection, and the highest arthritis index was reached on day 22. Arthritic and control rats were humanely killed on days 10, 15 and 22 after the adjuvant injection. Arthritis induced a decrease in body weight gain and in serum level of IGF-I as well as in its gene expression in the liver ($P < 0.01$). In the arthritic rats gastrocnemius relative weight was not modified on day 10, it decreased on day 15 ($P < 0.01$), and it was markedly lower on day 22 ($P < 0.01$). The muscular expression of myostatin decreased in the same way that gastrocnemius relative weight ($P < 0.05$). The gene expression of atrogen-1 was significantly increased in the skeletal muscle of arthritic rats on all days studied, specially on day 15 ($P < 0.01$). These data suggest that arthritis induced muscle loss can be mediated by changes in atrogen-1 and IGF-I, but not by an increase in myostatin gene expression in the skeletal muscle.

This work was supported by CYCYT (Ref: BEFI2003-02149, a grant to E. Castillero (Gobierno Vasco, BFI06.31) and a grant to M. Granado (FPU, AP2003-2564).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC126

The EP1 receptor antagonist GW848687X reduces trigeminal Fos expression

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Prostaglandins contribute substantially to neuronal sensitisation at both peripheral and central sites. The effects of prostaglandin E₂ (PGE₂) are mediated by G-protein-coupled EP (EP1-EP4) receptors. Activation of EP1 receptors increases intracellular calcium concentration and is crucial in the late phase of carrageenan-induced mechanical hyperalgesia (Nakayama *et al.* 2002). An EP1 receptor antagonist could block the positive feedback cascade mediated by PGE₂, resulting in inhibition of inflammation-induced hyperalgesia. Therefore selective EP1 antagonists are potential analgesics for inflammatory pain (Giblin *et al.* 2007). We have previously carried out detailed characterisation and identification of Fos expression within the trigeminal nucleus after tooth pulp stimulation in ferrets (Worsley *et al.* 2007). The aim of this study was to assess the ability of the EP1 receptor antagonist GW848687X (GlaxoSmithKline) to modify central neurone activation in response to stimulation of inflamed ferret tooth pulp. Eleven adult ferrets were prepared under anaesthesia (ketamine 25 mg/kg, xylazine 2 mg/kg, IM.) to allow tooth pulp stimulation, recording from the digastric muscle and intravenous injections at a subsequent experiment. Pulpal inflammation was induced in all animals by introducing human caries into a deep buccal cavity. Five days later, animals were re-anaesthetised (alphaxalone/alphadalone, induction: 6 mg/kg, maintenance: 6-8 mg/kg/h, IV) and the jaw opening reflex (JOR) threshold was measured. Animals were treated intravenously

with either GW848687X (bolus 10 mg/ml/kg, infusion 10 mg/10 ml/kg over 1 h, $n = 4$ and bolus 5 mg/ml/kg, infusion 5 mg/10 ml/kg over 1 h, $n = 4$) or vehicle (2% dimethyl sulfoxide and 5% glucose containing 10% hydroxypropyl- β -cyclodextrin, $n = 3$). Fifteen minutes after the initial bolus, tooth pulp stimulation was initiated at 10 times the threshold of the JOR for 90 minutes. All animals were perfused with fixative 120 minutes from the start of the stimulation and brainstems processed for Fos immunohistochemistry. Stimulation of inflamed tooth pulps induced ipsilateral Fos expression caudally in subnucleus caudalis (Vc) and rostrally in subnucleus oralis (Vo). Both doses of GW848687X reduced Fos expression in Vc (10 mg $P = 0.0003$, 5 mg $P = 0.002$, unpaired t-test with Bonferroni's correction) and Vo (10 mg $P = 0.02$, 5 mg $P = 0.02$). These results suggest that GW848687X reduces the number of trigeminal brainstem neurones activated by stimulation of the inflamed tooth pulp. Therefore GW848687X may have significant analgesic efficacy in trigeminal inflammatory pain.

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PC127

Membrane microdomains as signal transducers in inflammatory neurodegeneration

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Membrane microdomains (rafts and caveolae) represent multiprotein complexes assembling proteins involved in the regulation of signal transduction. We have evaluated the role of prion protein, cyclooxygenase 2 (cox-2) and prostacyclin synthase (PGIS) as components of rafts and caveolae in cultured neuronal cell (GN 11) and in human endothelial cells, demonstrating their role in the control of cell survival and angiogenesis (1-3). These proteins have also a role in triggering inflammatory processes. In particular the association COX-2 / PGIS within caveolae represents a powerful stimulus for inflammation. We have also evaluated criteria to select specific COX-2 inhibitors to control inflammation and neurodegeneration, confirming that while some COX-2 inhibitors as VIOXX are to be avoided because they inhibit PGIS, thus decreasing the synthesis of PGI₂, a powerful vasodilator, celebrex can be used more safely because has little effect on PGI₂ synthesis (Griffoni *et al.* Selective inhibition of prostacyclin synthase activity by rofecoxib, *J. Cell Mol. Med.* in press). We are evaluating the role of the conversion of prion protein to the form scrapie in triggering neurodegeneration preceded by inflammation and we are screening specific COX-2 inhibitors based on evaluation of PGIS inhibition. A second approach to control inflammatory neurodegeneration is based on the use of a selective micro RNA silencing cox-2 gene which we have recently identified and used to inhibit proliferation and invasiveness of colon cancer cells (4).

Our working hypothesis regards the involvement of modification of prion protein structure initiating a brain inflammatory process which in turn induces COX-2 gene sustaining chronic inflammation and neurodegeneration.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC128

The role of hydrogen sulphide in ischaemia/reperfusion injury of the rat kidney

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Hydrogen sulphide (H₂S) exerts beneficial effects in myocardial ischaemia (Sivarajah *et al.* 2006). H₂S is produced in the normal kidney by cystathionine gamma lyase (House *et al.* 1997), but the role of endogenous H₂S in the pathophysiology of ischaemia/reperfusion (I/R) injury of the kidney is unclear. Here, we have investigated the effects of topical administration of sodium hydrosulphide (NaHS), a H₂S donor, in a rat model of renal I/R injury.

Male Wistar rats weighing 200 to 320 g were subjected to 45 min of bilateral renal ischaemia followed by 6 h of reperfusion (under sodium thiopentone (120 mg/kg i.p.) anaesthesia, maintained by supplementary injections (~10 mg/kg i.v.) throughout the experiment) in the absence or presence of NaHS [100 µmol/kg, administered topically as a pre-treatment (15 min) and prior to reperfusion (5 min)].

Administration of NaHS to rats significantly attenuated I/R-induced renal dysfunction (increased serum creatinine), reperfusion injury (increased serum aspartate aminotransferase), glomerular dysfunction (decreased creatinine clearance), and tubular dysfunction (increased fractional excretion of sodium). In addition, Western blot analysis of kidneys taken at the end of

the experiment showed that administration of NaHS to rats significantly attenuated Bcl-2, Bid, cyclo-oxygenase-2, inducible nitric oxide synthase and intercellular adhesion molecule-1 when compared to rats subjected to I/R only.

These findings suggest that (i) pre-treatment with NaHS protects the rat kidney against I/R injury and (ii) this protective effect may act through anti-apoptotic and anti-inflammatory mechanisms.

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PC129

Fast diagnosis of *Schistosoma mansoni* infection in human serum and feces using polymerase chain reaction

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A fast, sensitive and specific detection of cercariae of human schistosomes is required for better definition of risk of infection. So, a novel method for the detection of *Schistosoma mansoni* in human samples that is based on the amplification of a highly repeated DNA sequence has been developed. By use of simple DNA extraction techniques and a rapid 2-step polymerase chain reaction (PCR), it was possible to amplify *S. mansoni* DNA in human fecal and serum samples. The high sensitivity of the approach enabled the detection of the parasite DNA in fecal samples containing as few as 3 eggs per gram of feces, which makes it 10 times more sensitive than the Kato-Katz examination. A detection limit of 1 fg of *Schistosoma* sp. DNA was determined when pure DNA was used as PCR template. The amplification reaction showed to be specific giving no cross-reaction with DNA from other helminths. The PCR assay developed in this study may constitute a valuable alternative for the diagnosis of the *Schistosoma* sp. infection.



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Many thanks for all people help me during this research.

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PC130

Immunology of human breast milk - a comparison of both fresh and banked human milk

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Human milk is bodily fluid which has proven to contain a variety of immune components such as antibodies, growth factors, cytokines, antimicrobial compounds and specific immune cells(1). Breast feeding has been shown to enhance the development of the immune system of the newborn as well as provide protection against enteric and respiratory infections (2). Excess milk can be donated by mothers to milk banks which are then subjected to pasteurisation to extend storage life as well as to prevent transmission of infections(3). However, little is known about the impact of this heat treatment on the immune and nutritional components. The concentrations of TGF-beta (Transforming Growth Factor), IL1 (interleukin1), IL10 (interleukin 10), RANTES (Regulated on activation, normal T cell expressed and secreted/ CCL5), IL8 (interleukin 8), IL6 (interleukin6) and sIL6R were measured by ELISA in both fresh and banked milk. Significant differences were noted in the concentrations of RANTES: fresh breast milk (1548 +/- 373 pg/ml, n=31, p=0.001), banked samples (875 +/- 1181 pg/ml, n=4, p=0.001). Similar differences were observed for TGF-beta between fresh milk samples (8047 +/- 4350 pg/ml, n=31, p=0.018) and banked samples (1989 +/- 321 pg/ml, n=4, p=0.018). No IL1 was detected in any of the banked samples, with an average of 1 pg/ml in the fresh samples. No significant difference between levels of IL10 in fresh and banked samples was observed. However, IL8 (p=0.004) as well as IL6 (p=0.008) levels were significantly reduced in banked samples when compared to the fresh samples. Solu-

ble IL6 receptor level was greatly reduced to a level of no detection in banked milk (24033 +/- 14125 pg/ml, n=20, p=0.04). These results suggest that pasteurisation may reduce levels of certain cytokines and chemokines in human breast milk and may therefore impair the immunological benefits of breast feeding.

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Supervisors: Dr Jewell, Dr Opara, Dr Michie. Kingston Milk Bank: Ms Anne Collet.

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PC131

Anti-xanthine oxidase, antioxidant and free radical scavenging activities of selected Algerian plants used in the treatment of inflammatory-related disorders

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Xanthine oxidase (XO)-inhibitory activity and total antioxidant capacity of extracts from two local medicinal plants, the leaves of Pistacia lentiscus used to treat gastro-intestinal inflammations and the bark of Fraxinus angustifolia used to treat arthritis and gout have been evaluated by various spectrophotometric methods. These include xanthine/XO test, inhibition of linoleic acid oxidation using the ferro-thiocyanate method and the capacity of the extracts to trap the radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H₂O₂).

Our results indicate that aqueous extracts (100 µg/ml) of Pistacia lentiscus and those of Fraxinus angustifolia showed a mixed type inhibition of 70% and 60%, respectively on XO activity. At a concentration of 50 µg/ml, extracts of Pistacia lentiscus inhibited linoleic acid oxidation by up to 97% whereas those of Fraxinus angustifolia showed an inhibition of only 75%, much less than the antioxidant of reference, butylhydroxyanisole (BHA) (87%). With the exception of the chloroform extract of Pistacia lentiscus, all the extracts showed high DPPH radical scavenging activity (90%), whereas Fraxinus angustifolia extracts presented an important activity which, however, remains lower than that of Pistacia lentiscus extracts. Pistacia lentiscus aqueous phases exhibited H₂O₂ scavenging activity of about 74% while that of Fraxinus angustifolia extracts is around 50%. Antioxidant capacity and scavenging activity against DPPH radical of Pistacia lentiscus are dose-dependant and more significant than many tested standards, including α-tocopherol. Column chromatography using silica gel allowed the isolation of the fractions responsible on inhibition of XO activity and linoleic acid oxidation. A positive correlation between various activities and the contents of total phenols was established for Pistacia lentiscus extracts.

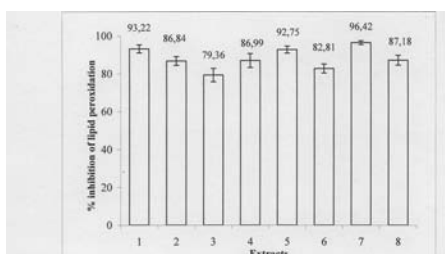


Figure 1: Inhibition of linoleic acid oxidation by extracts from *Pistacia lentiscus* at 50µg/ml (1: ethanol, 2: ethyl acetate, 3: aqueous ethyl acetate, 4: hexane, 5: aqueous hexane, 6: chloroform, 7: aqueous hexane, 8: BHA).

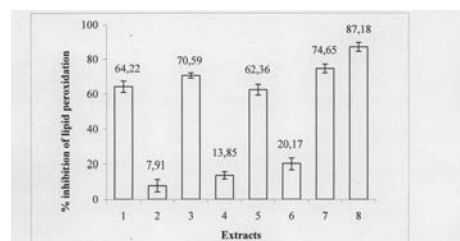


Figure 2: Inhibition of linoleic acid oxidation by extracts from *Fraxinus angustifolia* at 50µg/ml (1: ethanol, 2: ethyl acetate, 3: aqueous ethyl acetate, 4: hexane, 5: aqueous hexane, 6: chloroform, 7: aqueous hexane, 8: BHA).

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PC132

The kinetics of neutrophil migration through the basement membrane investigated in a human in vitro model

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The mechanisms by which migrating leukocytes penetrate the subendothelial basement membrane are poorly understood. We previously showed that prolonged culture of human umbilical vein endothelial cells (HUVEC) was associated with the deposition of a distinct basement membrane which modified neutrophil recruitment induced by inflammatory cytokines (Butler et al., 2005). Here, we modified this in vitro model to study the migration of neutrophils through the underlying basement membrane as well as the endothelial cell monolayer.

Confluent first-passage HUVEC were cultured for 4 or 20 days on 3µm pore transwell inserts and then treated with 0, 1 or 100U/ml tumour necrosis factor α (TNFα) or 50pg/ml interleukin-1β for 4 hours. Purified human neutrophils were added to the surface and their migration was assessed after 0.5, 1, 2 or 24h by collecting and counting neutrophils from the upper and lower chambers, and by fixing and staining the filters to quantify neutrophil number and location above and below the filter.

Neutrophil migration through the HUVEC and basement membrane increased with dose of cytokine and with time, but was consistently delayed for 20-day cultures compared to 4-day cultures. For example, $34.5 \pm 3.0\%$ of neutrophils added migrated through 4-day HUVEC treated with 100U/ml TNFα after 0.5h, compared to only $14.5 \pm 5.9\%$ migrated through 20-day HUVEC (mean ± SEM from 8 experiments; $P < 0.05$ by paired t test). If the chemoattractant interleukin-8 was added to the lower chamber, migration was increased but was still delayed for 20-day cultures compared to 4-day cultures. Blockade of neutrophil β2 integrins reduced neutrophil transmigration by >85%. Interestingly, antibody-blockade of platelet-endothelial cell adhesion molecule-1 (CD31) on neutrophils and endothelial cells had no effect on migration induced by either cytokine, at 4 or 20 days.

We have shown that the basement membrane produced by human endothelial cells delays neutrophil migration. Transmigration required β2-integrin engagement, but this could not be associated specifically with migration through the basement membrane. The model developed allows the further study of the molecular mechanisms by which neutrophils cross the distinct barriers presented by endothelial cells and their basement membrane.

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PC133

Modulatory effects of short chain fatty acids on neutrophil function

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Some studies have shown that SCFAs by acting on leukocytes modulate the inflammatory response. The aim of this study was to evaluate the effects of SCFAs (acetate, propionate and butyrate) on production of reactive oxygen species (ROS), chemotaxis and endothelial interaction of rat neutrophils. Neutrophils obtained from peritoneal cavity after 4 hours of intraperitoneal administration of oyster glycogen (1%). The cells were collected and incubated with acetate (Ac), propionate (Pr) or butyrate (Bt), in different concentrations, for 1 and 4 hours. Production of superoxide and hydrogen peroxide was evaluated in the presence or not of PMA. The interaction between leukocytes and vessel walls was evaluated by using intravital microscopy. Rats were anesthetized and the mesentery was exteriorized. After 10 minutes of the application of 20 µL SCFAs, the number of rolling and adhered leukocytes on the postcapillary venule wall was determined. In order to evaluate the chemotaxis and quantify L-selectin and β2-integrin expression, neutrophils were isolated from blood abdominal aorta and incubated with the SCFAs for 4 hours. Expression of L-selectin and β2-integrin was evaluated by flow cytometry and Real-Time PCR. Neutrophil chemotactic response was determined using 96-well disposable chemotactic plates. Bt (8 and 12 mM) reduced the production of superoxide and hydrogen peroxide by neutrophils stimulated with PMA, but did not affect the production by non stimulated cells. To evaluate the mechanism involved in this inhibitory action, expression of NADPH oxidase components (p22phox and p47phox) was examined by Real Time PCR. The SCFAs did not change the expression of p22phox and p47phox. However, Bt (12 mM) reduced the phosphorylation of p47phox as determined by Western blotting. Pre-incubation with Ac, Pr and Bt increased chemotactic capacity of unstimulated neutrophils, but had no effect in the presence of fMLP. Ac (25 mM) and Pr (24 mM) increased the number of rolling (86% and 87%, respectively, in comparison to control) and adhered leukocytes (59% and 49%, respectively, in comparison to control). SCFAs, mainly acetate and propionate, increased L-selectin mRNA and membrane migrated L-selectin in neutrophils, but had no effect on β2-integrin expression. Therefore, SCFAs can modulate important aspects of neutrophil physiology involved in the inflammatory response, such as ROS production and neutrophils migration. Bt had an inhibitory effect on ROS production by neutrophils that can partly result from the reduction in the p47phox phosphorylation. SCFAs, mainly acetate and propionate, stimulated the migration of neutrophils by increasing the chemotactic capacity of these cells raising the expression of L-selectin.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC134

Analysis of neutrophil migratory potential as a marker of human cardiac transplant rejection

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Introduction - Cardiac transplantation has become the standard treatment for end stage heart disease, yet allograft rejection has limited survival rates to approximately ten years. While the role of the T- Lymphocyte in this process has been elucidated, there is growing evidence to support the contribution of the neutrophil in the rejection process. Based on previous studies by our own group, we hypothesized that the extent of neutrophil infiltration varies individually and that this neutrophil migratory potential correlates with subsequent rejection grades. The aims of this study were to determine variations in neutrophil adhesion molecule expression following stimulation and that evaluation of this response pre-operatively could predict subsequent rejection grades in heart transplantation.

Materials and Methods - Whole blood was obtained from 11 patients prior to cardiac transplantation and stimulated with PMA (1 ng/ml) for 20 minutes, LPS (1 µg/ml) and fMLP (1 ng/ml) for 60 minutes and subsequently stained with antibodies to CD11b, CD62L and PSGL-1 and surface expression determined by flow cytometry. Intra - operative whole blood samples were also taken during the surgical procedure and stained as above. Endomyocardial biopsies were taken on day 10 post transplant and rejection grades pathologically assessed. Statistical analysis was carried out using the Pearson Correlation test.

Results - PMA, LPS and fMLP all stimulated differential expression of CD11b, CD62L and PSGL-1 with individual variation in their expression. fMLP stimulated CD11b expression pre-operatively correlated with the surgical CD11b response ($P = 0.013$). In the transplant patients, pre-operative CD11b response to LPS positively correlated with rejection grades on day 10 post surgery. ($P = 0.022$)

Conclusion - Individuals differ in their basal and stimulated expression of CD11b, CD62L and PSGL-1. Artificially stimulating neutrophils can simulate neutrophil responses to surgical trauma. In transplant patients stimulated expression of CD11b could predict the severity of rejection. These results not only implicate the neutrophil as a contributor to allograft rejection, but also advocate the possibility of using pre-operative neutrophil migratory potential as a marker of endomyocardial biopsy rejection grade. This would allow for earlier modulation of immunosuppressive therapy at the time of transplantation, before endomyocardial biopsy grades.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC135

Characterisation of CXCR7, CXCR3 and atypical on Mast Cells and other leukocytesI. Willox¹, J. Westwick², I. Lindley³, I. Mirkina³ and S. Ward¹¹University of Bath, Bath, UK, ²Norvatis, Horsham, UK and ³Norvatis, Vienna, Austria

Mast cells are multifunctional, tissue-dwelling cells that have been traditionally associated with the allergic response. There is a growing interest in the role of chemokines and their receptors in mast cell tissue localization and function. The chemokines CXCL9, CXCL10, and CXCL11 interact with CXCR3 which is highly expressed on mast cells and monocytes. CXCR3 is a pertussis toxin sensitive, G protein-coupled receptor that is known to exist in at least 2 other isoforms, namely CXCR3-B and CXCR3-alt. CXCR3-B is thought to be pertussis toxin insensitive and also interacts with another chemokine, CXCL4. We have evidence derived from the monocytic cell line model, THP-1, that CXCL4 induces chemotaxis and activates actin polymerisation in a pertussis toxin sensitive manner, suggesting that CXCR3-B is actually pertussis toxin sensitive.

Using cultured mast cells from CD133+ precursors derived from human cord blood we are investigating the expression of CXCR3 versus variant CXCR3 isoforms. To better understand how CXCR3 ligands can achieve selectivity, we are exploring differences in the phosphorylation profile of proteins mediated by the PI3K/PKB pathway and receptor tyrosine kinases using phosphoproteomic approaches as well as differences in Ca²⁺ influx/release. We are also exploring potential differences in functional responses such as degranulation and actin polymerisation. Recent evidence has discovered that in addition to CXCR3, CXCL11 also interacts with another chemokine receptor CXCR7. We have found that whilst CXCR7 is highly expressed on the mast cell line HMC-1, it is not detectable on the surface of human cord blood mast cells.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC136

Evidence of a PI3-K independent role for PKB/Akt in T Lymphocyte ChemotaxisA. Webb¹, D. Cronshaw¹, T. Crabbe² and S.G. Ward¹¹Pharmacy and Pharmacology, University of Bath, Bath, UK and ²UCB, Slough, UK

Chemokine directed migration of T lymphocytes is critical for the adaptive immune response. The phosphoinositide 3-kinase (PI3-K) signalling pathway is pivotally involved in directional cell migration of neutrophils and monocytes. However, data suggests that PI3-K is a dispensable signal in T lymphocyte migration. Protein kinase B (PKB)/Akt is a major proximal downstream component of the PI3-K dependent signalling cascade, however due to poor selectivity of inhibitors the role of PKB has been difficult to explore pharmacologically. In 2005, a new

class of PH domain-dependant inhibitors were described that show greater than 50-fold selectivity for Akt 1/2 over other closely related AGC family kinases. This inhibitor, Akti-1/2 (Calbiochem), was used to determine whether PKB/Akt was also dispensable in T lymphocyte signalling. Addition of the Akt inhibitor abrogated chemotaxis in primary and CEM cell-line models of PI3-K independent chemotaxis (IC₅₀ 9.15 µM and 7.3 µM respectively). Further pharmacological analysis using Ro-32-0432, which preferentially inhibits conventional and novel PKC isoforms, has revealed a potential role for protein kinase C in the phosphorylation of PKB/Akt. In summary, our data indicates a potential PI3-K independent role for PKB/Akt in T lymphocyte chemotaxis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC138

Sex differences in leukocyte flux: Implications for endothelium-derived hyperpolarising factorI.C. Villar¹, R.S. Scotland¹, A.J. Hobbs² and A. Ahluwalia¹¹William Harvey Research Institute, London, UK and ²University College London, London, UK

Introduction: Sex hormones underlie the lower incidence of cardiovascular disease in pre-menopausal women. Vascular inflammation is involved in the pathogenesis of several cardiovascular diseases and is dependent, in part, on immune cell recruitment, for which leukocyte rolling is a crucial step. We have demonstrated that a sex-specific prevalence of endothelium-derived hyperpolarising factor (EDHF) underlies the protection of females against hypertension (1). Herein, we assessed whether sex differences in leukocyte rolling might contribute to cardio-protection in females and the potential role of EDHF.

Material and Methods: Wild-type (WT; C57BL/6), endothelial NO synthase knockout (eNOS KO), cyclooxygenase-1 (COX-1) KO and eNOS/COX-1 double KO (dKO) mice, and ovariectomized (OVX) and sham-operated control (SHAM) animals (15–20g) were anaesthetised. The mesenteric vascular bed was exteriorised and prepared for intra-vital microscopy. Mice were pretreated with saline or interleukin-1β (IL-1β, 5ng.i.p.) 90min or 4h prior to analysis. One to three randomly selected post-capillary venules (diameter 20–40 µm, length > 100 µm) were observed for each mouse; measurements were taken 5–10min after exposure of the chosen vessels. The extent of basal and IL-1β-elicited leukocyte rolling was analyzed by counting the number of cells passing a fixed point per minute.

Results: Basal leukocyte rolling in male eNOS KO, COX-1 KO and dKO was elevated compared to female WT (30.3±5.5, 30.7±8.8 and 25±5.5 vs 12.6±2.8, respectively; n≥8; all P<0.05). The same profile was apparent in female COX-1 KO mice (24.3±2.9 vs 4.7±0.7; n≥6; P<0.05) whereas leukocyte rolling in female eNOS KO was not different to WT controls (7.4±1.4 vs 4.7±0.7; n≥6; P>0.05). In IL-1β-treated animals (90min), leukocyte rolling was significantly increased in male COX-1 KO mice compared to WT mice (72.6±16.2 vs 31.2±9.7; n≥4; P<0.05) while in female eNOS KO, COX-1 KO and dKO mice the rolling was

significantly reduced compared to males with the corresponding genotype (18.2 ± 3.6 vs 39.7 ± 11.3 , 20.1 ± 8.1 vs 72.6 ± 16.2 and 13.9 ± 6.1 vs 35.5 ± 12.5 , respectively; $n \geq 4$; $P < 0.05$). In IL-1 β -treated dKO, this difference persisted at 4h (male 13.2 ± 4.1 vs female 5.7 ± 0.7 ; $n \geq 4$; $P < 0.05$). OVX mice showed an increase in leukocyte rolling compared to control SHAM (26.1 ± 10 vs 10.4 ± 1.8 ; $n = 5$; $P < 0.05$).

Conclusion: These results indicate that females show a reduction in basal and IL-1 β -stimulated leukocyte rolling compared to males. Basally, leukocyte rolling in females is modulated by COX-1; however, in the presence of an inflammatory stimulus this control is exerted by EDHF. Thus, sex hormones might be implicated in female protection against vascular inflammation.

(1) Scotland RS *et al.* (2005). *Proc Natl Acad Sci USA* **102**, 14452-14457.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC139

Galectin-1: an endogenous inhibitor of neutrophil recruitment

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Galectins are structurally related proteins characterised by an affinity for β -galactosides. Galectin-1 (Gal-1), the prototype member of this family, decreases neutrophil recruitment to sites of inflammation *in vivo* (1) and inhibits neutrophil chemotaxis *in vitro* (2). Endothelial cells express Gal-1 in both cytosolic and membrane compartments, as well as releasing it into culture medium. This expression is increased upon exposure to pro-inflammatory cytokines (3). Thus endothelial-derived Gal-1 may function to limit leukocyte recruitment during inflammation. The objectives of this study were to investigate the effects of exogenous and endogenous Gal-1 on neutrophil recruitment under flow *in vitro* and leukocyte recruitment in the inflamed cremaster *in vivo*.

To investigate the effects of exogenous Gal-1, human umbilical vein endothelial cells (HUVEC) were stimulated with TNF- α (10ng/ml) for 4h. Freshly isolated neutrophils were incubated with human recombinant Gal-1 (0.04-4 μ g/ml) and perfused over the endothelial monolayers at a rate of 1 dyn/cm². To investigate the role of endothelial Gal-1, small interference RNA (siRNA) was used to deplete Gal-1 in HUVEC. Cells were transfected with non-targeting or Gal-1 specific siRNAs. Gal-1 expression, monitored by Western blotting was maximally suppressed by 75% at 48h post-transfection. HUVEC were then stimulated with TNF- α as described above and neutrophils were perfused over the monolayers at a rate of 1 dyn/cm². Six random fields were examined and the numbers of captured, rolling and adherent neutrophils were quantified off-line. To determine the effect of Gal-1 on leukocyte trafficking *in vivo*, Gal-1 null mice and their wild-type counterparts were treated with 30ng IL-1 β (intrascrotal) for 2-6 h followed by intra-vital microscopy of the cremaster (mice were anaesthetised with xylazine (7.5 mg/kg) and

ketamine (150 mg/kg) i.p.). The numbers of rolling, adherent and emigrated leukocytes were quantified in a minimum of three vessels/mouse.

Exogenous Gal-1 produced concentration-dependent effects with the lowest concentration (0.04 μ g/ml) significantly decreasing all three parameters measured ($p < 0.05$, $n = 4$). At 0.4 μ g/ml, neutrophil capture was significantly decreased by 49% ($p < 0.05$, $n = 4$), while at 4 μ g/ml, rolling was decreased by 94% ($p < 0.001$, $n = 4$). Conversely knock-down of endothelial Gal-1 resulted in significant increases in neutrophil capture and rolling by 89% and 66%, respectively ($p < 0.05$, $n = 3$). *In vivo*, a lack of Gal-1 resulted in significant increases in leukocyte emigration at 2 and 6h post IL-1 injection ($p < 0.05$, $n = 4-7$ /group). These data indicate a role for both exogenous and endogenous Gal-1 in limiting leukocyte recruitment during inflammation.

Rabinovich GA *et al.* (2000). *Eur J Immunol* **30**, 1331-1339.

La M *et al.* (2003). *Am J Pathol* **163**, 1505-1515.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC140

Characterisation of expression and functionality of atypical CXCR3 receptor on human T lymphocytes

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The chemokine receptor CXCR3 is predominantly expressed on activated T lymphocytes. Its agonists, IFN- γ - inducible chemokines; CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC are involved in Th1-type inflammatory responses such as multiple sclerosis, rheumatoid arthritis, psoriasis, and sarcoidosis. There is a growing body of evidence supporting the existence of splice variants and/or modified CXCR3 receptors such as CXCR3-b and CXCR3-alt which is a limitation in designing antagonists which block CXCR3 function.

Interaction of CXCR3 with its ligands induces cellular responses in leukocytes such as chemotactic migration and activation of multiple signaling pathways, including the PI3K pathway, which is thought to be important in directional movement towards a chemokine gradient. In addition, CXCR3 is internalised in response to interaction with its ligands, of which, CXCL11 has been shown to be the most potent inducer of internalisation. In contrast to CXCR3, CXCR3-b has been described as PTX insensitive and unable to induce calcium flux. CXCL4/PF4 has been found to be an additional ligand with unique selectivity toward CXCR3-b. We have detected CXCR3-b expression on primary human T cells at both mRNA and protein level but were unable to induce any chemotaxis towards CXCL4 in human T cells. However stimulation of T cells with CXCL4 resulted in phosphorylation of PKB/Akt as well as Erk1/2 pathways suggesting that CXCR3-b is coupled to downstream biochemical

machinery. Surprisingly the biochemical signals were sensitive to PTX treatment, suggesting that coupling is dependant on G alpha i/o proteins.

We are currently investigating ability of CXCL4 to cause receptor internalisation and whether distinguished signaling "finger-prints" are associated with CXCR3 and its variants.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC141

Annexin 1 presence in human PMN ectosomes correlates with inhibitory actions under flow

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It is now evident that cell activation is associated with rapid plasma membrane re-arrangements; the production of small particles, often released, would then occur: they have been named microvesicles or ectosomes. Importantly, they are not just by-products but, rather, they seem to display important biological functions. The recent notion that ectosomes from human PMN are anti-inflammatory whereas those obtained from other leukocyte sources are pro-inflammatory [1], prompted us to test whether this dichotomy was associated with presence of the endogenous annexin 1 (AnxA1). This pro-resolution and homeostatic factor induced by glucocorticoids, highly abundant in human PMN: ~60-70% of cell associated AnxA1 contained in subcellular organelles, acts an important brake signal on their activation and trafficking.

Ectosomes were produced by PBMC or PMN (50 million per experiment) incubation with HUVEC monolayers in the presence of 1 μ M fMLP (60 min at 37°C). Ectosomes were then separated from the cell supernatant by ultracentrifugation and their content analysed by FACS analysis, Western Blotting and Mass Spectrometry (Q-TOF) techniques. The presence of a specific signal for AnxA1 in PMN ectosomes, and not PBMCs, was observed by FACS analysis. Western blot analysis confirmed the presence of a distinct double band at 33 and 37 kDa, likely corresponding to the cleaved and uncleaved forms of the protein. Mass spectrometry further confirmed the presence of AnxA1 as the protein with the highest MASCOT score upon in-gel digestion and analysis of the 37kDa band.

Importantly, the anti-inflammatory effect of PMN-derived ectosomes was studied using a flow chamber apparatus, where a marked attenuation of cell rolling and adhesion was observed upon 5-10 min pre-incubation with 40-50 thousand ectosomes (>50% reduction; n=3, P<0.05). Of interest, this inhibitory effect was partially blocked by a neutralizing antibody against the AnxA1 receptor (also termed FPRL1) as well as by a peptide antagonist (peptide WRWWWW). These results indicate that AnxA1 is likely to be responsible for the inhibitory properties of PMN-derived ectosomes; we propose this represents a novel and exciting anti-inflammatory mechanism for the roles played by this endogenous anti-inflammatory mediator and may have potential clinical implications.

Gasser *et al* (2004). *BLOOD* 104, 2543-2548

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC142

Montelukast inhibits both resting and GM-CSF-stimulated eosinophil adhesion to VCAM-1 under flow conditions

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Introduction

Inappropriate accumulation of eosinophils and the subsequent release of their potent armoury of mediators are thought to contribute significantly to the airway inflammation underlying asthma pathogenesis. Montelukast (MLK) is a cysteinyl leukotriene receptor-1 (CysLT1) antagonist that is an effective treatment for asthma and other allergic conditions. However, MLK may possess anti-inflammatory effects independent of CysLT1 antagonism.

Aims

We assessed real-time resting and GM-CSF-stimulated eosinophil adhesive behaviour together with effect of MLK on eosinophil adhesion to recombinant human vascular cell adhesion molecule-1 (rhVCAM-1) at different rates of flow using a novel microflow system.

Methods

The flow system consists of a syringe pump and flow sensor controlled by a PC using dedicated software linked to a microfluidic biochip with 8-channels whose dimensions mimic the post-capillary venules [1]. The microfluidic syringe pump gives very accurate flow rates that are more reproducible and consistent than anything currently available and those used in this study (1-5 dyn cm⁻²) mimic those found in the post-capillary venules during inflammatory processes. Eosinophils were purified using our standard technique using dextran sedimentation and centrifugation on Percoll gradients followed by CD16-dependent negative immunomagnetic selection [2]. Resting or GM-CSF-stimulated eosinophils were pre-incubated with a concentration range of MLK before being pumped through the VCAM-1-coated channels at increasing rates of shear stress. Adherent eosinophils were microscopically recorded in real time and analysed using DucoCell software.

Results & Conclusions

At 1 or 2 dyn cm⁻² shear stress resting eosinophils either tethered immediately to rhVCAM-1, "rolled" along part of the channel until tethering took place, or roll without tethering. At flow rates greater than 2 dyn cm⁻², adherent eosinophils began to be displaced from rhVCAM-1. MLK (10nM and 100nM) significantly inhibited resting eosinophil adhesion to rhVCAM-1 at 2 dyn cm⁻² shear stress (40.5±9.9% and 47.8±9.4% inhibition respectively; P<0.05). GM-CSF-stimulated eosinophil adhesion at 2 dyn/cm² was characterised by

greater cell flattening with significant inhibition by 10nM MT ($22.2 \pm 5.4\%$, $P < 0.05$ compared to control). These findings demonstrate inhibition of eosinophil adhesion to VCAM-1 by MLK under shear stress at physiologically relevant concentrations indicating a potential anti-asthmatic action independent of CysLT1 antagonism.

[1] Williams et al, J Assoc Lab Automation, 7,135;2002

[2] Wu et al, Clin Exp Allergy, 35,1073,2005

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC143

Characterisation of heteromeric Kir4.1/Kir5.1 channels from *Xenopus tropicalis*

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Potassium channels are formed from four subunits, each of which contributes to the K⁺-selective pore. In mammals, the inwardly-rectifying (Kir) potassium channel subunits Kir4.1 and Kir5.1 co-assemble to form novel pH-sensitive heteromeric channels which show unique single channel bursting behaviour and sub-conductance states.

In this study, we have identified orthologs of Kir4.1 and Kir5.1 from *Xenopus tropicalis* (XT) where the XTKir4.1 shares >90% identity with rat Kir4.1, and XTKir5.1 an overall 63% identity with rat Kir5.1, which increases to >80% if the more divergent distal termini are excluded.

Electrophysiological analysis of co-expressed XTKir4.1/XTKir5.1 channels revealed them to have an almost identical pH-sensitivity to the mammalian channels, but that their rate of time-dependent activation at hyperpolarising potentials was markedly different. However, the principal difference is in their kinetic behaviour. We found that the subconductance states in XTKir4.1-XTKir5.1 heteromeric channels have a much longer duration. In particular, they have a long S1 sublevel opening which is almost 15 times of the average dwell time duration of the S1 sublevels observed in the rat Kir4.1-Kir5.1 channels (7.5ms vs 0.5ms). We then created a ratKir4.1-XTKir5.1 dimer in order to assess whether this difference is conferred by the XTKir5.1 subunit. We found that ratKir4.1-XTKir5.1 heteromeric channels also exhibit these markedly long S1 sublevels, thus confirming that the XTKir5.1 subunit is responsible for this difference.

These novel channels will therefore provide a greater insight into the mechanism of K⁺ channel gating at the single molecule level and help in the identification of the domains and/or residue(s) responsible for controlling the transitions between these states. This will have a major impact on our understanding of the structural basis of the sublevel transitions which occur during channel opening in this and other K⁺ channels.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC144

TWIK-2 expression in human intrauterine tissues

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Potassium (K⁺) channels comprise the largest ion channel family with channel structure, function and modulation exhibiting considerable diversity. One particular subfamily, characterised by the presence of two conduction pores and four transmembrane domains (2P/4TM; K2P), generates a leak conductance, which contributes to repolarisation and maintenance of resting membrane potential.

A number of K2P channels are expressed in the human placenta (Bai et al., 2005a) and uterus (Bai et al., (2005b) but there is little information on the tissue distribution of TWIK-2 between the non-pregnant and pregnant state. During pregnancy, paracrine and autocrine interactions maintain the uterus in an electrically inexcitable state in order to promote fetal growth and development. We have studied the tissue distribution of TWIK-2 (Tandem of pore domains in Weak Inward rectifier K⁺ channels-2), a particular K2P that is expressed in peripheral tissues and is sensitive to internal acidification but insensitive to the classical K⁺ channel blockers TEA and 4-AP.

Myometrial samples and placentae were obtained at elective caesarean section. Placentae were immediately separated into amnion, chorion, decidua and villous constituents. Endometrium and myometrium from non-pregnant, normally cycling women were taken at hysterectomy. All samples were rapidly snap-frozen for western blotting investigations. Patients gave fully informed written consent and the study had local ethics approval.

Crude tissue lysates were prepared from uterine and placental tissues following homogenisation and centrifugation. Proteins were then resolved by SDS-PAGE and transferred onto nitrocellulose. After blocking in 7% non-fat milk and washing, membranes were probed using an anti-TWIK-2 antibody (1:250, Alomone Labs, Israel) followed by incubation in alkaline-phosphatase linked secondary antibody and detection by chemiluminescence (Immunstar, BioRad, UK).

Our results demonstrate the presence of a 34 kDa band consistent with expression of TWIK 2. Highest expression was observed in non-pregnant and pregnant myometrium with endometrium, decidua, chorion and cytotrophoblast also exhibiting significant amounts. TWIK-2 protein was undetectable in amnion. The presence of large amounts of TWIK-2 in myometrium suggests that it may be important in dampening contractions until labour thereby mediating gestational quiescence. Its presence in other intrauterine tissues may indicate a role for it as a molecular sensor of pH. Studies are underway in order to characterise TWIK-2 function in these tissues.

Bai et al., (2005) J Soc Gynecol Invest 12, 77-83.

Bai et al., (2005) Reproduction 129, 525-530.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

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PC145

Resolution of block by apamin and extracellular acidosis of expressed wild type and mutant SK2 and SK3 channels

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Macroscopic whole-cell current and inside-out patch recording under isotonic K⁺ conditions from tsA-201/HEK cells transiently expressing homomeric rSK2 and hSK3 channels was used to study block by apamin and extracellular acidosis. Inside-out patches from cells expressing rSK2 channels were bathed in a solution containing 1 mM Ca²⁺ and displayed two amplitude levels corresponding to conductances of approximately 10 and 18 pS, with the larger amplitude being more common. Rare transitions between amplitude classes were apparent, suggesting that the lower amplitude was a sub-conductance state. Channels exhibited bi-exponential open time distributions with time constants of ~ 1.8 and 12 ms and closed time distributions best described by 3 exponentials (τ 's ~1.5, 18 and 100 ms). The prevalence of low amplitude openings was enhanced by extracellular acidosis suggesting that protonation of a residue in the outer pore reduced conduction through the channel.

Inclusion of a supra-maximal concentration of apamin (100 nM) in the electrode solution produced patches that displayed a lower activity, which resulted from the recruitment of an additional closed state (τ ~750–1000 ms). In addition, remaining channel openings were predominantly of low amplitude (10 pS) that displayed prolonged open times. Mutation of a residue in the outer pore of rSK2 produced a channel that displayed only low amplitude openings of approximately 10 pS conductance. This corresponded to the low conductance state of the wild type rSK2 channel promoted by apamin, implying an impairment of the high amplitude conductance pathway that mimicked the effect produced by acidosis. Whole cell currents evoked from cells expressing mutant rSK2 were insensitive to acidosis or apamin indicating this residue was critical for block of macroscopic current. hSK3 inside out patches displayed openings of similar conductance levels to rSK2. Comparison of homomeric hSK3 and rSK2 whole cell currents revealed a similar level of block by apamin (~55%), but significant differences in the block by acidosis. This suggested that SK3 may possess an additional interaction site. Apamin and extracellular protons reduced the macroscopic current by interacting with an outer pore residue in rSK2 and hSK3, which is proposed to be involved in stabilising the conduction pathway. In addition, data is presented suggesting that SK3 has an extra block under acidosis due to an additional interaction site.

PC146

Proarrhythmogenic effects of Kir2.1 and KvLQT1 familial gene mutations in human atrium: a computational study

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Proarrhythmogenic effects of two gene mutations were evaluated computationally on genesis and maintenance of familial atrial fibrillation (AF). These were the Kir2.1 V93I mutation (GM1) and the KvLQT1 S140G mutation (GM2), that produce gain-in-function of the inward rectifier K⁺ current, I_{K1}, and of the slow delayed rectifier K⁺ current, I_{Ks} respectively. Electrophysiological changes induced by GM1 in I_{K1} (Xia et al. [1]) and GM2 in I_{Ks} (Chen et al. [2]) were incorporated into a cellular model of the human atrial action potential [3]. Changes in action potential duration (APD), APD restitution (APDR) and effective refractory period (ERP) were quantified by standard S1-S2 protocols. Rate-dependence of intra-atrial conduction velocity (CV) and the temporal vulnerability (VW) were computed in 1D models. Using 2D models, the dynamical behaviour of reentrant waves was characterised by tip meander, stability and lifespan.

With both GM1 and GM2, atrial APD and ERP were remarkably abbreviated. The computed APD decreased from 323 ms in control to 258 ms (30% reduction) with GM1 and 22.2 ms (93% reduction) with GM2 (Figure 1). APDR was flattened: maximal slopes were 2.3, 0.46 and 0.011 in control, GM1 and GM2 respectively. Intra-atrial CV with a solitary wave was decreased from 0.27 m/s in control to 0.255 m/s with GM1 and 0.259 m/s with GM2. Both mutations facilitated high rate atrial excitation/conduction – the measured maximal rate of atrial excitation/conduction was 196, 240 and 1000 beats/min for control, GM1 and GM2, respectively (Figure 2). Both mutations reduced the VW (from 16.5 ms in control to 13.7 ms with GM1 and 7.59 ms with GM2), but sustained reentrant excitation. In 2D simulations, reentry self-terminated in control (LS, ~1400 ms), but persisted with GM1 and GM2. With GM1, a reentrant spiral wave degenerated into multiple stable spiral wavelets with a maximum of seven co-existing wavelets. With GM2, a reentrant spiral wave presented as a stable and persistent mother rotor with its tip meandering in a small tissue region. We conclude that both mutations reduce atrial APD and slow down intra-atrial conduction (producing a shortened wavelength of atrial excitation waves), which helps to stabilise atrial reentry and enable co-existence of multiple wavelets. Though the tissue's vulnerability to genesis of reentry is decreased by both mutations, the lifespan of reentry is remarkably increased leading to persistent AF. This study provides insights into understanding the genesis and maintenance of chronic AF in patients with the two gene mutations.

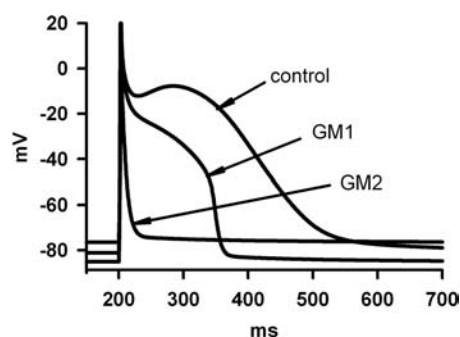


Figure 1. Action potential profiles in control, GM1 and GM2.

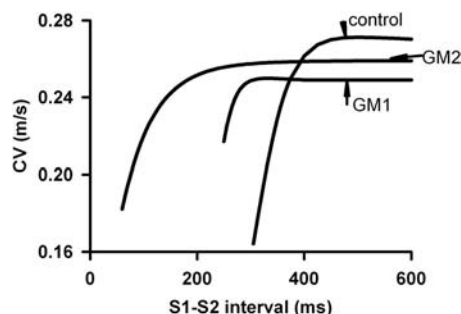


Figure 2. CV restitution curves for control, GM1 and GM2.

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PC147

Screening BKCa channel splice variants using voltage-sensitive dyes

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Large conductance voltage and calcium-activated potassium (BKCa) channels link electrical excitability with cellular signalling. A single gene (KCNMA1 or slo) encodes the α -subunits that form the native BKCa channel pore. Alternative pre-mRNA splicing of the α -subunits determines the channel phenotype, including its intrinsic voltage dependence and calcium sensitivity (Chen et al., 2005). The identification and characterization of BKCa channel splice variants depends on the use of low throughput patch clamp electrophysiology. Here we present a robust, high throughput assay to screen BKCa splice variants, which was initially optimized using the voltage-sensitive dye

Membrane Potential Red (Molecular Probes), the automated fluid-handling fluorometer FlexStation II®, and HEK 293 cells transiently or stably transfected with the stress-related exon (STREX) BKCa channel splice variant. As BKCa channels are activated by elevation of intracellular free calcium, ionomycin (a calcium ionophore) was used to stimulate channel activity via influx of calcium ions. Ionomycin (1 μ M) induced a significant prolonged hyperpolarisation in HEK cells transfected with the STREX variant but not in untransfected controls. The hyperpolarizing response was attenuated by the removal of extracellular calcium and was blocked by the BKCa channel inhibitor paxilline with an IC₅₀ of $0.23 \pm 0.05 \mu$ M (n=15). The robustness of the assay allowed us to ask whether we could discriminate between BKCa channel splice variants with known differences in calcium sensitivities. To test this we used the STREX, ZERO, and e22 splice variants that differ in the alternatively spliced inserts present at splice site 2 in the c-terminus of the α -subunit and as a result display a range of calcium sensitivities. In accordance with previous electrophysiological assays the voltage-sensitive dye assay revealed that expression of the STREX variant produced the greatest hyperpolarizing response to ionomycin. e22 and ZERO produced significantly smaller responses, of $64 \pm 17\%$ and $45 \pm 13\%$ respectively, when compared to the STREX response (taken as 100%) with the rank order in agreement with electrophysiology assays (n=8, data are mean $\pm$ sem p<0.001 ANOVA with Tukey post-hoc test). Expression of the different BKCa channel splice variants had no significant effect on the ionomycin-induced calcium influx in HEK293 cells as measured using Calcium FLIPR 3A (Molecular Probes).

This approach allowed reproducible high throughput data to discriminate between BKCa channel splice variants that can be applied to the analysis of BKCa channel splice variant regulation, cell surface expression and pharmacology.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC148

Extracellular H62 and D197 mediate functional inhibition of P2X₇ receptor by zinc

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One of unique features that distinguish P2X₇ receptor from the other P2X receptors is that divalent cations such as zinc potently inhibit its functional responses (North, 2002). This has been long and widely interpreted, in the case of calcium and magnesium, as ATP⁴⁻ being the efficient agonist form (Tatham et al., 1988) although not conclusively proved. There is suggestive evidence that the inhibition by divalent cations is via direct binding and

allosteric modulation of the receptor (Virginio et al., 1997). To provide direct molecular evidence for the latter mechanism, this study was to aim to identify the residues mediating functional inhibition of the P2X₇ receptor by zinc.

There are fourteen potential residues in the extracellular domain that are conserved among the P2X₇ but other P2X receptor subunits. Alanines were singly substituted by site-directed mutagenesis into each position of the rat P2X₇ receptor subunit. Wild-type (WT) and mutant P2X₇ receptors were expressed in human embryonic kidney (HEK) 293 cells and studied by whole cell patch-clamp technique with a holding potential of -60 mV. At WT receptor, zinc (0.1 µM to 100 µM) inhibited the currents evoked by 30 µM 2'-3'-(O)-(4-benzoyl) benzoyl ATP (BzATP) in a dose-dependent manner with an IC₅₀ value of 4.1 ± 0.6 µM and Hill coefficient (n_H) of 0.8 ± 0.1 (n = 5). Interestingly, strong rebound currents appeared when simultaneously washing zinc and BzATP, suggesting that zinc directly binds P2X₇ receptor and that zinc dissociates from P2X₇ receptor much faster than BzATP. Zinc at 30 µM inhibited all the mutants to a similar degree as the WT receptor except H62A and D197A. The inhibition at H62A and D197A was respectively 29.7 ± 3.5% (n = 7, p < 0.001, unpaired Student's t-test) and 55.4 ± 3.4% (n = 5, p < 0.05), significantly less than that at WT receptor (88.0 ± 2.5%, n = 5). For the double mutant, H62A/D197A, there was no significant inhibition by zinc: the currents in the presence of 30 µM and 100 µM zinc were 315.3 ± 160.2 pA and 321.7 ± 161.4 pA, respectively, which were not different from the control (352.3 ± 158.9 pA, n = 5; paired Student's t-test). In addition, the rebound currents were significantly reduced or completely abolished at D197A, H62A and H62A/D197A mutants. These results indicate that H62 and D197 are the key residues mediating the functional inhibition. Therefore the study provides clear evidence supporting that the zinc inhibition of P2X₇ receptor results primarily from direct binding and allosteric modulation instead of the long-standing assumed mechanism of reducing the effective agonist concentration by its chelating ability.

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PC149

Properties of voltage-dependent Mg²⁺ block in rat recombinant chimaeric NMDA receptors expressed in *Xenopus laevis* oocytes

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N-methyl-D-aspartate receptors (NMDARs) are composed of NR1 and NR2 subunits most likely arranged in a dimer of dimers configuration (Furukawa et al. 2005; reviewed in Chen & Wyllie, 2006). These receptor-channels exhibit characteristic voltage-dependent channel block by Mg²⁺ ions, the sensitivity of a

particular receptor combination being determined by the identity of the NR2 subunits present, with NR2A-containing NMDARs being the most sensitive and NR2D-containing NMDARs being the least sensitive to Mg²⁺ block (Monyer et al. 1994; Kuner & Schoepfer, 1996). In this study we have examined voltage-dependent Mg²⁺ block using two-electrode voltage-clamp recordings from *Xenopus laevis* oocytes expressing wild-type NR2A subunits or chimaeric NR2A-NR2D subunits where either the S1 and S2 ligand-binding domains (LBD) of the NR2A subunit were replaced by the S1 and S2 LBDs of the NR2D subunit or the first three membrane associated regions of the NR2A subunit were replaced by the equivalent regions of the NR2D subunit. All NR2 subunit constructs were co-expressed with the NR1a subunit. Concentrations of Mg²⁺ ions required to inhibit glutamate-evoked responses by 50 % (IC₅₀ values) at holding potentials of -80 mV, -60 mV and -40 mV were determined for wild-type and chimaeric receptor combinations and the results obtained shown in Table 1 below. The data indicate that when the M1-M3 regions of the NR2A subunit are substituted with the corresponding regions of the NR2D subunit there is a reduction in the sensitivity of such receptors to Mg²⁺ ions. Intriguingly, expression of the LBD of the NR2D subunit in the NR2A subunit increases Mg²⁺ potency compared to wild-type NR2A-containing NMDARs (P < 0.01). Current-voltage plots for glutamate-evoked currents, in the presence of 1 mM Mg²⁺, showed the typical J-shape profile for each of the constructs examined. Thus, in addition to the well-described role of the pore-forming regions, it appears that the LBD of different NMDAR subunits also influences the potency of Mg²⁺ block at these receptor-channels.

Table 1.

Receptor combination	IC ₅₀ (-80 mV) (n)	IC ₅₀ (-60 mV) (n)	IC ₅₀ (-40 mV) (n)
NR2A(WT)	34 ± 4 µM (15)	157 ± 24 µM (15)	701 ± 84 µM (15)
NR2A(S1S2NR2D)	21 ± 2 µM (24)	81 ± 7 µM (24)	325 ± 23 µM (24)
NR2A(M1-M3NR2D)	335 ± 27 µM (11)	1.3 ± 0.2 mM (11)	4.7 ± 0.5 mM (11)
NR2A(S1S2M1-M3NR2D)	197 ± 31 µM (15)	548 ± 90 µM (15)	1.7 ± 0.4 mM (15)

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PC150

Discrete regions of the P2X₇ receptor confer differential sensitivity to ATP and BzATP

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P2X₇ is an ATP-gated cation channel expressed primarily in cells of the immune system, where brief activation couples to pro-inflammatory cytokine release and prolonged activation leads

to cell death. Uniquely for P2X₇, the agonist 2', 3'-(benzoyl-4-benzoyl) ATP (BzATP) is more potent than ATP; in addition, striking differences in ATP and BzATP potency are observed between receptors from different species. To address the basis of differential agonist sensitivity, we firstly compared the ATP- and BzATP-induced currents of wild-type rat and mouse P2X₇ expressed in HEK-293 cells. Mouse P2X₇ displayed an 8-fold lower sensitivity to ATP (EC₅₀ values of 936±21µM compared to 123±4µM) and an 80-fold lower sensitivity to BzATP than rat P2X₇ (EC₅₀ values of 285±16µM compared to 3.6±0.2µM). Secondly, we analysed the agonist sensitivities of rat/mouse receptor chimaeras. We found that transposition of two segments of the ectodomain of rat P2X₇ (115-136 and 282-288) into mouse P2X₇ converted mouse P2X₇ agonist sensitivities to those of rat P2X₇. Finally, single point mutagenesis within these regions revealed two critical amino acids involved in agonist sensitivity. Substitution of asparagine-284 of the rat receptor into mouse P2X₇ (D284N) restored the level of ATP sensitivity to that of rat P2X₇ (EC₅₀ of 146±11µM). We demonstrated that this mutation increased the apparent molecular mass of the receptor from that of mouse P2X₇ (75kDa) to that of rat P2X₇ (78kDa), due to the incorporation of an additional N-glycosylation acceptor sequence (already present in rat P2X₇). We suggest that N-glycosylation at this position stabilises the structure of the receptor, and effects an increase in ATP potency through more efficient channel gating. Substitution of lysine-127 into mouse P2X₇ (A127K) enhanced BzATP but not ATP sensitivity (EC₅₀ values of 80±7µM and 815±56µM), suggesting a direct role for this residue in BzATP binding. A mouse P2X₇ construct bearing both mutations (A127K/D284N) was indistinguishable from rat P2X₇ in terms of ATP and BzATP sensitivity (EC₅₀ values of 112±11µM and 5±1µM respectively), demonstrating that lysine-127 and asparagine-284 are sufficient to confer rat-like ATP and BzATP sensitivity to mouse P2X₇.

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PC151

Gating of a ClC chloride channel by voltage-dependent proton influx into the selectivity filter

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Membrane channels and transporters involved in moving ions into and out of cells are believed to function in fundamentally different ways. Ion channel function requires a continuous aqueous pore through the protein to allow ion migration by electrodiffusion plus some mechanism for opening and closing this pathway, the gate. Transporters, on the other hand, require at least two gates never simultaneously open that temporarily trap the ion within the pore. As might be expected from their functional differences, ion channels and transporters belong to different protein families. An exception to this rule occurs in the

ClC transport protein family that, surprisingly, has both Cl⁻ ion channel and H⁺-coupled Cl⁻ transporter members. The strong conservation in structure within the ClC family suggests a degree of conservation in their molecular mechanisms (Dutzler, 2006). Available ClC structures reveal a selectivity filter providing a transmembrane pathway which is obstructed at its external opening by a highly conserved glutamate residue side-chain. A displacement of the selectivity filter glutamate clears the pathway to ion passage and is believed to be the process gating the pore in ClC channels. In the transporters, the equivalent residue serves to capture a proton to be translocated in exchange for Cl⁻ across the plasma membrane in a cycle of conformational changes not yet well defined. Here we show what appears like a partial reaction of such cycle accounting for the gating of ClC-2, a Cl⁻ channel member of the ClC family (Niemeyer *et al.*, 2003; Yusef *et al.*, 2006). We demonstrate that ClC-2 channels, which are activated by hyperpolarisation, require protonation of the gate glutamate (E207 in ClC-2) in order to open. A Woodhull-type analysis suggests that E207 lies well within the electrical field of the membrane, making its neutralisation markedly voltage-dependent consistent with H⁺ entering the selectivity filter to effect activation, but not permeating across. Intracellular Cl⁻, which also has an effect upon gating, acts in a permissive rôle, modulating the pH-dependence of channel opening in a voltage-independent manner. Our data are consistent with a model in which hyperpolarisation activates ClC-2 Cl⁻ channels by promoting H⁺ partial crossing of the selectivity filter to neutralise the gate glutamate residue thus allowing Cl⁻ permeation. This model is consistent with the idea that ClC channels might have evolved from their exchanger counterparts conserving partial reactions of their transporter cycle.

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PC152

An intracellular P2X receptor required for osmoregulation in amoeba

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P2X receptors are cell membrane ion channels gated by extracellular ATP¹. They are widely distributed in vertebrates, having roles in pain and inflammatory processes, but previously unidentified in protozoa. We identified a weakly related gene (*p2xA*) in the genome of the social amoeba *Dictyostelium discoideum*, which putatively encoded a P2X receptor with less than 8% identity compared to its vertebrate counterparts. Whole-cell and outside-out patch recordings from HEK293 heterologously expressing

p2xA revealed robust desensitising inward currents evoked by ATP (EC_{50} $176 \pm 5 \mu\text{M}$) with a unitary conductance of 8.2 pS (-100 mV), demonstrating that the gene encoded a functional P2X receptor. The receptor displayed characteristics of a non-selective cation channel, being permeable to Ca^{2+} ($P_{\text{Ca}}/P_{\text{Na}} = 1.5 \pm 0.3$, $n = 8$) but relatively impermeable to NMDG ($P_{\text{NMDG}}/P_{\text{Na}} = 0.21 \pm 0.2$, $n = 8$). The receptor was activated by α,β -methyleneATP, β,γ -methyleneATP, β,γ -imidoATP and benzoyl-benzoylATP (EC_{50} 147 ± 8 , 515 ± 12 , $14 \pm 3 \mu\text{M}$, and $> 1 \text{ mM}$ respectively), but was insensitive to usual P2X antagonists (PPADS, suramin, TNP-ATP), though blocked by nanomolar copper (IC_{50} $40 \pm 5 \text{ nM}$, $n = 5$). Despite the weak homology, mutagenesis revealed essential conservation of key structure-function relations; however, some regions deduced to have been critical for ATP binding and/or channel activation in mammalian P2X receptors were clearly not essential². Surprisingly, in *D. discoideum*, the receptor was found on intracellular membranes, with prominent localisation to an osmoregulatory organelle, the contractile vacuole. Mutant amoeba with a disrupted *p2xA* gene showed no regulatory cell volume decrease in response to hypotonic stress over a 60 min time period ($n = 30$ cells). In wild-type cells, regular contractile voiding events occurred with a cycle of $80 \pm 5 \text{ s}$ ($n = 30$ cells). In contrast, mutant cells had a reduced frequency of voiding and a prolonged cycle ($182 \pm 10 \text{ s}$, $n = 30$ cells). The osmoregulatory defect in mutant cells could be rescued by over-expression of the P2X receptor, and furthermore, the mutant phenotype could be recapitulated in wild-type cells by the addition of copper. These results suggest that loss of the amoeba P2X receptor leads to a compromised contractile vacuole, an inability to osmoregulate, and ultimately cell swelling. These findings demonstrate a hitherto unrecognised role of P2X receptors on intracellular organelles, in this case in osmoregulation.

¹ North RA. (2002). *Physiol. Rev.* **82**, 1013-1067 (2002).

² Roberts JA. *et al.* (2006) *Pflugers Arch.* **452**, 486-500.

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have characterised the opener properties of BKOEt₁, an example of this chemical series, on a HEK293 cell line expressing the zero variant of the human BK_{Ca} alpha subunit.

Whole-cell and inside-out patch clamp configurations were used. Currents were recorded using voltage step protocol (-100 to $+100 \text{ mV}$) in symmetrical potassium (140 mM) with $0.3 \mu\text{M}$ free Ca^{2+} in the bath and pipette solutions. Compounds were applied by continuous perfusion into the bath. Normalised conductance vs voltage (G-V) curves were plotted and V_{50} values calculated. For single channel analysis currents were recorded for 60s at each voltage (-30 mV to $+80 \text{ mV}$) from which open pore probability (NfPo) was calculated.

In whole-cell configuration, BKOEt₁ (3 , 10 , 30 and $100 \mu\text{M}$) produced significant ($p < 0.001$) leftward shifts in V_{50} of $77.5 \pm 0.7 \text{ mV}$, $43.9 \pm 6.3 \text{ mV}$, $27.7 \pm 1.4 \text{ mV}$ and $15.9 \pm 0.4 \text{ mV}$ respectively ($n = 6-9$) in G-V curve from control ($V_{50} = 90.1 \pm 2.0 \text{ mV}$). The effects of BKOEt₁ ($30 \mu\text{M}$) were abolished in the presence of the BK_{Ca} channel blocker paxilline ($10 \mu\text{M}$). In inside-out configuration, BKOEt₁ ($30 \mu\text{M}$) did not alter significantly the single-channel conductance ($278.9 \pm 2.3 \text{ pS}$, $n = 4$) relative to control ($267.1 \pm 3.3 \text{ pS}$, $n = 4$). BKOEt₁ (10 , 30 and $100 \mu\text{M}$) enhanced the activity of BK_{Ca} by increasing NfPo in a concentration-dependent manner as indicated by leftward shift in V_{50} from control ($79.5 \pm 0.4 \text{ mV}$) of $40.1 \pm 1.7 \text{ mV}$, $28.3 \pm 1.3 \text{ mV}$, $-3.9 \pm 0.9 \text{ mV}$ respectively ($n = 4-6$). At $+40 \text{ mV}$ the NfPo was significantly increased from 0.058 ± 0.02 in control to 0.364 ± 0.169 , 0.813 ± 0.098 , and, 1.00 ± 0.09 ($n = 4-6$) in BKOEt₁ (10 , 30 and, $100 \mu\text{M}$) respectively. The observed increase in NfPo by BKOEt₁ was abolished by paxilline ($10 \mu\text{M}$).

In conclusion, BKOEt₁ is a novel BK_{Ca} channel opener and that activation of the channel is brought about through increasing NfPo with no effect on single channel conductance.

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Lawson K & McKay N (2006) *Curr Pharm Design* **12**, 459-471.

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PC153

Identification of the N-arylbenzamide BKOEt₁ as a novel BK_{Ca} channel opener

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BK_{Ca} channels are fundamental in the control of cellular excitability (Lawson and McKay 2006). Thus, compounds that activate BK_{Ca} channels could provide potential therapies in the treatment of pathologies of the cardiovascular and central nervous system (Calderone 2002). We have previously demonstrated that a series of novel N-arylbenzamide compounds exhibit BK_{Ca} channel opening properties using an Rb⁺ efflux assay (Kirby R *et al* 2006). Using whole cell and excised patch clamp electrophysiology we

PC154

Assessment of a series of novel compounds as BK_{Ca} channel openers using a Rubidium (Rb⁺) efflux assay

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BK_{Ca} channels are fundamental in the control of cellular excitability and have been identified as potential therapeutic targets in many pathological conditions (Lawson and McKay 2006). We have previously optimised a non-radioactive Rb⁺ efflux assay for the assessment of BK_{Ca} channel activity (Kirby R *et al* 2006). This assay was used to characterise a series of novel N-arylbenzamide compounds for BK_{Ca} channel opener activity (Calderone V, 2006) using a recombinant HEK293 cell line expressing either the human α -subunit or co-expression with the $\beta 1$ -subunit of BK_{Ca} channel.

Cells were incubated in RbCl (5.4mM) buffer for 4 hours then washed prior to incubation in (5.4mM) KCl buffer containing either test compound (0.1-100 μ M) or vehicle (DMSO, 0.3%) for 10 minutes. The supernatant and lysate samples were collected and the Rb⁺ content was determined by atomic absorption spectrometry and Rb⁺ efflux (%) was calculated. Data are presented as mean values ($\pm$ SEM).

All novel compounds tested on the BK_{Ca} channel α -subunit cell line evoked concentration related increases in Rb⁺ efflux. The maximum increase in Rb⁺ efflux (E_{max}) evoked by N-aryl-benzamide compounds ranged between 53-91% (n=8) above that the baseline of 22.2 $\pm$ 1.1% (n=10-12); that achieved with NS1619 (reference BK_{Ca} channel opener) was 62.5 $\pm$ 4.8% (n=8). The series of compounds demonstrated a 40-fold potency range (EC₄₀: 0.12-2.95 μ M); EC₄₀ for NS1619 was 0.35 $\pm$ 0.40 μ M (n=8). In the presence of the BK_{Ca} channel blocker, iberiotoxin (0.1 μ M), the compounds (100 μ M for 10 minutes) failed to increase Rb⁺ efflux.

All compounds tested on the BK_{Ca} channel α - and β 1 subunit evoked significant concentration related increases in Rb⁺ efflux. The E_{max} for the compounds was in the range of 50-87%, and that achieved for NS1619 was 65.3 $\pm$ 2.7% (n=8). The compounds demonstrated a 220-fold range in potency (EC₄₀: 0.44-96.0 μ M) and that for NS1619 was 0.68 $\pm$ 0.37 μ M (n=8). The increase in Rb⁺ efflux due to the compounds (100 μ M) in cells expressing the α - and β 1 subunit was abolished by Iberiotoxin (0.1 μ M). In conclusion, the N-aryl-benzamide compounds tested exhibit properties consistent with the activation of BK_{Ca} channels. Preliminary data indicate discrimination, based on efficacy and potency, to activate BK_{Ca} channels composed of α -subunit only or in the presence of β 1 subunit by this series of compounds. Such agents will assist in the development of pharmacophore models to give insight into the receptor site for openers within the BK_{Ca} channel protein.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC155

Intracellular coiled-coil domain engaged in subunit interaction and assembly of TRPM2

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TRPM2 channels, activated by adenosine diphosphoribose and related molecules, are assembled as oligomers and most likely tetramers. However, the molecular determinants driving the subunit interaction and assembly of the TRPM2 channels are not well defined. Here we examined, using site-directed mutagenesis in conjunction with co-immunoprecipitation and patch clamp recording, the role of a coiled-coil domain in the intracellular C terminus of TRPM2 subunit in subunit interaction and channel assembly. Deletion of the coiled-coil domain resulted in severe

disruption of the subunit interaction and substantial loss of the adenosine diphosphoribose-evoked channel currents. Individual or combined mutations to glutamine of the hydrophobic residues at positions a and d of the abcdef heptad repeat, key residues for protein-protein interaction, significantly reduced the subunit interaction and channel currents; the mutational effects on the subunit interaction and channel currents were clearly correlated. Furthermore, deletion of the coiled-coil domain in a pore mutant subunit abolished its dominant negative phenotypic functional suppression. These results provide strong evidence that the coiled-coil domain is critically engaged in the TRPM2 subunit interaction and such interaction is required for assembly of functional TRPM2 channel. The coiled-coil domain, which is highly conserved within the TRPM subfamily, may serve as a general structural element governing the assembly of TRPM channels.

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PC156

Role of pre-synaptic kainate receptors in Mossy Fiber LTP: 2-photon microscopy, patch-clamp electrophysiology and calcium imaging

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Long-term potentiation (LTP) is a well-established experimental model used to investigate the synaptic basis of learning and memory. The present study concerns the induction of mossy fiber (MF) LTP in hippocampal brain slices. This form of synaptic plasticity is particularly interesting because it is NMDA receptor-independent and requires activation of pre-synaptic kainate receptors. Previous electrophysiological data ^[1] from our lab has demonstrated a role for intracellular Ca²⁺-induced Ca²⁺ release (CICR) in both the induction of MF LTP and the pronounced synaptic facilitation that occurs during high-frequency stimulation. These experiments also provided evidence that, under physiological conditions, Ca²⁺ permeation through kainate receptors acts as the trigger for intracellular CICR. Our present study extends this research by using a technique ^[2] that combines 2-photon microscopy, patch-clamp electrophysiology and calcium imaging to directly investigate the role of pre-synaptic calcium in MF LTP. Granule cells of the dentate gyrus are held in the whole cell patch-clamp configuration and experiments are conducted in current-clamp mode. The patch pipette contains a red morphological marker (to identify pre-synaptic giant MF boutons) and a calcium indicator dye. Cells are held for >2hrs prior to calcium imaging to allow for dye diffusion and equilibration. Pre-synaptic calcium signals are generated by evoking orthodromic action potentials (via current injection into the patch pipette: train of 5 pulses; 2ms; 25Hz) and are recorded by

line-scanning individual MF boutons in the presence and absence of kainate receptor agonists and antagonists.

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PC157

The involvement of potassium channels in the vasodilator responses of cyanopindolol and CGP 12 177 in the rat mesenteric artery

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In addition to β_1 -, β_2 -adrenoceptors, the existence of vasodilatory, propranolol-insensitive atypical β -adrenoceptors has been suggested in rat and human vessels. They are activated by non-conventional partial β -adrenoceptors agonists – cyanopindolol (CYP) and CGP 12 177 (CGP) in an endothelium-dependent manner [1, 2]. The aim of the present study was to examine the endothelium-dependent relaxant mechanism of CYP and CGP in the rat mesenteric artery. For comparison, the β_2 -adrenoceptor agonist fenoterol (FEN) was used. Male Wistar rats (8-week-old/ 280-350g) were killed by decapitation. Mesenteric arteries pre-constricted with phenylephrine (1 μ M) were relaxed in a concentration-dependent fashion by CYP ($pD_2=5.29\pm0.04$; $E_{max}=105.2\pm1.3$; $n=12$), CGP ($pD_2=4.5\pm0.08$; $E_{max}=103.9\pm1.5$; $n=18$) and FEN ($pD_2=4.42\pm0.1$; $E_{max}=98.4\pm3.1$; $n=11$). Relaxations were either insensitive (CYP, CGP) or sensitive (FEN) to propranolol (0.3 μ M). In endothelium-intact vessels the inhibitor of nitric oxide synthase L-N^G-nitroarginine methyl ester (L-NAME; 300 μ M) diminished the relaxation evoked by FEN, but not by CYP and CGP. Vasodilatory effects of all agonists were insensitive to the inhibitor of cyclooxygenase indomethacin (INDO; 10 μ M) but they were inhibited by tetraethylammonium (TEA; 300 μ M) and 4-amidopyridine (4-AP; 1mM), calcium activated (K_{Ca}) and voltage-gated (K_V) potassium channels blockers, respectively. The blocker of ATP-sensitive potassium channels glibenclamide (10 μ M) reduced the relaxation elicited by FEN but not by CYP and CGP. All agonists were less potent in arteries pre-constricted with 60 mM KCl compared to phenylephrine-pre-constricted vessels. Combined pre-incubation with L-NAME, INDO, Ba^{2+} (the inhibitor of inward rectifying potassium channel; 30 μ M) and ouabain (the inhibitor of Na^+/K^+ -ATP-ase; 1mM) increased the basic level of vessel tone and attenuated the vasorelaxant response to CYP.

Thus, our results demonstrate that CYP and CGP evoked an endothelium-dependent relaxation via activation of K_{Ca} and K_V , but not K_{ATP} , perhaps via propranolol-insensitive β -adrenoceptors. Kozłowska H *et al.* (2003). *Br J Pharmacol* **140**, 3-12.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC158

Suppressive effect of human TRPM4 channels on the store-operated Ca^{2+} entry induced by UTP in HEK 293 cells

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TRPM4 has been described as the nonselective cationic channel regulated by internal Ca^{2+} ($[Ca^{2+}]_i$) and required for regulating Ca^{2+} oscillations. Here we examined whether and how TRPM4 channels are involved in store-operated Ca^{2+} (SOC) entry in the HEK 293 cell by using RFP-tagged full length TRPM4 and its variant TRPM4a, which was deleted the first part of 174 amino acid residues in N-terminus. Confocal microscopic data showed that TRPM4 was predominantly localized in plasma membrane and TRPM4a was rarely detected in the membrane but densely in endoplasmic reticulum and Golgi apparatus. SOC influx was examined by exposure to 2.5 mM Ca^{2+} following stimulation by 100 μ M UTP for 2-3 min in order to deplete the internal Ca^{2+} store via endogenous P2Y receptor- $G_{q/11}$ - $InsP_3$ signaling pathway in HEK 293 cell. Externally applied 100 μ M UTP caused $[Ca^{2+}]_i$ transients in non-transfected and both TRPM4- and TRPM4a-expressed cells under the Ca^{2+} -free condition. Subsequent exposure to Ca^{2+} elicit a second $[Ca^{2+}]_i$ rise in non-transfected and TRPM4a expressed cells but not in TRPM4-expressed cells. Nonselective whole-cell currents were hardly detected in TRPM4-expressed cells, suggesting that TRPM4 channels interfere with SOC entry pathway(s) such as Ca^{2+} -permeable TRP channels endogenously expressed in HEK 293T cells. Taken together, these results show that TRPM4 can possibly inhibit store-operated Ca^{2+} entry and its first part of N-terminus is critical for the translocation of TRPM4 to the membrane.

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PC159

Respiratory Syncytial Virus inhibits Na^+ current in H441 human bronchial epithelial cells

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There is a growing body of evidence suggesting that respiratory pathogens can alter the ion transport and hence electrical properties of the respiratory epithelium. Severe respiratory syncytial virus (RSV) infection disturbs the fluid balance in the lower airways, implying an effect of the virus on the ion transport in these tissues. In the present study the physiological consequences of RSV infection were investigated in a cell culture model using the H441 human bronchial epithelial cell line. Cells were grown to confluent monolayers on semi-permeable filters in the presence of dexamethasone. Initial studies have shown that in this

configuration, the H441 cell line develop a Na⁺ absorbing phenotype. Cells are then infected, at a multiplicity of infection of four, with either a live or UV-inactivated RSV strain. The UV-inactivated strain is capable of binding to and entering cells, but is unable to undergo viral replication. Viral infection was confirmed

though confocal microscopy. 24 hours post infection, a statistically significant decrease in total current was observed for both strains; live 12.6 ± 3.1 to 5.2 ± 3.1 $\mu\text{A}/\text{cm}^2$ ($p < 0.02$), UV-inactivated 13.4 ± 4.3 to 7.0 ± 2.7 $\mu\text{A}/\text{cm}^2$ ($p < 0.05$), with no statistically significant change in control (11.0 ± 2.2 to 11.8 ± 2.0 $\mu\text{A}/\text{cm}^2$, $p > 0.05$). These results suggest that RSV infection (though a live or UV-inactivated strain) inhibits Na⁺ transport in the H441 cell line within 24 hours post infection.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC160

STIM1, STIM2 and Orai1 contribute to calcium entry of vascular smooth muscle cells

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Calcium entry mechanisms have important roles in vascular smooth muscle cells, regulating contraction, phenotype, proliferation and motility. Recent studies suggest two types of relatively unexplored membrane protein, STIM1 and Orai1, function together in calcium release-activated calcium (CRAC) channels of lymphocytes and cell lines (Liou et al. 2005; Feske et al. 2006; Spassova et al. 2006; Mercer et al. 2006). Here we explore the expression and function of STIM1, Orai1 and their homologues in vascular smooth muscle cells from discarded saphenous vein samples obtained with ethical approval during coronary artery bypass surgery. Messenger RNA species encoding STIM1, STIM2, Orai1, Orai2 and Orai3 were detected in order of abundance: Orai3>Orai1>Orai2>STIM1>STIM2. siRNA probes were screened for efficacy against each RNA species and probes causing >75% specific reduction of RNA abundance were used for further experiments. Calcium measurements were made on a real-time fluorescence 96-well plate reader. Knock-down of RNA encoding STIM1 or STIM2 suppressed calcium entry after store depletion. Ca²⁺-content of intracellular stores, as determined by agonist-evoked Ca²⁺-release, was unaffected by STIM1 siRNA. Antibody targeted to STIM1 also inhibited calcium entry. Anti-STIM1 staining was localized to small spots that were reduced by siRNA targeting STIM1. The number of spots did not change significantly in response to store-depletion. Knock-down of RNA encoding Orai1 also suppressed calcium entry but no effects relating to RNA encoding Orai2 or Orai3 were detected. The data suggest two new families of membrane protein with functional importance for calcium entry in vascular smooth muscle.

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PC161

Membrane targeting and distribution of neuronal voltage-gated calcium channel subunits

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Calcium entry through voltage-gated calcium channels (VGCCs) plays a pivotal role in diverse cellular processes including muscle contraction, neuronal excitability and gene expression. Multiple VGCC subtypes exist, that differ in their biophysical properties and distributions within and between tissues, thereby, allowing cells to tailor calcium influx to discrete functional demands. However, the mechanisms underlying this process are not yet understood. VGCCs are multi-subunit complexes that comprise a pore-forming alpha1 and two regulatory subunits: alpha2-delta and beta. Of these, the alpha2-delta subunit is an attractive candidate to drive targeting and distribution of VGCCs. Alpha2-delta subunits enhance surface expression and modify the biophysical properties of VGCCs (Bernstein and Jones, 2007). Moreover, structurally, they are similar to integrin receptors that mediate bidirectional signalling between intra- and extracellular compartments. ECM-integrin interactions are known to control growth factor-dependent signalling in sensory neurones using the major VGCC regulatory ERK1/2 pathway (Fitzgerald, 2000; Woodall et al., 2005). Thus, we hypothesize that a key role of alpha2-delta subunits may be to direct VGCCs to specific ECM-associated signalling complexes.

Using a library of functional VGCC subunits each separately tagged with red, green or blue protein fluorophores, we have tested interactions between the ECM and discrete VGCC complexes. High resolution fluorescence imaging of COS-7 and HEK-293 cells transiently transfected with select VGCC complexes has confirmed the requirement for the presence of a beta subunit (beta 3) in directing exit from the endoplasmic reticulum and subsequent surface expression, of the pore-forming alpha1 subunit (Cav2.2, N-type). Alpha2-delta-1 appears to show a distinct pattern of labelling, indicative of its high cell surface expression and regionalized distribution. Polystyrene beads coated with ECM proteins (laminin-1 and fibronectin) cause a marked redistribution of VGCC complexes containing alpha2-delta-1 subunits. Ongoing experiments are defining the basis for these effects in terms of the precise subunits and regions driving these interactions and their functional consequences.

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PC162

BK channels are expressed and functional in human osteoblast-like cells

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We have previously used single channel patch-clamp and RT-PCR to show that a number of channels are present in both MG63 and SaOS2 osteoblast-like cells. Large conductance Ca^{2+} -activated K^{+} channels are amongst the most prevalent channels observed in these cells. The precise subunit composition of the native BK channel and its role remain however unknown. BK channels normally exist as a tetramer of 4 α subunits associated with β subunits. The subunit composition, specifically the type of β subunit co-assembled with the α subunits, modifies both the voltage- and Ca^{2+} -sensitivity and the pharmacological fingerprint (e.g. sensitivity to iberitoxin) of BK channels¹. Here we attempt a preliminary pharmacology of the channel, test its role in proliferation and try to specify the subunit composition of BK channels in MG63 cells.

Single channel activity in osteoblast-like MG63 cells was measured in cell-attached, excised outside-out and inside-out patches. Cell numbers were measured by the MTS assay. RNA extraction, followed by RT-PCR was carried out to investigate the expression of α (KCNMA) and β (KCNMB) subunits of the BK channels.

BK channels were readily observed in cell-attached patches at the resting membrane potential. Channel activity was voltage-dependent, activity increasing markedly on depolarisation, there often being up to 7 channels in some patches. External or internal tetraethylammonium chloride (TEA) reduced reversibly channel activity in excised patches. For example, in outside-out patches bath-application of 500 μM TEA typically reduced the open probability, producing an apparent reduction in unitary current, due to flickery channel block. More BK-selective blockers such as iberitoxin (IbTX- 5-60 nM) and tetrandrine (5-30 μM) also produced reversible block of the channel in excised outside-out patches. Internal paxilline (10 μM) blocked reversibly the channel in excised inside-out patches. The channel was activated by external isopimaric acid (10 μM). RT-PCR showed that the α (KCNMA) subunit and the $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 4$ (KCNMB1-4) subunits were present. Interestingly, TEA and tetrandrine had a dual effect on cell numbers. Treatment for 72 hours with TEA and tetrandrine increased significantly cell numbers at low concentrations (1 mM and 3 μM respectively), and reduced cell numbers at high concentrations (>10 mM and >10 μM). IbTX (10 - 300 nM) did not alter cell numbers.

Given these data suggest a possible role for BK channels in MG63 cell growth, these channels may therefore represent a target for therapeutic intervention. Future work aims to characterise fully

the pharmacology of the BK channel, to define the native subunit composition, and the role of the channel in mineralization and secretion in osteoblast-like cells.

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PC163

Microtubule involvement in potassium channel surface expression

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Many stages in the transportation of membrane proteins rely on the actin and/or microtubule based cytoskeletons. This is particularly well established for transport between the ER and Golgi, and in transport between the surface membrane and cytoplasmic organelles. Little is known, however, regarding the specific machineries utilized by the various cardiac potassium channels. Here we examine the effects of disruption of microtubules or of the dynein-dynactin retrograde motor complex on the functional expression of several potassium channels. Exposure of rat ventricular myocytes to the microtubule depolymerising agent nocodazole increases the magnitude of the transient outward current (I_{TO}) and decreases the inward rectifying (I_{K1}) current, resulting in a decrease in action potential duration. In heterologous expression systems, nocodazole treatment leads to changes in surface expression of several channels, and disruption of dynein motor function by p50 overexpression leads to time-dependent changes in expression in a subset of these. Thus, it is likely that different channels utilize different pathways in moving from the ER through the Golgi and on to the surface, as well as in post-internalization trafficking from the surface.

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PC164

Protein kinase C and recycling of the pancreatic ATP-sensitive potassium channel

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ATP-sensitive K^{+} (K_{ATP}) channels couple the metabolic state of a cell to its membrane potential. This property allows pancreatic β -cells to regulate insulin secretion in accordance with blood glucose levels. Genetic mutations associated with persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) and permanent neonatal diabetes mellitus (PNDM) have been shown to affect forward trafficking and endocytosis of pancreatic K_{ATP} channels respectively [1 & 2]. These findings indicate a possible role for changes in K_{ATP} channel cell surface density in the physiological

and pathophysiological response of the β -cell to blood glucose fluctuations. Recent work from our laboratory has demonstrated that the pancreatic K_{ATP} channel undergoes rapid ($t_{0.5} \sim 5$ min) endocytosis in a constitutive fashion [2]. In addition, previous studies have found that inhibition of protein kinase C (PKC) leads to an increase in K_{ATP} channel cell surface density, an effect attributed to inhibition of channel endocytosis [3]. Here we report similar findings in the β -cell derived INS1e cell line. However, in addition to an inhibition of endocytosis it is possible that the increase in cell surface K_{ATP} channel density could be explained by increased channel recycling. As there were no data to support this hypothesis we first had to establish whether internalised K_{ATP} channels were able to recycle back into the plasma membrane. In order to investigate this possibility, K_{ATP} channels comprising SUR1 and extracellular HA-tagged Kir6.2 subunits were expressed in a variety of mammalian cell lines. Cell surface channels were allowed to bind and internalise anti-HA antibodies for 2 hrs at 37° C; after removing excess antibodies at 4° C, the cells were incubated at 37° C to permit recycling. Recycled and non-recycled channels were stained with fluorophore-conjugated secondary antibodies and visualised by confocal microscopy. Internalised K_{ATP} channels enter the perinuclear compartments enriched in the cation-independent mannose-6-phosphate receptor, from where they are rapidly recycled to the plasma membrane; this recycling is enhanced by the PKC inhibitor chelerythrine. Unexpectedly, however, endocytosis of K_{ATP} channels *per se* was unaffected by chelerythrine. Further studies using dominant negative constructs of Rab proteins demonstrated that chelerythrine inhibits exit of K_{ATP} channels from early endosomal compartments (EEA1 and Rab4 positive) into perinuclear compartments, promoting recycling of channels via the Rab4 dependent route. In conclusion, our data suggest that when PKC is activated, endocytosed K_{ATP} channels largely enter the perinuclear compartments and that their recycling back to the cell surface via a Rab4 dependent rapid recycling pathway is inhibited, thereby accounting for the observed decrease in cell surface channel density resulting from PKC activation.

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PC165

Altered expression of ion transport genes may partly underlie heart rhythm disturbances in the streptozotocin-induced diabetic rat heart

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Recent *in vivo* biotelemetry studies have demonstrated rapid reductions in heart rate following administration of the diabetogenic agent streptozotocin (STZ) to young rats and these

bradycardic effects of STZ-induced diabetes mellitus (DM) are partially normalized with insulin replacement (Howarth *et al.*, 2005 & 2006). Reductions in beating rate have also been reported in spontaneously beating isolated perfused heart and in atrial preparations suggesting that arrhythmias in diabetes mellitus may be partly caused by intrinsic alteration of sinoatrial node (SAN) function (Howarth & Qureshi, 2006; Kofo-Abayomi & Lucas, 1988). The effects of diabetes on electrical activity and expression levels of mRNA for specific ion channel and gap junction proteins in the SAN have been investigated. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg) administered to young male Wistar rats (200-250g). Experiments were performed 8-10 weeks after treatment. SAN action potentials were measured in spontaneously beating Langendorf perfused heart with a suction electrode. Conduction time and pacemaker cycle length were measured in sino-atrial node preparations with extracellular electrodes. Experiments were performed at 36-37 °C. Expression levels of mRNA for Kv4.2 (Ito4.2), Kv4.3 (Ito4.3), ERG (IKr), KVLQT1 (IKs), NCX1 (INa/Ca), Gja5 (Cx40), Gja1 (Cx43) and Gja7 (Cx45) were measured in SAN and compared with right atrium and right ventricle with real-time quantitative reverse transcription-polymerase chain reaction (real-time RT-PCR). Diabetes was confirmed by a significant elevation of blood glucose (356±21 mg/dl) compared to age-matched controls (66±2 mg/dl). Spontaneous heart rate was significantly ($P<0.05$) lower in diabetic rats (181±7 BPM) compared to controls (248±12 BPM). SAN action potential duration, measured during repolarization at 50 % and 70 % from peak action potential, were significantly prolonged in diabetic heart compared to controls. The pacemaker cycle length and the sino-atrial conduction time were significantly prolonged in SAN from diabetic heart (415±43 and 12±2 ms) compared to controls (255±7 and 7±1 ms), respectively. Expression levels of mRNA for Kv4.2 (Ito4.2) was significantly reduced and for Kv4.3 (Ito4.3) was significantly increased in right ventricle from diabetic heart. Expression levels of mRNA for Gja7 (Cx45) was significantly increased in SAN from diabetic heart compared to controls. Altered expression of ion transport genes may partly underlie electrophysiological defects in STZ-induced diabetic rat heart. Howarth FC *et al.* (2005). *Exp Physiol* **90**, 237-245.

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PC166

Function and mRNA expression of a large conductance Ca^{2+} -activated K^+ channels in rat aortic smooth muscle cells under ionized irradiation

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Title only

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PC167

Mutation of two positively-charged pore residues abolish the effect of polyunsaturated fatty acids on C-type inactivation of the K⁺ channel, Kv1.4

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Kv1.4 is known to encode a transient outward K⁺ current in the heart, including in the human. The ω -3 polyunsaturated fatty acids, eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA), found in fish oils, are known to play a protective role against the development of ventricular fibrillation accompanying myocardial ischaemia. On the other hand, there is known to be an accumulation of the ω -6 polyunsaturated fatty acid, arachidonic acid (C20:4n-6; AA) in the ischaemic myocardium (there can be a >3-fold increase in free AA to ~30 μ M). We have studied the effect of AA, EPA and DHA on Kv1.4. A mutant Kv1.4 channel that lacks N-type inactivation (fKv1.4 Δ 2-146) was expressed in *Xenopus* oocytes, and currents were recorded using the two-electrode voltage clamp technique. AA, EPA and DHA dramatically enhanced fKv1.4 Δ 2-146 C-type inactivation with a K_D of 43, 15 and 18 μ M, respectively (n=10, 7 and 5). Replacement of either of two positively-charged pore residues with a cysteine residue (H508C; K532C) abolished the enhancement caused by AA and EPA (n = 5 and 5). It is known that these mutations cause a loss of sensitivity of Kv1.4 to pH and extracellular K⁺ (pH and extracellular K⁺ also affect C-type inactivation of Kv1.4). We observed an interaction among the effects of AA, pH and [K⁺]_o; in the presence of 30 μ M AA, the effect of pH on C-type inactivation of Kv1.4 was markedly reduced (n = 5), while raising extracellular K⁺ abolished the effect of AA on C-type inactivation (n = 5). We conclude that AA, DHA and EPA cause a dose-dependent enhancement of C-type inactivation of Kv1.4 via a mechanism it shares in common with extracellular pH and K⁺ and involving extracellular positively-charged pore residues. This inhibition of Kv1.4 by polyunsaturated fatty acids may involve a binding of the negatively charged polyunsaturated fatty acids to the positively charged residues (H508 and K532) in the extracellular mouth of the channel.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC169

Properties of a Na⁺-dependent inward rectifying K⁺ current in rat atrial myocytes overexpressing Kir3.4

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We have shown previously that overexpression of Kir3.4 using adenoviral gene transfer in adult atrial myocytes results in formation of homotetrameric channel complexes with weaker

inward rectification than endogenous ACh-activated current (I_{K(ACh)})¹. In the present study, properties of current carried by homomeric Kir3.4 channels were investigated and compared with endogenous I_{K(ACh)} using whole cell voltage clamp. In Kir3.4 overexpressing myocytes intracellular Na⁺ ≥ 15 mM, supplied via the patch pipette, resulted in concentration-dependent activation of a background inward-rectifying current (I_{bir}) without saturation up to 60 mM. In contrast to reports based on oocyte studies, endogenous (Kir3.1/Kir3.4) heteromeric channels were completely insensitive to [Na⁺]_i in this range of concentrations. Myocytes expressing a mutant Kir3.4 subunit (D223N), which lacks the putative Na⁺ binding aspartate residue, were devoid of Na⁺-dependent activation of I_{bir}. [Na⁺]_i-dependent background inward-rectifying current was neither affected by Pertussis toxin nor by GDP-β-S, suggesting its activation to be independent of the G protein cycle. Using a protocol to induce PIP₂ depletion via endogenous PLC-coupled α₂ adrenergic receptors, native Kir3.1/3.4 channel currents were inhibited by about 75%. In contrast, inhibition of I_{bir} amounted to < 20 %. Kir3.x channel currents can be selectively blocked by Tertiapin-Q (T-Q). Inhibition of I_{K(ACh)} by T-Q can be described using an EC₅₀ of 12 nM, in line with published data. Sensitivity of I_{bir} to the blocking venom was almost 20 fold higher (EC₅₀: 0.7 nM). These data clearly suggest, that [Na⁺]_i-dependent gating has no relevance to endogenous Kir3.1/Kir3.4 channels in cardiac myocytes. Furthermore, our results identify I_{bir} as a homotetrameric Kir3.4 channel current with novel properties of regulation and pharmacology. I_{bir} shares some properties in common with a background current recently described in atrial myocytes from an animal model of atrial fibrillation and human AF patients^{2,3}.

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PC170

Differences in correlation between mitochondria and various types of calcium released channels in secretory cells of chironomus plumosus larvae salivary glands

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Mechanisms of coupling between Ca²⁺-released and mitochondria Ca²⁺ uptake in secretory cells is interesting problems of modern cell physiology. The aim of this study was examine correlation between mitochondria and Ca²⁺-released channels in secretory cells of Chironomus plumosus L. larvae salivary glands.

Experiments were done on isolated salivary glands that were incubated with saponin (0.1 mg ml⁻¹, 10 min) for cell permeabilisation. After that, saponin-treated glands were incubated during 30 min at 25° C in control solution that contained (mM):

15.3 NaCl, 129.94 KCl, 0.35 Na₂HPO₄, 0.44 KH₂PO₄ and 5.55 glucose; pH 7.0. Other components (Ins(1,4,5)P₃, ryanodine, ruthenium red) were added to this control solution. Changes in common calcium content in gland tissue were measured using the metallochromic dye arsenazo III. Changes of membrane-bound Ca²⁺ were measured using chlortetracycline. This method is based on property of chlortetracycline to fluorescence in hydrophobic environment after binding Ca²⁺. So, fluorescence changes of complex chlortetracycline-Ca²⁺ inside the membranes reflect shifts of ionized Ca²⁺ concentration close to membrane.

The simultaneous presence of ruthenium red (10⁻⁵M) and Ins(1,4,5)P₃ (10 μ M) in incubation medium did not cause any statistically significant changes in common calcium content in comparison with control medium. In contrast to simultaneous presence of ruthenium red (10⁻⁵M) and ryanodine (5nM) evoked statistically significant ($p=0.023$, $n=6$) decreased of calcium content in comparison with control medium by $37.65\pm9.65\%$. Alone action of Ins(1,4,5)P₃ decreased common calcium content by $42.94\pm11.82\%$ ($p=0.046$, $n=8$), ryanodine – by $59.31\pm13.32\%$ ($p=0.045$, $n=6$), ruthenium red – by $32.84\pm7.71\%$.

We observed increasing of membrane-bound Ca²⁺ at ryanodine presence in incubation medium by 28.5% ($p=0.01$, $n=8$), also Ins(1,4,5)P₃ caused statistically authentic increasing of membrane-bound Ca²⁺ by 40% comparison to control. Simultaneous presence of ruthenium red (10⁻⁵M) and ryanodine (5nM) in incubation medium did not cause any statistically significant changes of membrane-bound Ca²⁺. In contrast to simultaneous presence of ruthenium red (10⁻⁵ M) and Ins(1,4,5)P₃ (10 μ M) evoked statistically significant ($p=0.0014$, $n=7$) increasing of calcium content in comparison with control medium by $34.74\pm12.90\%$.

We made conclude, that are difference relations between mitochondria and RyRs and between mitochondria and IP₃Rs in salivary glands secretory cells of *Chironomus plumosus* larvae L.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC171

Regulation of N-type calcium channel transcript stability by the stargazin-related protein $\gamma 7$

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The role of the novel putative stargazin-like γ -subunits in relation to voltage-gated calcium channel function is controversial. We have previously cloned a member of this family, $\gamma 7$, and found that it markedly reduced functional expression of Ca_v2.2 channels. Here we have shown the degradation rate of Ca_v2.2 mRNA is increased in the presence of human $\gamma 7$ (after 9 hours with Actinomycin D: $45.8 \pm 14\%$, $n=11$, vs $11.9 \pm 3.6\%$, $n=7$, with $\gamma 7$ and under control conditions, respectively; mean $\pm$ SEM, $P<0.05$, Student's *t*-test). This process regulates the physiological level of Ca_v2.2 transcripts, since knockdown of endogenous

$\gamma 7$ with short hairpin RNA constructs in the neuron-like PC12 cell line markedly increased their endogenous Ca_v2.2 mRNA level. Interestingly, the knockdown of endogenous $\gamma 7$ also affects neurite outgrowth of differentiated PC12 cells and native DRG neurons. We have shown that the reduction of neurite outgrowth is associated with modification of F-actin and GAP-43 distribution in growth cones. Our results support the hypothesis that $\gamma 7$ is involved in the stability of specific mRNAs such as Ca_v2.2 and that this function is involved in neurite outgrowth.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC172

Deoxycholic acid chronically inhibits colonic epithelial chloride secretion *in vitro*

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Background: Cl⁻ secretion is the primary driving force for fluid secretion in the intestine. In patients with bile acid malabsorption, pathophysiologically high (mM) concentrations of dihydroxy bile acids enter the colon and stimulate Cl⁻ secretion, thereby causing diarrhea. However, under normal physiological circumstances, deoxycholic acid (DCA) is the predominant luminal bile acid and is present at lower (μ M) concentrations; its role in regulating secretion under such conditions is unclear. Thus, the aim of this study was to investigate the effects of physiological DCA concentrations on colonic epithelial secretory function. **Methods:** Cl⁻ secretion was measured as changes in short circuit current (I_{SC}) across voltage-clamped monolayers of T84 colonic epithelial cells in Ussing chambers. Protein expression was measured by Western blotting. **Results:** Acute exposure to high concentrations of DCA (1 mM) rapidly stimulated Cl⁻ secretion across T84 cells; an effect that was associated with epithelial injury as evidenced by decreases in transepithelial resistance (TER) and increased lactate dehydrogenase (LDH) release. Acute exposure to low concentrations (100 μ M) of DCA did not alter basal Cl⁻ secretion, however prolonged exposure (24hrs) inhibited secretory responses to both the Ca²⁺- and the cAMP-dependent agonists, carbachol (CCh; 100 μ M) and forskolin (FSK; 10 μ M) to $39.4 \pm 7.7\%$ ($n=10$; $p<0.001$) and $73.3 \pm 5.9\%$ ($n=5$; $p<0.01$) of those in control cells, respectively. Under such conditions, DCA did not alter TER or LDH release. This chronic antisecretory effect of DCA occurred with an EC₅₀ of 75 μ M, was apparent within 3 hrs and was maximal by 6 hrs after exposure to the bile acid. Further investigation revealed that DCA did not alter CCh-induced mobilization of intracellular calcium, implying its antisecretory actions are not due to alterations in the generation of 2nd messengers. Chronic exposure to DCA did not alter the expression of NKCC1 or the Na⁺/K⁺ ATPase pump at the protein level, nor did it alter apical Cl⁻ channel or basolateral K⁺ channel conductances. However, the antisecretory effects of DCA were blocked by the inhibitor of protein translation, cycloheximide. **Conclusion:** By virtue of its ability to stimulate secretion at high concentrations and to inhibit secretion

at low concentrations, we propose a novel role for DCA as a colonic "osmosignal" that regulates the fluidity of the luminal contents. The antisecretory effects of DCA are mediated by *de novo* protein synthesis but do not appear to involve alterations in the activity or expression of key transport proteins of the Cl⁻ secretory mechanism.

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PC173

Sodium-calcium exchange in human skin pigmentation

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The fifth member of the potassium-dependent sodium calcium exchanger (NCKX) family, SLC24A5, was recently found to be critical for normal pigmentation in zebrafish melanophores and retinal pigmented epithelium through the cloning of the golden locus (1). Independently, as part of a whole genome scan in a population with ancestry from the Indian sub-continent, we have identified 2 single nucleotide polymorphisms (SNPs) that are strongly associated with natural variation in human skin colour. Genotype analysis revealed that the ancestral allele (A111) of a non-synonymous SNP in SLC24A5 (pA111T, rs1426654) is highly associated with dark skin (allele frequency difference = 38.7%; alternative allele freq = 49.13% in dark subjects compared with 10.43% in light subjects). Together with data from the human HapMap (2) and African-American and African-Caribbean populations (1) our results indicate that SLC24A5 plays a key role in human skin pigmentation.

SLC24A5 was the predominant SLC24 transcript detected in human skin biopsies and cultured primary human melanocytes by real-time PCR. Immuno-localisation using affinity purified anti-NCKX5 peptide antibodies suggest that the antibody recognises an intracellular epitope in cultured melanocytes, confirmed using siRNA-mediated knockdown of NCKX5 protein.

The nsSNP (pA111T) lies in a region of NCKX5 amino acid sequence that is highly conserved between different NCKX family members. Site-directed mutagenesis studies on a heterologously expressed NCKX2 with the 177T SNP demonstrated that the light skin variant has reduced ion exchange function compared to the A177 SNP, with no significant variation in ion dependencies. These data suggest that SLC24A5-mediated ion exchange may affect skin pigmentation, but the mechanism remains to be elucidated.

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PC174

Altered cell biology of CLC-5 by Dent's disease-causing missense mutations

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CLC-5 is a member of the family of voltage-gated Cl⁻ channels and Cl⁻/H⁺ antiporters [1,2]. It is expressed in the proximal tubule of the kidney where it plays a role in the reabsorption of albumin from the glomerular filtrate. CLC-5 is mainly localised to endosomal membranes and has been proposed to provide a conductance through which Cl⁻ ions can pass into the vesicle lumen to counterbalance the influx of H⁺ pumped by H⁺-ATPase. Mutations of CLC-5 have been linked to Dent's disease, an X-linked disorder characterised by proteinuria, hypercalcaemia, and nephrolithiasis [3].

Dent's mutants have previously been described as those that completely ablate CLC-5 currents or those that give either increased or decreased currents. However, the role of surface membrane CLC-5 in the proximal tubule is unclear and nothing is known about the properties of mutant CLC-5 in organelles, where it is thought to play a key physiological role. We therefore examined both the electrophysiology and cell biology of several known disease-causing missense mutations in an attempt to further our understanding of the precise CLC-5 defect in Dent's disease. Wild type and mutant EYFP-CLC-5 were transiently transfected into HEK-293 cells and studied by patch clamp electrophysiology, confocal imaging, and protein biochemistry. Whole cell currents were recorded from fluorescing cells using CsCl-based solutions, holding the cell at -30mV and applying 10ms pulses from -100 to +200mV.

Compared with wild-type CLC-5, two of the mutations, G57V and R280P, reduced the current amplitude whilst no significant whole-cell currents were recorded from cells expressing the mutants S270R, G513E, R516W, I524K, and E527D. These properties correlated with the sub-cellular distribution of the wild-type and mutant EYFP-CLC-5: mutations that reduced whole-cell currents were predominantly located in intracellular endosomes and to varying extents at the cell surface whilst mutants that abolished whole-cell currents displayed a diffuse pattern of staining consistent with ER-localisation and retention. One exception was E527D which did not pass current when examined by electrophysiology, but was located predominantly in punctuate endosomal structures.

All of these mutations result in proteinuria in Dent's patients, but there exists a functional heterogeneity of disease-causing mutations that correspond to a structure-function relationship. Further studies are required to determine the physiology of wild-type and mutant CLC-5 in both surface and organelle membranes to understand their role in normal and diseased kidney function.

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PC175

Identification of an evolutionary-conserved threonine residue critical for the surface expression of the Kv1.4 voltage-gated potassium channel

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A fundamental factor in controlling membrane excitability is the precise and dynamic expression of voltage-gated ion channels at the cell surface. Voltage-gated potassium channels act as potent modulators of neuronal excitatory events and effect the shape, size and duration of action potentials(1,2,3). These electrophysiological properties conferred on the cell are affected by both channel surface density and exact subunit composition of Kv1 channels. Previous studies have shown that regulation of export of Kv1 channels from the ER to the plasma membrane is highly complex and may serve as a means to regulate both channel density and the deliverance of physiologically appropriate channel complexes. We investigated the role of a single amino acid in the S1-S2 linker in the trafficking of Kv1.4 using immunofluorescence microscopy, patch-clamp and biochemical techniques. We demonstrated that a single threonine residue in the S1-S2 linker is necessary for the correct trafficking of Kv1.4 to the cell surface. Mutation of this residue to alanine or to a phosphomimetic aspartic acid prevented cell surface expression by retaining channels in the ER, a phenomenon rescued by mutating to a serine residue. Of note is that this is a highly conserved residue within all VGK families and mutations of this residue in both Kv2.1 and Kv3.1 revealed similar results. These results demonstrate a critical role for a threonine residue in the biogenesis and/or trafficking of voltage-gated potassium channels to the cell surface.

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PC176

Transporting a transporter – regulation of the Shaker family by the potassium channel regulator

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With over seventy potassium channel genes in our genome and many implicated in pathophysiology, potassium channels and

their multi-molecular complexes have taken centre stage in physiological and signaling research. Several of these genes, including those from the Shaker family i.e. Kv1.1, 1.2 and 1.4, have been cloned and their electrophysiological properties well characterized.

Being one of the most well studied groups of proteins, these molecular marvels can selectively conduct potassium close to diffusion limits. Their concern however just does not end at aiding the remarkably regulated process of nerve conduction. Today, research into cell growth, division and signaling has opened avenues to explore the multi-faceted functions of these conduction pores. Over the years advancements in our understanding of potassium channel regulating proteins has convinced us of their multi-tier regulation strategy. It is not only the opening and closing of channels that is regulated, their surface densities are critically controlled for optimum cellular function.

One such regulator, KCNRG (potassium channel regulator) is a potential tumour suppressor and our experiments with this protein in mammalian cells have implicated it to be an ER resident protein which tends to oligomerise. The lab has also succeeded in establishing that KCNRG produces no significant differences in the total levels of Kv proteins in a cell. Also, it does not affect generic cellular processes like endocytosis (clathrin dependent and independent) or exocytosis. However, further investigation with microscopy has revealed that it brings about a selective down-regulation of surface protein levels in the case of the Shaker family member Kv 1.4. In contrast, no such effect is observed in the Shab family member, Kv 2.1. KCNRG manages this process by interactive retention of the potassium channel in the endomembrane of the cell i.e. ER. Such an interaction has been conclusively shown using cellular co-localization and immunoprecipitation. This association is speculated to be through the T1 domain of the Shaker family. Our work focuses on determining the details of this process and the mechanism this protein employs to bring about regulation of this family of potassium channels. Ivanov DV *et al.* (2003). *FEBS Lett.* 539,156-60.

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I would like to thank Anna Baranova from the George Mason University for providing me the cloned KCNRG gene for my experiments. Dr. MM Tamkun from the Colorado State University, for providing the fluorescent tagged potassium channels, which made this study possible. Also Prof. Satyajit Mayor from NCBS-TIFR for advice and resources to carry out the microscopy experiments.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC177

Increased spontaneous activity in diabetic overactive bladder

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The myogenic basis of bladder overactivity has recently become a focus of interest and may involve increased detrusor muscle spontaneous activity (Brading, 1997) and changes in potassium (K) channel function (Andersson, 1992). The pathophysiological mechanisms underlying this remain unclear. This study aims

to assess the spontaneous phasic activity (SA) in the detrusor muscle of bladders from a diabetic rat model.

Male Wistar rats (200-250g) were administered streptozotocin (STZ, 65mgkg⁻¹, i.p.). Strips of detrusor smooth muscle were isolated from bladder of rats 1, 4, 8 and 12 weeks after STZ or from weight matched controls. Tissues were mounted in tissue baths containing Krebs-bicarbonate solution (in mM, 118.3 NaCl, 11.7 D-Glucose, 24.9 NaHCO₃, 4.7 KCl, 1.15 MgSO₄, 1.15 KH₂PO₄, 1.9 CaCl₂) with 5μM indomethacin at 37°C and gassed with 95% O₂/5% CO₂. Isometric tension was measured, via UF1 force transducers connected to a Powerlab using Chart software. After equilibration (2g of tension, 60 minutes), tissues were assessed for changes in basal SA expressed as mean area under the curve (AUC) and the effect of muscarinic modulation with carbachol (CCH) (0.05-0.5μM) was analysed. The effects of the large conductance Ca²⁺ activated K channel (BK) opener, NS1619 (1-30μM), on the SA in presence of 0.5μM CCH (1-30μM) of this drug was assessed. Then the effects of K channel blockers, iberiotoxin (IBTX) (BK channel blocker) TRAM34 (IK channel blocker), apamin (SK channel blocker) (0.01-0.1μM) and glibenclamide (K_{ATP} channel blocker) (1-10μM) were tested on the basal SA. Differences between control and diabetic tissues were analysed using Students *t*-test. Basal SA was seen in over 50% of the detrusor strips from 1 (AUC=98.321±12.53, *p*<0.05), 4 (AUC=119.29±17.86, *p*<0.05) and 8 weeks (AUC=112.23±24, *p*<0.05) diabetic rats compared to controls (AUC=59.56±5.52), but not in strips from 12 week diabetic rats. Initial results in 1-week and 8-week diabetic tissues show significantly higher SA to muscarinic modulation compared to controls (table 1) NS1619 (30μM) showed reduced AUC (%) at 1 (44.25±5.1%) and 8 weeks (19.65±17.1) similar to control (31.7±10.4%). IBTX on its own was the only K channel blocker which could increase basal SA. In conclusion, detrusor muscle from STZ diabetic rat bladders shows increased SA up to 8 weeks following induction of diabetes. The SA can be modulated by muscarinic stimulation and diabetic bladders are more sensitive to this. BK channels also appear to be involved in the SA, but further investigation is needed.

	0.05μM CCH(AUC)	0.1μM CCH(AUC)	0.5μM CCH(AUC)
controls(n=8)	4.86±5.02	21.744±5.16	51.24±10.60
1 week(n=4)	10.04±7.38	52.97±17.14*	252±49.58***
8 week (n=4)	25.30±8.11*	103.39±33.26**	205.87±38.7***

p*<0.05,*p*<0.01,****p*<0.001

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Effect of NS1619 on inhibition of muscarinic stimulated responses in diabetic overactive bladder

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Potassium channels have re-emerged as targets for overactive bladder and other urological diseases in recent years (Gopalakrishnan & Shieh, 2004).

Large conductance Ca²⁺ activated potassium (BK) channels are thought to have a prominent role in urinary bladder smooth muscle function and have become a potential treatment target of overactive bladder (Meredith et al, 2004).The aim of this study was to investigate the role of BK channels in inhibiting the muscarinic stimulated contractions of bladder smooth muscle in the streptozotocin-induced diabetic rat.

Male Wistar rats (200-250g) were administered streptozotocin (STZ, 65mgkg⁻¹, i.p.). Strips of detrusor smooth muscle were isolated from bladder of rats 1, 4, and 12 weeks after STZ or from weight matched controls. Tissues were mounted in tissue baths containing Krebs-bicarbonate solution (in mM, 118.3 NaCl, 11.7 D-Glucose, 24.9 NaHCO₃, 4.7 KCl, 1.15 MgSO₄, 1.15 KH₂PO₄, 1.9 CaCl₂) with 5μM indomethacin at 37°C and gassed with 95% O₂/5% CO₂. Isometric tension was measured via UF1 force transducers connected to a Powerlab using Chart software. After equilibration, (2g of tension for 60 minutes), tissues were stimulated with 1μM carbachol (CCH) twice with a fifteen minutes wash in between. The tissues were then stimulated with CCH in the presence of increasing concentrations of the BK channel opener, NS1619 (1-30μM) or the vehicle (DMSO) to measure the inhibitory effect. Differences between control and diabetic tissues were assessed using Students *t*-test.

NS1619 significantly (*p*<0.01 and *p*<0.05) inhibited the CCH induced contractions only at 30μM in control (19.62.80±3.2%) (n=18), 1 (22.25±6.0%) (n=7), 4 (15.82±3.6%) (n=12), and 12-week (27.79±4.7%) (n=12) diabetic tissues, producing a similar level of inhibition in all groups. However, in 12-week diabetic tissue, NS1619 also significantly inhibited the contractions at 10μM (14.37.37±3.1%) (*p*<0.05) showing higher sensitivity. In conclusion, NS1619 did have a minor inhibitory effect on agonist induced contractions only at its highest concentration (30μM) in control and diabetic groups. But it seems that 12-week diabetic tissues are more sensitive to this inhibition which requires further investigation. These results suggest that BK channel modulation can affect detrusor contractions in this rat model. But the mechanisms underlying this modulation need further investigation.

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PC179

Effects of orexins/hypocretins on excitability of isolated rat sensory neurones

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Orexins A and B (or Hypocretins 1 and 2) are two new neuropeptides synthesized by neurones in the lateral hypothalamus and recently characterized as ligands of two "orphan" receptors.

They play important role(s) in wide variety of physiological functions including food intake, regulation of the neuroendocrine system, energy metabolism, control of blood pressure, body temperature, and the sleep-wake cycle. Additionally, widespread projections of their axons to superficial dorsal horn of the spinal cord and expression of both orexin-A and orexin-B receptors in dorsal root ganglion (DRG), lamina X surrounding the central canal and on C-fibres in the spinal cord led to the idea, and emerging evidence suggests that orexins modulate pain transmission. It is apparent from the evidence available that agonists specific for orexin-A and/or orexin-B receptors could represent a novel class of analgesic agents for the treatment of pain.

The aim of this study was to investigate the effects of orexin-A and orexin-B on the excitability of cultured rat sensory neurones. Since the DRG contains cell bodies of primary sensory neurones that convey information about a wide variety of sensory signals including noxious stimuli, we have used isolated DRG cells as a nociceptive neuronal model.

DRGs were removed from 1-2 day old neonatal rats after decapitation and DRG neurones were isolated by enzymatic and mechanical procedures. After establishing whole-cell mode of the patch clamp technique, resting membrane potentials were recorded under current clamp recording conditions.

Brief application of orexin A induced dose-dependent biphasic subthreshold changes from resting membrane potential. The mean depolarisation of membrane potential was 0.6 ± 0.2 mV ($n=7$), 2.2 ± 0.4 mV ($n=7$), 6 ± 0.7 mV ($n=12$) and 7.7 ± 1.5 mV ($n=7$) after extracellular application of 20, 30, 100 and 200 nM orexin A, respectively. There were hyperpolarizing response to 100 nM ($n=3$) and 200 nM ($n=2$) orexin A application. The orexin A-induced depolarisations were not pertussis toxin-sensitive (100 nM, $n=4$). A considerable number of cells (11 out of 36 cells) were also non-responder to orexin A. Orexin B (100 nM) also caused reversible depolarisation of membrane potential in 3 out of 5 cells studied.

Since both calcium activated chloride, nonselective cation and potassium conductances were active under our recording conditions it was difficult to interpret the obtained results.

Results from this preliminary study warrants further investigations involving voltage clamp experiments and fluorescence calcium imaging to clarify the mechanism of actions of orexins in this model of nociceptive neurones.

Keywords: Orexin A and B, pain, patch clamp, sensory neuron

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC180

Why are partial agonists partial?

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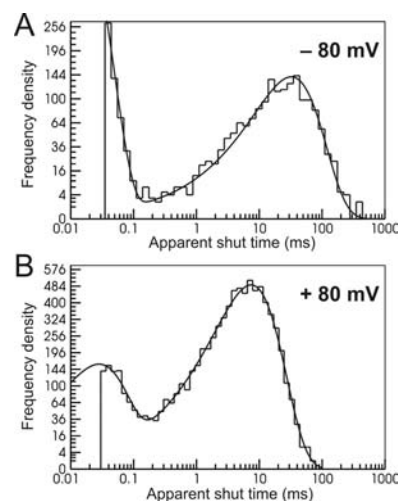
Advances in our understanding of the structure of ligand-gated ion channels have given new relevance to the question of what

biophysical properties determine the efficacy of an agonist. In addition to that, series of agonists of different efficacy are useful in the investigation of the molecular effects of channel mutations. Because of this, we have analysed the properties of single-channel activity elicited by choline on human recombinant muscle nicotinic acetylcholine receptors.

Choline is a particularly interesting example, as it is thought to have a low efficacy (gating constant ≈ 0.05) because of a slow channel opening rate constant (Grosman & Auerbach, 2000). This makes it particularly attractive as a tool in the characterization of mutations that facilitate gating and increase the channel opening rate constant beyond its wild-type value of about 50000 s^{-1} for ACh. It has been used widely for this purpose (e.g. Mitra *et al.*, 2005).

The quantitative understanding of choline activation of ACh receptors poses special challenges, because choline blocks the channel pore at concentrations that are similar to those that activate it, and it produces a very fast channel block.

According to the conventional view that choline has a slow opening rate, it should not produce openings in bursts. Both the slow opening rate constant, and the rapid dissociation that is expected of a low affinity agonist like choline, should prevent the channel from re-opening. The single channel record should, therefore, contain very few short shut times, of the sort that separate re-openings of the channel with more potent agonists (Colquhoun & Sakmann, 1985). However we see a clear component of brief shut times that are not predicted by this conventional view (Figure 1). These brief shuttings cannot all result from brief channel blockages because they are clearly seen at positive membrane potentials. The conventional view of the action of choline is clearly wrong, and some possible explanations will be discussed. For example, partial agonism could arise not because of low efficacy for the gating step, but because of low efficacy for a pre-opening conformation change of the sort that has been invoked for glycine receptors (Burzomato *et al.*, 2004). Alternatively the low maximum response evoked by choline could result from occurrence of a more complex form of channel block than the obvious very fast block. Preliminary evidence from fast concentration jumps does not support the latter view. Figure 1. Shut time distributions, 10 mM choline on recombinant human nicotinic receptors.



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PC181

A novel α -conotoxin, ArIB, is a potent ligand selective for $\alpha 7$ nicotinic acetylcholine receptors

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Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand gated ion channels composed of either heteromeric (non- $\alpha 7$) or homomeric ($\alpha 7$) subunits. To date, 12 neuronal subunit genes have been identified and the multiple combinations of subunits create a diverse family of nAChRs. $\alpha 7$ nAChRs have high relative permeability to Ca^{2+} and are implicated in disease states including schizophrenia and Alzheimer's disease (Cassels *et al.*, 2005).

Subtype-selective tools are critical for dissecting the roles of particular nAChR subtypes. The α -conotoxins are neurotoxic peptides first identified in the venom of poisonous marine cone snails. They are small disulphide-rich peptides that target nAChRs to antagonise agonist activation. Subtype-selective α -conotoxins have proved to be valuable tools for investigating the structure, function and the diversity of nAChRs (McIntosh *et al.*, 1999). In this study, we examined the selectivity of a novel α -conotoxin, an analogue of a clone obtained from *Conus arena-tus*, for its activity at native $\alpha 7$ nAChRs compared with non- $\alpha 7$ nAChRs.

The α -conotoxin ArIB competed for [¹²⁵I]- α -bungarotoxin binding to rat brain P2 membranes with $\text{IC}_{50} = 50\text{nM}$. This indicates a competitive interaction with $\alpha 7$ nAChRs. Functional antagonism by ArIB was investigated with respect to agonist-evoked increases in intracellular Ca^{2+} in PC12 cells. Cells were loaded with Fluo-3 and changes in the intracellular Ca^{2+} levels were monitored as described previously (Dickinson *et al.*, 2007). The $\alpha 7$ nAChR-selective agonist, choline (1-10mM), elicited small increases in fluorescence consistent with an increase in intracellular Ca^{2+} . This response was amplified by preincubation (2min) with PNU 120596 (10 μM ; an $\alpha 7$ nAChR-selective positive allosteric modulator; Hurst *et al.*, 2005) and could be blocked with α -bungarotoxin (100nM, 20min), confirming that it is mediated by $\alpha 7$ nAChRs. The response to choline and PNU 120596 was concentration-dependently blocked by ArIB, with complete blockade achieved with 300nM ArIB. The specificity of ArIB for $\alpha 7$ nAChRs was supported by its failure to inhibit Ca^{2+} increases mediated by non- $\alpha 7$ nAChRs (induced by nicotine or 5-iodo-A-85380) or KCl. Thus ArIB is a potentially useful selective antagonist for $\alpha 7$ nAChRs.

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PC182

Diversity of nicotinic acetylcholine receptor subtypes modulating dopamine release in striatum and prefrontal cortex *in vitro*

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The subtypes of presynaptic nicotinic acetylcholine receptors (nAChRs) that modulate dopamine (DA) release in the striatum have been well defined [1]. Evidence from pharmacological tools coupled with immunoprecipitation and transgenic mice indicate diverse nAChR containing $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ and/or $\beta 3$ subunits on DAergic terminals. There is also evidence for an indirect contribution from $\alpha 7$ nAChR, in slice preparations [2]. The local nicotinic modulation of DA release in the PFC has been less well studied. Here, the aim is to characterise the nAChR subtypes mediating DA release in rat PFC slices, for comparison with striatum.

Chopped prisms (150 μm) of striatum or PFC were loaded with 50nM [³H]DA and release assayed by filtration [2,3]. The tissue was distributed onto a 96 well filter plate and incubated with buffer alone or plus antagonist (mecamylamine, 30 μM ; dihydro-beta-erythrodine (DH β E), 10 μM ; α -conotoxin MII ($\alpha\text{CnTxMII}$), 120nM; or α -bungarotoxin (αBgt), 40nM) for 5 min. Buffer with or without antagonist was replaced with buffer containing agonists ((-)-nicotine, 0.01-100 μM ; choline, 1-10mM; 5-IA85380, 0.001-1 μM ; PNU120596, 10 μM). After 5min incubation, stimulated release was collected and samples counted for radioactivity. [³H]DA remaining in the tissue was also estimated to determine fractional DA release.

Nicotine evoked concentration-dependent [³H]DA release from striatal slices ($\text{EC}_{50} 177 \pm 10\text{nM}$); this was abolished by mecamylamine, a broad spectrum nAChR antagonist. Similar responses were observed for the PFC slices but the maximum response was 75% of that from striatum. The $\beta 2^*$ selective agonist 5-IA85380 evoked concentration-dependent [³H]DA release from both tissues and this was inhibited by the selective $\beta 2^*$ nAChR antagonist DH β E. The $\alpha 6^*$ component of DA release was dissected using $\alpha\text{CnTxMII}$, previously shown to inhibit responses by $\alpha 6^*$ nAChRs [4]. In contrast to striatum, immunoprecipitation experiments confirm that the $\alpha 6$ subunit does not contribute to nAChRs in the PFC. The $\alpha 7$ nAChR agonist choline evoked [³H]DA release from striatum and this was amplified by addition of the $\alpha 7$ positive modulator PNU120596 and abolished

by α Bgt. This drug combination revealed a contribution of pre-synaptic $\alpha 7$ nAChR to [^{3} H]DA release in the PFC.

This analysis is consistent with differences in the nAChR subunit composition of mesocorticolimbic versus nigrostriatal projections to PFC & striatum, respectively.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

This suggests that $A\beta_{1-42}$ potentiates Ca^{2+} influx downstream of nAChR, by increasing the activity of VOCC.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC183

Acute exposure of amyloid- β_{1-42} potentiates Ca^{2+} influx stimulated by nicotine or KCl

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Amyloid- β_{1-42} ($A\beta_{1-42}$) is believed to play a central role in the etiology of Alzheimer's disease due to its accumulation in the brains of patients and its toxic effect on neurons. Reactive oxygen species, membrane perturbation and loss of ionic homeostasis, particularly that of Ca^{2+} , have been implicated in the mechanism of toxicity (Mattson *et al*, 1992). $A\beta_{1-42}$ interacts with a number of receptors and channels, including those permeable to Ca^{2+} , such as nicotinic acetylcholine receptors (nAChR; Dougherty *et al*, 2003) and voltage operated calcium channels (VOCC; Ueda *et al*, 2003). Upon activation, nAChR allow Ca^{2+} and Na^{2+} entry. Amplification of this Ca^{2+} signal can occur by the depolarization that can open VOCC or by calcium-induced calcium release, depending on the nAChR subtype (Dickinson *et al*, 2006). $A\beta_{1-42}$ has been variably reported to act as an agonist or an antagonist at nAChR (Liu and Wu, 2006). $A\beta_{1-42}$ also directly affects VOCC to increase Ca^{2+} signals, when applied chronically (Ueda *et al*, 2003). This study investigates the acute effects of $A\beta_{1-42}$ on intracellular Ca^{2+} produced from nAChR activation in the PC12 cell line.

$A\beta_{1-42}$ was treated with trifluoroacetic acid for 30 min to encourage oligomer formation, replicating the proposed toxic form. Toxicity was confirmed by treating PC12 cells with 10 μ M $A\beta_{1-42}$ for 24 h: this produced a 30 % decrease in MTT reduction when compared with vehicle-treated controls ($n=6$, $p<0.001$). When applied acutely to cells loaded with Fluo-3, $A\beta_{1-42}$ (0.1 pM – 100 nM) alone produced no increase in intracellular Ca^{2+} signal, even in the presence of PNU 120596, an allosteric modulator used to amplify nAChR activity. This suggests that $A\beta_{1-42}$ does not affect Ca^{2+} levels directly when applied acutely. However, a 10 min incubation with $A\beta_{1-42}$ potentiated nicotine-evoked increases in Ca^{2+} (2.5-fold, $n=3$, $p>0.05$). This was fully blocked by mecamylamine (20 μ M) and by the VOCC-antagonist verapamil (10 μ M). An interaction with VOCC was supported by the potentiation of KCl-evoked increases in Ca^{2+} (1.9-fold, $n=3$, $p<0.01$) that was also blocked by verapamil (10 μ M).

PC184

Subtype selectivity and pharmacological characterisation of novel nicotinic acetylcholine receptor agonists and potentiators

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Mammalian nicotinic acetylcholine receptors (nAChRs) have been implicated in several neurological disorders and are increasingly being seen as important target sites for therapeutic drug discovery. The identification of subtype-selective nicotinic ligands (including agonists, antagonists and potentiators) is currently a major research goal, both for pharmaceutical drug development and as tools for the pharmacological characterisation of neuronal nAChRs. We have used a variety of molecular pharmacological and electrophysiological techniques to examine the nAChR subtype-selectivity of novel and recently identified nicotinic agonists and potentiators. Heterologous expression of chimeric and mutated subunits is being used to identify the influence of subunit domains and of individual amino acids upon the potency and selectivity of nicotinic ligands. TMAQ (5-(Trifluoromethyl)-6-(1-methyl-azepan-4-yl)methyl-1H-quinolin-2-one) is a $\beta 4$ subunit-selective nicotinic agonist which also exhibits remarkable species selectivity. Studies with recombinant nAChRs expressed in mammalian cell lines and in *Xenopus* oocytes have revealed that TMAQ is a potent agonist of nAChRs containing the human $\beta 4$ subunit but exhibits little or no agonist potency on recombinant nAChRs containing the rat $\beta 4$ subunit. In contrast, other nicotinic receptor agonists such as epibatidine show little or no difference in their affinity or agonist potency for human and rat $\beta 4$ -containing nAChRs. With the aim of identifying subunit domains and individual amino acid contributing to the species-selectivity of TMAQ, a series of human-rat $\beta 4$ subunit chimeras were constructed. In addition, individual amino acids within the human and rat $\beta 4$ subunits were altered by means of site-directed mutagenesis. Recombinant receptors containing wild-type, chimeric or mutated $\beta 4$ subunits were examined by radioligand binding, intracellular calcium assays (using a fluorometric imaging plate reader) and by electrophysiological recording. Two adjacent amino acids located close to loop-D in the extracellular domain of the $\beta 4$ subunit (N57 and V58 in human $\beta 4$ subunit and S57 and I58 in rat $\beta 4$)

have been identified as playing a critical role in the binding and agonist potency of TMAQ. These findings provide further evidence that non- α subunits can have a substantial influence upon the pharmacological properties of neuronal nAChRs. More recent studies have been aimed at examining the mechanism of action and subtype selectivity of potent nAChR potentiators.

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PC185

Influence of subunit domains upon targeting, trafficking and functional properties of neuronal nicotinic acetylcholine receptors

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels which exhibit considerable subunit diversity. In addition to nAChR subunits expressed at the neuromuscular junction ($\alpha 1$, $\beta 1$, γ , δ and ϵ), twelve neuronal nAChR subunits ($\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$) have been identified which coassemble to generate a diverse family of nAChRs. Neuronal nAChRs are expressed both in the central and peripheral nervous system and have been implicated in processes such as synaptic transmission and modulation of neurotransmitter release. Neuronal nAChRs have been implicated in several neurological disorders and are seen as important targets for therapeutic drug discovery. 5-hydroxytryptamine type 3 receptors (5HT3Rs) are related members of the same superfamily. In an attempt to overcome problems of inefficient expression of nAChRs in heterologous expression systems, chimeric subunits were created from both nAChRs and 5HT3Rs. A series of subunits chimeras has been constructed which contain regions from nAChR and 5HT3R subunits. By expression of wild-type and chimeric subunits, we have examined the influence of nAChR and 5HT3R subunit domains upon receptor targeting, trafficking and functional properties. Studies in mammalian cell lines have demonstrated the importance of transmembrane domains for efficient subunit folding, receptor assembly and cell-surface expression. Electrophysiological studies of receptors assembled from $\alpha 7/5HT3A$ subunit chimeras have revealed the influence of subunit domains upon ion channel properties such as rectification, desensitisation and conductance. Evidence has been obtained to indicate that both extracellular and cytoplasmic domains exert a dramatic and significant influence upon single-channel conductance. These data support a role for regions other than hydrophobic transmembrane domains in determining ion channel properties. A further series of subunit chimeras has been constructed with the aim of investigating the influence of the large cytoplasmic loop domain (between transmembrane regions M3 and M4) on receptor targeting and trafficking. Evidence from expression in polarised cells has shown that the intracellular loop region of several nAChR subunits can markedly influence subunit folding, assembly, cell-surface expression and intracellular targeting.

Additionally, these intracellular loop chimeras have provided further evidence that this non-transmembrane region exerts a dramatic influence upon functional properties such as single-channel conductance. These M3-M4 loop chimeras are useful tools with which to investigate the influence of subunit domains upon interactions with intracellular proteins.

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PC186

Permeation and rectification of the human CLC-5 channel/antiporter: implications for its role in the kidney

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Members of the CLC family comprise Cl^- channels and Cl^-/H^+ antiporters that conduct outwardly, inwardly, and non-rectifying currents. They also have different surface and organelle membrane localisation. Mammalian CLC-5 is a Cl^-/H^+ antiporter [1, 2] that is expressed in the kidney, the proximal tubule in particular, and is involved in the process of albumin reabsorption from the glomerular filtrate. CLC-5 is thought to provide a conduit through which Cl^- can flow from the cytosol into the endosome to balance the H^+ pumped by the H^+ -ATPase to acidify the contents: an electrical shunt. Various mutations in human CLC-5 result in Dent's disease, which is characterised by the appearance of low molecular weight protein in the urine.

We investigated the properties of CLC-5 by overexpressing EYFP-CLC-5 in human embryonic kidney cells (HEK293). Confocal imaging showed that CLC-5 was localised to both surface and endosomal membranes, consistent with CLC-5 immunohistochemistry in native tissue [3]. Membrane currents were recorded by whole cell patch clamp using CsCl-based solutions, holding the cell at -30mV and applying 10ms steps from -100 to +200mV. Large and sustained outwardly rectifying currents were recorded at potentials positive to +30mV with fast activation (1ms) kinetics. Tail currents were recorded over the same voltage range following a prepulse to +200mV. At voltages that induced an outward current these currents were sustained, but inward currents were followed by a rapid (0.2ms) and complete deactivation. Extrapolating these properties to endosomal membranes this means that Cl^- ions may only flow from the endosome into the cytosol, coupled with the transport of H^+ into the endosome. To account for changes during endosomal ionic homeostasis and acidification the biophysical properties were investigated using a range of Cl^- and H^+ gradients. CLC-5 exhibited strong outward rectification under all conditions tested. The inhibition of CLC-5 by reduced extracellular pH could be explained by a shift in activation kinetics that is related to the Cl^-/H^+ coupling. Only mutating the "gating glutamate" (E211A), which uncouples H^+ transport, removed rectification and permitted the flow of currents in the opposite direction.

Our results suggest that CLC-5 has both channel and transporter-like properties, has a non-conducting role at the surface membrane, and does not provide an electrical shunt that facilitates

endosomal acidification by H⁺-ATPase. Instead CLC-5 may be involved in a complex of membrane proteins involved in endocytosis [4] and could provide a parallel endosomal acidification mechanism by exchanging Cl⁻ for H⁺ in early endocytotic vesicles.

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PC187

Identification of a region of TASK3 two pore domain K channels critical for their modulation by methanandamide

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The TASK family (TASK1 and TASK3) of two pore domain potassium (K₂P) channels underlie leak K currents in a number of different CNS neurons. These channels are regulated by a variety of physiological mediators such as changes in extracellular pH and activation of G protein coupled receptors and by a number of pharmacological agents such as volatile anesthetic agents, ruthenium red, zinc ions and methanandamide (see Kim 2005, Mathie 2007).

Using whole-cell electrophysiological recordings of currents through TASK channels expressed in tsA 201 cells, we have found that human TASK1 and TASK3 channels are both sensitive to block by (*R*)-methanandamide (a stable analogue of the endogenous cannabinoid anandamide), despite this compound once being regarded as a relatively selective TASK1 inhibitor (Maingret *et al* 2001). Methanandamide (3 μ M) produced inhibitions of $81 \pm 2\%$ ($n = 11$, mean $\pm$ SEM) and $64 \pm 4\%$ ($n = 8$) for TASK3 and TASK1 channels, respectively. For either TASK1 or TASK3, block by methanandamide was virtually irreversible, unless cells were washed with BSA (1 mg/ml).

Previously, G protein-mediated inhibition and volatile anesthetic-mediated enhancement of TASK1 and TASK3 channels has been shown to require the presence of six amino acids (VLRFLT for TASK3) at the interface between the final transmembrane domain and the cytoplasmic C terminus (Talley & Bayliss 2002). We have confirmed these observations for TASK3 channels with a construct where these six amino acids have been deleted. Currents through channels in this deletion mutant were significantly ($p < 0.05$, Student's *t*-test) reduced in amplitude from 67 ± 9 pA/pF ($n = 18$) to 26 ± 2 pA/pF ($n = 29$), when measured as the difference current between -40 mV and -80 mV. These channels were no longer inhibited by activation of muscarinic receptors (0.1 μ M muscarine producing $6 \pm 2\%$ inhibition, $n =$

3) nor by co-transfection of cells with constitutively active G α_q (24 ± 3 pA/pF, $n = 13$). The mutant channels were, however, inhibited as normal by zinc (Clarke *et al* 2004) with 100 μ M giving $88 \pm 5\%$ inhibition ($n = 5$). Methanandamide no longer inhibited and, instead, now slightly enhanced current through this VLRFLT deletion mutant ($13 \pm 4\%$ ($n = 9$) enhancement by 3 μ M methanandamide).

Thus methanandamide converges on the same intracellular gating pathway as G protein-mediated inhibition and volatile anesthetic-mediated enhancement of TASK3 channels. It remains to be determined, however, whether this region in TASK channels is involved in the gating of the channel *per se*, the binding of modulatory molecules, or in the transduction of the signal to the gate following binding.

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PC188

Acidosis inhibits I_{Ca} and I_{Kr}, but not I_p in myocytes isolated from the rabbit atrioventricular node

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The atrioventricular node (AVN) is normally the only site at which electrical activity can pass from the atria to the ventricles of the heart, so that changes in AVN conduction may be proarrhythmic or protective. Previous work has shown that acidosis, which occurs in a number of clinical conditions, slows the rate of action potential conduction through the node (Aberra *et al.*, 2001; Walker *et al.*, 2004). However, although conduction depends critically on ionic currents, little is known about the effects of acidosis on the electrophysiology of the AVN. The aim of the present study was to investigate the effect of acidosis on membrane currents recorded from myocytes isolated from rabbit AVN as described previously (Hancox *et al.*, 1993). Ionic currents were recorded at 37 °C using the whole-cell patch-clamp technique. Acidosis was produced by reducing the pH of the HEPES buffered bathing solution from pH 7.4 to pH 6.3; intracellular [Ca²⁺] and pH were buffered by inclusion of 5 mM BAPTA and 10 mM HEPES respectively in the pipette solution. Membrane potential was held at -40 mV and test pulses applied to a range of voltages between -120 and +50 mV at 5 s intervals. AVN cells could be distinguished from atrial and ventricular cells on the basis of size, small or absent I_{K1} and high membrane resistance (Hancox *et al.*, 1993): AVN cells had a membrane capacitance of 34.8 ± 1.6 pF (mean $\pm$ SEM, $n = 29$) and input resistance of 681.8 ± 60.4 M Ω at -40 mV (the approximate zero-current potential). I_{Ca} and I_{Kr}, identified based on previous work (Howarth *et al.*, 1996), and I_f were recorded. Acidosis decreased

the amplitude of I_{Ca} at all potentials (from 17.7 ± 1.8 to 12.5 ± 1.4 pA/pF at 0 mV; $p < 0.01$, paired t -test, $n = 29$), and increased its time to peak (from 3.8 ± 0.3 to 4.8 ± 0.4 ms at 0 mV; $p < 0.01$, $n = 29$). Acidosis also decreased I_{Kr} tail current (from 1.7 ± 0.1 to 1.1 ± 0.2 pA/pF at +20 mV; $p < 0.01$, $n = 19$). In 7 cells that exhibited I_p this current was unaffected by acidosis (-2.7 ± 0.7 pA/pF at pH 7.4; -2.7 ± 0.7 pA/pF at pH 6.3, recorded at -120 mV; $p > 0.05$). We conclude that I_f is unlikely to be involved in the response of the AVN to acidosis, but that inhibition of I_{Ca} and I_{Kr} by acidosis is likely to play a significant role in action potential repolarisation and conduction in the AVN.

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PC189

Single-channel study of recombinant rabbit RyR2 identifies a novel inactivating property of ATP

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Although the cDNA for the cardiac ryanodine receptor (RyR2) has been available for more than 15 years, a thorough characterisation of the recombinant RyR2 has yet to be undertaken. We established a stable inducible HEK293 cell line expressing the full-length rabbit RyR2 cDNA and assessed the functionality of the recombinant RyR2 by single-channel analysis.

Sarcoplasmic reticulum (SR) membrane vesicles isolated from sheep hearts obtained from an abattoir or from HEK293 cells were incorporated into planar lipid bilayers for single-channel studies as previously described (1). Similar experiments were performed with purified sheep RyR2 (2).

The single-channel conductance of the recombinant RyR2 with Ca^{2+} as the permeant ion was similar to that of channels isolated from cardiac muscle as was the response of the channel to caffeine, micromolar Ca^{2+} and ryanodine. Likewise, the activation portion of the dose-response relationship of the recombinant RyR2 to ATP was essentially identical to that previously described for RyR2 isolated from cardiac muscle (1). However, in contrast to the effects observed with RyR2 isolated from cardiac muscle, application of ≥ 5 mM ATP led to complete inactivation of the recombinant channel (15 out of 16 channels compared with 1 out of 16 sheep RyR2). Similarly, purified sheep RyR2 channels also displayed complete inactivation in the presence of ≥ 5 mM ATP (4 of 4 channels).

These results may be highly significant from a physiological perspective. 5 mM ATP is within the physiological concentration

range of cellular ATP (3). However, as inactivation by 5 mM ATP was rarely observed in RyR2 isolated from cardiac muscle, these results raise the possibility that the consistent inactivation observed with the recombinant RyR2 and purified RyR2 could be the result of missing accessory proteins and/or co-factors that protect against ATP-dependent inactivation.

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PC190

Ca²⁺/Calmodulin-dependent facilitation of P2X7 receptor currents

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The ATP-gated P2X7 receptors are electrophysiologically unique among the members of the P2X ionotropic receptors family by the fact that they exhibit cationic currents that facilitate when brief agonist applications are repeated. The aim of this study was to elucidate the molecular basis of this facilitation phenomenon recorded by the whole-cell patch clamp configuration in HEK293 cells transiently expressing rat P2X7 receptors. Repeated application of maximum ATP concentration (5 mM, 10 s duration applied at 40 s intervals) resulted in a 2.5-fold increase in current density over the first four applications and then remained at this facilitated level for all subsequent applications (up to 4 min). Facilitation was highly dependent on the intracellular calcium activity and was significantly decreased by the calmodulin-binding peptide (CBP) while co-expression of the dominant-negative calmodulin (CaM1234) was less effective to inhibit facilitation. By searching in a calmodulin target database, we identified an IQ-independent putative calmodulin binding site, made by 17 consecutive amino acid residues from I541 to R557, in the P2X7 cytosolic C-terminus. A double-mutant receptor (I541T, S552C) in this region, generated in order to remove the putative calmodulin binding site, showed no Ca^{2+} /calmodulin dependent facilitation. We also demonstrated by co-immunoprecipitation experiments that calmodulin interacts with the wild-type receptor and that this interaction is increased when receptors are activated by ATP. Calmodulin did not co-immunoprecipitate with the I541T, S552C double-mutant receptor. These results suggest that calmodulin is not pre-bound to the receptor but associates rapidly as the Ca^{2+} concentration increases in the cell. This study presents, for the first time, evidence that calmodulin associates with a purinergic receptor, the P2X7 receptor, and is responsible for its facilitation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC191

Population patch clamp analysis of human recombinant GABA_A $\alpha 1\beta 3\gamma 2$ channels using IonWorks Quattro

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γ -Amino butyric acid (GABA)_A-activated Cl⁻ channels are the targets for a range of clinically used drugs. However, in the absence of well characterised fast Cl⁻-sensitive fluorophores, configuring meaningful high throughput, functional assays for these channels remains a challenge. In this study we have explored population patch clamp (PPC) using the IonWorks Quattro planar array electrophysiology platform (Finkel *et al*, 2006) as a possible approach to this end.

HEK293 cells stably co-expressing human GABA_A $\alpha 1, \beta 3$ and $\gamma 2$ subunits were used as a model system throughout. GABA-evoked currents were recorded 8s post-drug addition, in a PBS external solution (containing 138mM NaCl) and K-gluconate (140mM), MgCl₂ (5mM) based internal, containing 100 μ M amphotericin (theoretical Cl⁻ E_{rev} -66mV). Confounding leak currents were minimised by measuring currents at 0mV (John *et al*, 2007). GABA (30 μ M), evoked currents in GABA_A (969 $\pm$ 208pA, mean $\pm$ SD, n=384) but not wild-type HEK293 cells (<50pA), that reversed close to E_{Cl} (-56mV). Concentration-response curve analysis yielded a pEC₅₀ value of 5.4 $\pm$ 0.1 and n_H of 1.5 $\pm$ 0.3 (n=12). Muscimol and isoguvacine also elicited currents with pEC₅₀ values of 5.9 $\pm$ 0.1 (n=10) and 4.4 $\pm$ 0.1 (n=10), respectively. Positive modulators augmented the GABA EC₂₀ response (1 μ M) with pEC₅₀ and % max potentiation values for diazepam of 7.0 $\pm$ 0.2 and 291 $\pm$ 51% (n=10), zolpidem of 6.8 $\pm$ 0.2 and 378 $\pm$ 48% (n=10), pentobarbital of 4.5 $\pm$ 0.2 and 811 $\pm$ 81% (n=10) and tracazolate of 5.3 $\pm$ 0.1 and 571 $\pm$ 99% (n=2). GABA responses were inhibited by bicuculline, picrotoxin and gabazine with pIC₅₀ values of 4.8 $\pm$ 0.1 (n=6), 5.6 $\pm$ 0.1 (n=6) and 5.7 $\pm$ 0.1 (n=6), respectively. Bicuculline and gabazine produced concentration-dependent, parallel rightward displacement of GABA curves with pA₂ and slope values of 5.7 and 1.0 for bicuculline and 6.7 and 1.0 for gabazine, indicating competitive antagonism. Picrotoxin depressed the GABA response with minimal shift in the pEC₅₀ value implying a non-competitive antagonism.

In conclusion, despite the inability to resolve the peak GABA responses, PPC can quantify agonist, modulator and antagonist pharmacology in the GABA_A cell line. Agonist and modulator potencies were 3-10-fold greater than expected, with steep Hill slopes, presumably reflecting concentration-dependent desensitisation that serves to underestimate the true response curve asymptote. This is a significant technical limitation. For antagonists and blockers, where such kinetic constraints are less important, our Schild analysis and ability to discern competitive and non-competitive antagonism are very encouraging. These data suggest that PPC may be a valuable approach for screening GABA_A receptors and other slow ligand-gated ion channels.

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PC192

Evidence for chloride channels in differentiated 3T3-L1 adipocytes?

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The primary metabolic functions of lipogenesis and lipolysis in white fat adipocytes are regulated by catecholamines and insulin. These agents have been shown known to affect the resting membrane potential of adipocytes. Nonetheless, the electrophysiology of white adipocytes is poorly studied. Non-selective cation channels and voltage-dependent K⁺ channel have been identified in rat white adipocytes. With regards to anion-selective (Cl⁻) channels, these have been described in brown adipocytes (Pappone and Lee, 1995), but their presence in white adipose cells has not been previously reported. Thus, we have used the cell-attached patch-clamp technique to explore the existence of Cl⁻ channels in differentiated 3T3-L1 adipocytes, an established experimental model of white fat adipocytes.

With Cl⁻ as the major anion in the pipette solution, multiple amplitude single-channel currents were to be found to be spontaneously active in 80 out of 86 patches. Currents were outward at pipette potentials positive to -20 mV and had an ohmic relationship with holding potential. In normal high K⁺ pipette solution, the median single-channel slope conductance was 13.8 $\pm$ 0.8 pS (mean $\pm$ S.E.M, n=8), similar slope conductances were observed in pipette solutions where the major cation was either Na⁺, TEA⁺, NMG⁺, or K⁺ (with or without Mg²⁺ or Ca²⁺) with Cl⁻ as the anion. On the contrary, when Cl⁻ in the pipette solution was substituted for aspartate, the occurrence of single-channel currents was significantly reduced to only 4 in 20 patches (χ^2 p<0.0001). Under these conditions the median single-channel conductance was 3.9 $\pm$ 1.9 pS (n=4).

In conclusion, the major ion channel isoform active in the plasma membrane of 3T3-L1 adipocytes appears to be Cl⁻ selective; further studies are needed to determine its existence in rat adipocytes and also clarify its function and regulation.

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The Royal Thai Government Scholarship

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC193

Activation of large-conductance background potassium channels and membrane hyperpolarization by arachidonic acid in murine immature B lymphocytes

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In addition to the voltage-gated K⁺ current (I_{Kv}) and Ca²⁺-activated K⁺ current (I_{KCa}) that are commonly expressed in various

lymphocytes, the immature B cell line (WEHI-231) and mouse splenic B cells express phosphatidylinositol 4,5-bisphosphate (PIP₂)-inhibited large conductance background K⁺ channels (LK_{bg}). The activity of LK_{bg} is low in cell-attached conditions. After making inside-out patches, LK_{bg} is spontaneously activated while inhibited by the application of ATP via PIP₂ formation. In the immature B lymphocytes, generation of arachidonic acid (AA) by phospholipase A₂ has been suggested to be involved in the apoptosis and growth arrest by antigen receptor stimulation. In this study, effects of AA on the ion channels were investigated in WEHI-231 cells, mainly focusing on LK_{bg}. The application of AA initially suppressed I_{Kv} and I_{KCa} while subsequently increased background-type K⁺ currents. In single channel recordings, LK_{bg} were activated by AA and other unsaturated fatty acids, which became weaker as the acyl chain is more saturated; AA > oleic acid > palmitic acid. AA showed partial activating effects on LK_{bg} directly inhibited by PIP₂. The activation of LK_{bg} by AA was fully reversible and unaffected by the treatment with inhibitors of metabolizing enzymes for AA. Consistent with the activation of LK_{bg}, the application of AA induced strong hyperpolarization of WEHI-231 and a subgroup of mouse splenic B lymphocytes. The hyperpolarization by AA was resistant with charybdotoxin, a selective blocker for I_{KCa}. AA also activated small amplitudes of nonselective cationic current in WEHI-231 cells. The AA-induced activation of background K⁺ current and hyperpolarization was rarely observed in Bal-17, a mature B cell line of mice. In summary, the LK_{bg} highly expressed in the immature B lymphocytes are potentially activated by AA while other types of K⁺ channels commonly expressed in mature B lymphocytes are suppressed. The strong hyperpolarization and efflux of electrolytes (KCl) with water might play a role in the decrease of cell volume during apoptosis in immature B lymphocytes.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC194

Increased spinal reflex inhibitory effect of the central muscle relaxant agent, tolperisone, in the presence of dextromethorphan

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Tolperisone is a centrally acting muscle relaxant drug, which inhibits segmental spinal reflexes via a blockade of voltage gated sodium and calcium conductances (Kocsis *et al.* 2005). The rat hemisectioned spinal cord model was used for characterizing spinal reflex inhibitory effects of drugs *in vitro*. Spinal cords were isolated from 6-day-old rats anesthetized with ether and cooled down by crushed ice until their respiration stopped. After removal, the spinal cord was hemisectioned along the midline, and transferred to a recording chamber. Tolperisone inhibited the

monosynaptic component of the dorsal root stimulation-evoked ventral root reflex in this preparation with an IC₅₀ value of 52±8 µM. In the presence of the antitussive agent dextromethorphan (0.25 µM) tolperisone was significantly more potent (IC₅₀=24±2 µM; p<0.05) than alone.

Further, we analyzed the possible mechanisms underlying the potentiation of the pharmacological actions of tolperisone by dextromethorphan. Dextromethorphan has multiple pharmacological actions; among others it inhibits serotonin transporters. In a binding study on membranes isolated from rat brain, dextromethorphan displaced [³H]sertraline with a K_i of 26 nM.

In a recent study, we found that several selective serotonin reuptake inhibitors, such as paroxetine or fluoxetine, enhanced the sodium channel inhibitory action of a variety of blockers, among them tolperisone, via involvement of 5-HT₂ receptors and PKC (Thán *et al.* 2007). To investigate if similar mechanisms play a role in the action of dextromethorphan, we investigated this interaction in the presence of the 5-HT₂ receptor antagonist ketanserin (1 µM). The enhancement of the reflex inhibitory effect of tolperisone by dextromethorphan, as well as by paroxetine and fluoxetine, were prevented by ketanserin, suggesting similar mechanisms.

In whole cell patch clamp recordings on dorsal root ganglion cells, tolperisone inhibited tetrodotoxin-sensitive voltage gated sodium currents concentration-dependently (IC₅₀=193 µM). Activation of 5-HT₂ receptors with the receptor agonist DOI (10 µM) enhanced the effect of tolperisone (IC₅₀=80 µM). This positive interaction was prevented by blockade of protein kinase C (PKC) with staurosporine (1 µM; intracellular application), suggesting the involvement of 5-HT₂ receptor mediated PKC dependent phosphorylation of sodium channels.

In summary, an increased serotonin tone due to inhibition of the transporter, followed by activation of 5-HT₂ receptors and PKC seem to underlie the modulation of tolperisone's action by dextromethorphan.

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Thán M *et al.* (2007). *Neurochem Int* **50**, 642-652.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC195

Investigation of the role of lysophosphatidylcholine in receptor activation of the TRPC5 channel

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TRPC5 is a member of the Transient Receptor Potential protein family and is widely expressed, including in vascular smooth muscle cells (Xu *et al.*, 2006). It has calcium channel properties and is activated by different stimuli, showing polymodal behaviour (Zeng *et al.*, 2004). In this study our aim was to investigate the mechanism linking G-protein coupled receptors to TRPC5 activation. Our hypothesis is that the coupling involves generation of LPC via the activation of Ca²⁺-independent PLA₂ (iPLA₂), which catalyze the hydrolysis of the sn-2 position of membrane

glycerophospholipids to liberate a free fatty acid (e.g. arachidonic acid) and 2-lysophospholipid. LPC is suggested as a convergence point for some stimulatory signals (Wolf *et al.*, 1997) and we recently showed extracellular and intracellular activation by LPC, but not arachidonic acid (Flemming *et al.*, 2006). Receptor agonist activating TRPC5 include carbachol (CCh) and sphingosine-1-phosphate (S1P) (Xu *et al.*, 2006, Zeng *et al.* 2004). We now find that priming TRPC5 channels with LPC abolishes the stimulatory effect of CCh consistent with LPC being involved in the receptor-activation mechanism. To further investigate this effect we firstly treated cells with 25 μ M of bromoenol lactone (BEL), an irreversible inhibitor of iPLA₂. Significant reduction in TRPC5 activation by CCh or S1P was observed in BEL-treated cells. However LPC activation of TRPC5 was not affected. Secondly we used an iPLA₂ specific siRNA transfection method to suppress iPLA₂ gene expression, confirming specific knock down by real time RT-PCR. Again CCh and S1P activation of TRPC5 were inhibited, whereas LPC activation was not. The data support the idea that receptor-activation of TRPC5 occurs via iPLA₂ and the generation of LPC, which then acts relatively directly on TRPC5.

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Wellcome Trust

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC196

A role for TASK-1 channels in the regulation of resting membrane potential and spontaneous contractions in rat urinary bladder

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The mammalian urinary bladder detrusor smooth muscle exhibits spontaneous activity *in vivo* and *in vitro*. Generation of spontaneous action potentials and subsequent contractions is dependent on Ca²⁺ influx through voltage-gated calcium channels. This is determined by the smooth muscle cell resting membrane potential (Em). Recently, two-pore domain K⁺ channels have been implicated in the setting of Em in vascular and gastrointestinal smooth muscle cells (Gardener *et al.*, 2004; Cho *et al.*, 2005). However, their role in detrusor smooth muscle remains unknown. Our aim was to investigate the expression and function of two-pore domain K⁺ channels in the rat detrusor.

RT-PCR analysis of rat detrusor homogenates detected mRNAs for TWIK-1, TWIK-2, TREK-1, TASK-1 and THIK-1. TREK-2, TRAAK, TASK-2, and TASK-3 mRNAs were not detected.

Subsequent experiments focused on acid-sensitive TASK-1 channels. Using an anti-TASK-1 antibody (Alomone Labs, Israel), Western blot analysis confirmed the expression of TASK-1 protein in rat detrusor homogenates. Immunohistochemistry showed that TASK-1 protein was expressed in both the detrusor

smooth muscle and urothelium of the rat bladder. HEK 293 cells expressing TASK-1 and TASK-2 channels were used as controls for both techniques.

The role of TASK-1 channels in the modulation of spontaneous activity was investigated. Male Wistar rats were killed by overdose of CO₂ followed by cervical dislocation. For membrane potential recordings, strips of bladder were superfused with HEPES-buffered Tyrode's solution at 37°C and myocytes were impaled with a sharp microelectrode (50-80 M Ω) filled with 3M KCl. Isometric tension recordings were performed on bladder strips that were incubated in HEPES-buffered Tyrode's solution bubbled with 100% O₂ at 37°C. Strips were set at a tension of 10mN and allowed to equilibrate for 1 hour at which point the strips displayed spontaneous contractions.

Changing bath solution pH from 7.4 to 8.4 hyperpolarised Em of the myocytes (-53.8 ± 1.1 mV to -59.5 ± 0.9 mV, $P < 0.01$) with a change to 6.4 producing a depolarisation (-59.5 ± 0.9 mV to -41.1 ± 0.9 mV, $P < 0.001$). pH 6.4 also produced an increase in the integrated force (AUC) and baseline tone of the spontaneous contractions ($P < 0.05$). Similarly, the TASK-1 inhibitor anandamide (10 μ M; Maingret *et al.*, 2001) produced a depolarisation of Em (-53.8 ± 0.4 mV to -44.6 ± 1.0 mV, $P < 0.01$) and an increase in the AUC and baseline tone of spontaneous contractions ($P < 0.05$).

These results confirm the expression of two-pore domain K⁺ channel subunits in the rat detrusor and implicate TASK-1 channels in the setting of Em and subsequent regulation of spontaneous electrical and contractile activity in the rat detrusor.

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Gardener, M. J. *et al.* (2004) Functional evidence of a role for two-pore domain potassium channels in rat mesenteric and pulmonary arteries. *B J Pharmacol* 142: 192-202.

Maingret, F. *et al.* (2001) The endocannabinoid anandamide is a direct and selective blocker of the background K⁺ channel TASK-1. *EMBO J* 20: 47-54.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC197

Bupivacaine-sensitive K⁺ channels in human airway cells

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The depth and composition of the airway surface liquid (ASL) must be strictly regulated to maintain an environment that is optimal for the activity of a variety of innate airway defence mechanisms that help to keep the lungs sterile. One way in which ASL is regulated is through the absorption of Na⁺ across the airway epithelium. Much previous work has investigated the mechanisms by which the Na⁺ channel (ENaC) itself is regulated, but less emphasis has been placed on the role of basolateral K⁺ channels, which maintain the electrical driving force for Na⁺ absorption by maintaining the membrane potential during

absorption. We recently showed, for the first time, that K^+ channels of the K2P subtype maintain resting levels of Na^+ absorption in the H441 cell line (Inglis, S.K., et al., A Ba^{2+} -resistant, acid-sensitive K^+ conductance in Na^+ absorbing H441 human airway epithelial cells. Accepted for pub. Am. J. Physiol., 2007). The aim of the current study was to investigate whether K2P channels maintain resting levels of ion transport in primary cultures of human nasal epithelial cells. Nasal cells were harvested from individuals undergoing unrelated surgery using a small nylon brush in a procedure approved by the local ethics committee. Cells were grown to approximately 70% confluence in a flask before being seeded onto collagen-coated porous supports (Snapwells, Corning, Inc.). After 14 days cells had formed resistive monolayers and were mounted in an Ussing chamber. Transepithelial potential (V_t) was monitored using a voltage clamp (WPI) attached to a computer via Powerlab interface (ADInstruments). One stable, V_t was clamped to 0mV and short-circuit current (I_{sc}) recorded. Mean V_t , I_{sc} and resistance (R_t) were 2.2 ± 2.5 mV, $12.5 \pm 2.9 \mu A cm^{-2}$ and $285 \pm 112 \Omega cm^2$ respectively ($n=11$). Amiloride and bumetanide (both $10 \mu M$) inhibited approximately 80% and 0% of I_{sc} respectively ($n=4$). After imposing a basolaterally-directed K^+ gradient and permeabilising the apical membrane with amphotericinB ($200 \mu M$) bupivacaine ($3 mM$) inhibited $24.4 \pm 5.1\%$ of basolateral K^+ current ($P < 0.05$; $n=3$). Subsequent addition of Ba^{2+} ($1 mM$) had no effect. Bupivacaine inhibited $15.4 \pm 2.4\%$ of I_{sc} in intact monolayers ($n=3$), this was not significant. These results suggest that primary cultures of human airway cells possess K2P channels that may help to maintain basal levels of ion transport.

Wellcome Trust

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC198

Evidence for heteromeric assembly among Slo α potassium channel subunits

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The potassium channel subunits Slo α 1, Slo α 2.1, Slo α 2.2, and Slo α 3 are encoded by the genes KCNMA1, KCNT2, KCNT1, and KCNU1, respectively. Each subunit contains 6 or 7 transmembrane domains, a signature potassium channel G(Y/F)G re-entrant pore loop, and a large intracellular C-terminal domain that comprises >65% of the protein. The best understood subunit is Slo α 1, which encodes the tetrameric large-conductance Ca^{2+} -activated K^+ channel, which is activated by a combination of membrane depolarisation and intracellular Ca^{2+} and Mg^{2+} . In contrast, Slo α 2.1, α 2.2, and α 3 are not activated by these divalent cations but are instead regulated by different intracellular ions (Na^+ , Cl^- , ATP^{4-} and H^+ [1]). Despite these differences, there are strong structural similarities between these subunits, in particular the presence of RCK domains that in Slo α 1 are important in Ca^{2+}/Mg^{2+} activation [2] and also in tetramerisation [3].

We have studied human Slo α 1, Slo α 2.2, and mouse Slo α 3 expressed in *Xenopus* oocytes using two-electrode voltage clamp. Consistent with previous studies Slo α 2.2 and α 3 expressed poorly and required 100 to 1000-fold more RNA than α 1 to obtain significant currents. We hypothesised that these subunits represent a discrete subfamily of K^+ channels, that the conserved RCK domains enable heteromultimerisation when co-expressed, and that this may be important in native tissue where Slo α 2.x and Slo α 3 may not readily form homomeric channels. Indeed a novel current was observed when Slo α 1 and Slo α 2.2 was co-expressed in a previous study [4]. To test this we employed a dominant negative approach by mutating the G(Y/F)G motif to AAA [3] in Slo α 1, α 2.2, and α 3 and co-injecting with wild-type Slo α 1. Each of the dominant negative subunits (dnSlo α X) almost completely abolished Slo α 1 currents when co-injected into oocytes, but had no effect on Kv1.1, a member of a different K^+ channel subfamily. The dnSlo α 2.2 and dnSlo α 3 exerted their effects on Slo α 1 with low RNA doses with which wild-type subunits fail to generate significant Slo α 2.2 and α 3 currents. Furthermore, fluorescently labelled subunits colocalised when transfected into HEK cells and studied by confocal microscopy.

The high levels (50ng) of Slo α 2.2 and α 3 RNA that are required to generate macroscopic currents suggest that they do not readily form functional homomeric channels. At low RNA doses (1ng) dnSlo α 2.2 and dnSlo α 3 subunits are synthesised and incorporate into tetramers with Slo α 1 to exert a dominant-negative effect. These data suggest that the different Slo α subunits have the potential to heteromultimerise when co-expressed and that Slo α 2.x and Slo α 3 subunits are unlikely to exist as homomultimers *in vivo*.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC199

Functional TRPM3 channel in human saphenous vein smooth muscle cells

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TRPM3 is a TRPM subfamily member reported to be activated by reduced extracellular osmolarity [1] and sphingolipids closely related to, and including, *D-erythro*-sphingosine [2]. While conventional TRP channel inhibitors such as lanthanides and 2-APB have been used to characterise TRPM3, these agents are non-specific. Using previously described methods to target antibodies to the third extracellular loop in the channel structure [3]

we developed the subtype specific antibody TM3E3. Targeting of the antibody near to the channel pore allows for inhibition of TRPM3 channel activity. Preincubation with TM3E3 produced about a 40% block of calcium influx through the TRPM3 channel, as measured by 96-well plate calcium fluorimetry, and direct application of TM3E3 during patch-clamp recording. PreadSORption of TM3E3 to its antigenic peptide prevented its blocking effect. TM3E3 had no effect on the closely related channel TRPM2, or other TRP family members TRPC5 and TRPV4, confirming its specificity for TRPM3. Recently, novel activators for TRPM3 have been suggested. In conjunction with TM3E3, these modulators have been used to study native TRPM3 in human saphenous vein smooth muscle cells (HSV cells). Messenger RNA encoding TRPM3 was detected by RT-PCR and peptide-specific labelling of the cells occurred with TM3E3. Sphingosine and its analogues evoked calcium responses in HSV cells that could be inhibited by gadolinium or 2-APB, and sphingosine-evoked calcium entry was partially suppressed following preincubation with TM3E3. This inhibitory effect was mimicked by siRNA that suppressed TRPM3 gene expression.

The data show a novel TRP channel tool for studies of endogenous channels and suggest TRPM3 is a functional calcium-entry channel of vascular smooth muscle cells.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC200

Development of tools for high-throughput screening of voltage gated sodium channels

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Voltage gated sodium channels (NaV channels), of which there are multiple members (NaV1.1-1.9), play an essential role in the initiation and propagation of action potentials in neurons and other electrically excitable cells. There is considerable evidence that drugs able to specifically act on these channels could have numerous clinical uses, such as in the development of pain relieving drugs and the treatment of epilepsy.

Screening for inhibitors and modulators is typically achieved using mammalian cells stably expressing specific NaV channels. We have developed a range of cell lines expressing NaV channels that overcome common limitations experienced when using stable mammalian cell lines, such as slow growth rates and low levels of expression. We show high through-put fluorescent assay data and confirmatory electrophysiological data to validate our alternative approaches.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC201

Brn-3a promotes transcription of the cardiac Nav1.5 sodium channel's neonatal isoform

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Nav1.5, the predominant sodium channel in the mammalian heart, is the main component of the upstroke of the cardiac action potential. Accordingly Nav1.5 dysfunction (via mutation or aberrant expression) underlies numerous inherited cardiac arrhythmias and has a role in cardiac hypertrophy and ischemic heart disease.

Heart sodium currents change significantly when cardiomyocytes lose the ability to proliferate in postnatal heart remodelling. Thus, electrophysiological recordings from foetal myocytes exhibit markedly slower kinetics than adult cells (Jacques *et al.*, 1997). We have recently demonstrated the existence of adult and foetal ('neonatal') alternative splice forms of Nav1.5 (Fraser *et al.*, 2005). This splicing is regulated postnatally in the heart, with the 'neonatal' exon of Nav1.5 transcripts rapidly replaced by the 'adult' exon. The kinetics of 'neonatal Nav1.5' are markedly slower than those of the adult form suggesting that alternative splicing may be a major mechanism underlying postnatal changes in heart currents (Mattis *et al.*, 2007).

Two *SCN5A* promoters, differentially utilised pre- and postnatally, have recently been found suggesting that *SCN5A* transcriptional and alternative splicing regulation may be coupled (Shang and Dudley, 2005). Here we show that the Brn-3a transcription factor (expressed in the foetal heart and DRG sensory neurons like Nav1.5) can regulate foetal Nav1.5 expression *in vitro* and *in vivo*. Using real-time RT-PCR methods we found Nav1.5 mRNA levels in DRG and whole heart extracts from E13.5 Brn-3a *-/-* mice were significantly reduced (~2-fold) compared to wild-type litter-mates. This reduction was probably due to Brn-3a acting to promote the transcription of *SCN5A* since over-expression of Brn-3a in human SH-SY5Y sensory neuron/neuroblastoma cells *in vitro* increased endogenous Nav1.5 mRNA levels ~2-fold. Importantly, although both neonatal and adult isoforms could be detected only the levels of neonatal Nav1.5 changed with Brn-3a manipulations. This appeared to be due to Brn-3a acting specifically upon the upstream 'foetal' *SCN5A* promoter since the activity of a 'foetal' *SCN5A* promoter-luciferase fusion construct was increased ~2-fold in both primary myocytes and SH-SY5Y cells over-expressing Brn-3a. In conclusion, we show that in the pre-natal heart and DRG Brn-3a regulates the expression of neonatal Nav1.5 specifically by acting upon the 'foetal' *SCN5A* promoter. Our data suggest that *SCN5A* provides a further example of developmental-specific transcription and alternative splicing coupling (Kornblihtt, 2005).

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PC202

Activation of K⁺ currents during hyposmotic stress in neuronal CAD cells

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Hypotonic stress causes the activation of both K⁺ and Cl⁻ channels in cells, allowing ions to leave the cell followed by osmotically obliged water. The cell returns to its physiological volume, by a process known as regulatory volume decrease (RVD). There are two main types of K⁺ channels; voltage-gated and Ca²⁺-activated. Voltage-gated channels comprise the delayed rectifier and A-type channels while the Ca²⁺-activated channels comprise BK, IK and SK channels. Both channel types have been identified in neuronal cells, however the K⁺ channel responsible for RVD in CAD cells is not known. This study investigated the K⁺ channel activated in response to hypotonic stress using the whole cell patch clamping technique. K⁺ currents were recorded under isotonic and hypotonic conditions at 20°C. Cells were held at a holding potential of -60mV for 4.64ms and were subjected to a step protocol (-60mV to +60mV in 20mV increments), each pulse lasting 100ms. In the presence of both intracellular and extracellular Ca²⁺ the K⁺ current increased from 20.13±1.9pA/pF to 33.06±2.3pA/pF (n=5) upon hypotonic stress. In the absence of extracellular Ca²⁺ the K⁺ current increased from 24.82±1.3pA/pF to 30.68±1.5pA/pF (n=5). Experiments in the absence of intracellular Ca²⁺ also showed channel activation with current increasing from 25.13±1.3pA/pF to 28.15±1.6pA/pF (n=4). Removal of both extracellular and intracellular Ca²⁺ resulted in a decrease in K⁺ current from 29.43±2.8pA/pF to 27.87±3.2pA/pF (n=3). Inhibition of the hypotonically induced current from 31.0±4.2pA/pF to 16.7±4.0pA/pF using the SK inhibitor apamin (100nM) suggested a role for this channel. The K⁺ current activated in the presence of both intracellular and extracellular Ca²⁺ was significantly greater than that observed in the absence of either extracellular or intracellular Ca²⁺ or the addition of apamin (ANOVA and Tukey HSD).

Under Ca²⁺ free conditions a significant reduction in current is observed when compared to the current activated under control conditions, suggesting that whilst intracellular Ca²⁺ can activate the K⁺ channel, extracellular also plays a role. The removal of intracellular Ca²⁺ also reduced the amount of current through the channel while the removal of both intracellular and extracellular Ca²⁺ resulted in the abolition of channel activation. These results suggest that intracellular Ca²⁺ plays a greater role in channel activation compared to extracellular Ca²⁺. The abolition of current in the absence of Ca²⁺ and the reduction of current in the presence of apamin suggest the SK Ca²⁺-activated K⁺ channel is responsible for the K⁺ efflux following hypotonic exposure in neuronal CAD cells.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC203

Dexbrompheniramine maleate inhibits the putative cough receptor, TRPV1 and effectively treats chronic cough

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American guidelines support the use of antihistamines as a first line treatment for some causes of chronic cough. The capsaicin receptor TRPV1 is thought to be one of the primary sensory receptors for cough. We therefore sought to determine whether the antihistamine Disofrol (dexbrompheniramine maleate) could antagonise TRPV1 responses to capsaicin and resiniferatoxin and could be used to treat chronic cough. Dexbrompheniramine (100µM and 1mM) significantly ablated capsaicin and resiniferatoxin triggered activation (calcium influx) of TRPV1 permanently expressing HEK293. Dexbrompheniramine was selective since it failed to inhibit PAR2 or endothelin evoked responses. Interestingly two related antihistamines diphenhydramine and fexofenadine had negligible effect on TRPV1 activation by capsaicin. Similar results were also obtained for rat TRPV1 expressing HEK and Pro5 cells. In cultured rat dorsal root ganglia neurons, dexbrompheniramine (30µM, 100µM and 300µM) also exhibited an inhibitory effect on capsaicin evoked responses. Finally, 76 patients referred to Hull cough clinic with intractable cough were recruited and dispensed dexbrompheniramine (Disofrol) twice daily for four weeks. Dexbrompheniramine improved cough in 48 (63%) of patients. Overall the mean (standard deviation) visual analogue cough score following treatment (4.82 ± (2.6)) was significantly lower than before treatment (7.63 ± (1.42), p<0.001). The results therefore suggest that dexbrompheniramine may inhibit TRPV1 and may be a safe and effective treatment for chronic cough.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC204

Different PKC isoenzymes are involved in inhibition of rat mesenteric arterial voltage-gated K⁺ channels by endothelin-1 and angiotensin II

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Voltage-gated K⁺ (Kv) channels of arterial smooth muscle modulate arterial tone, and are inhibited by vasoconstrictors. In rat

mesenteric arterial smooth muscle cells both angiotensin II (Ang II) and endothelin-1 (ET-1) inhibit Kv channels through PKC (Hayabuchi et al., 2001; Rainbow et al., 2006) and here we have investigated the PKC isoenzymes involved. Smooth muscle cells were isolated enzymatically from rat mesenteric arteries and expression of PKCs α , δ , ϵ and λ/ζ was detected using western blotting. Kv currents were recorded in physiological K^+ concentrations using conventional whole-cell recording where the pipette solution contained 100 nM Ca^{2+} (buffered with 10 mM EGTA), 1 mM Mg^{2+} , 1 mM ATP and 0.5 mM GTP. Ang II (100 nM) and ET-1 (10 nM) inhibited Kv currents by similar amounts ($52.6 \pm 6.7\%$ and $53.2 \pm 7.7\%$, $n = 5, 6$). However, PKC ϵ inhibitor peptide, added either by inclusion in the patch pipette or externally in the form of a membrane permeable Tat-linked peptide, reduced inhibition by Ang II to $10.6 \pm 2.1\%$ ($p < 0.001$ vs control) and $5.3 \pm 2.8\%$ ($p < 0.001$) respectively. However, we saw little effect of PKC ϵ inhibitor peptide (included in the pipette) on the inhibition of Kv currents by ET-1. In contrast, Gö6976, an inhibitor of conventional PKCs did not significantly affect Kv inhibition by angiotensin, but reduced that by ET-1 to $17.0 \pm 2.8\%$ ($p < 0.001$). To investigate the possible involvement of PKC β , we tested the PKC β inhibitors LY333531 and PKC β inhibitor peptide, neither of which reduced Kv inhibition by endothelin. We also investigated the roles of PKC isoenzymes in the contractile effects of these vasoconstrictors using myography of small mesenteric arterial segments. PKC ϵ inhibitor peptide (Tat-linked to allow intracellular access) substantially inhibited contractions to angiotensin, but did not affect those to ET-1, while Gö6976 inhibited contractions to ET-1 and also reduced those to Ang II by a lesser extent. Our results confirm that Ang II inhibits Kv channels through PKC ϵ , and suggest that ET-1 acts primarily through a conventional PKC, most likely PKC α . These results are also consistent with Kv inhibition contributing to the contractile actions of these vasoconstrictors.

Hayabuchi Y et al (2001) *Am J Physiol* 281, H2480.

Rainbow RD et al (2006) *J Physiol* 575, 833.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC205

Development of a 96-well filtration assay for monitoring TRPV1-induced CGRP release from adult spinal cord

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The transient receptor potential channel of the vanilloid receptor type, subtype 1 (TRPV1) is a member of the TRPV family of ion channels. TRPV1 is a non-selective cation channel, with high permeability to calcium and acts as an integrator of noxious stimuli including heat, low pH and capsaicin. TRPV1 is also activated by endogenous lipid agonists and sensitized by inflammatory mediators. It plays an important role in pain signaling as demonstrated through the use of TRPV1 knockout mice and selective antagonists.

TRPV1 is functionally expressed on the terminals of primary afferent fibers terminating in the superficial layers of the dorsal spinal cord, with activation of the receptor leading to, amongst other things, release of CGRP. In the current study, we have examined the ability of agonists (endogenous & synthetic) of the TRPV1 receptor to evoke CGRP release from homogenate of adult rat spinal cord using a 96-well filter plate assay with results showing a rank order of potency of Resiniferatoxin > Capsaicin > Arvanil > Olvanil > NADA > OLDA > Anandamide. In addition, we have investigated the potency of a variety of TRPV1 antagonists in blocking capsaicin-induced CGRP release (BCTC > Neurogen > A425619 > AMG 49a > AMGEN I > Renovis > JNJ17203212 > Capsazepine > GSK705498 > AMG9810 > SB366791). These antagonists were also profiled for activity against the sensitized TRPV1 receptor.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC206

Acid/base transport across the apical membrane of Calu-3 airway epithelial cells

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The Calu-3 cell line, derived from human airway submucosal glands, expresses high levels of CFTR and is a good model for investigating Cl^- and HCO_3^- secretion in an epithelium that is adversely affected by cystic fibrosis (Hug et al. 2003). Particular interest focuses on the role of CFTR in HCO_3^- secretion, the loss of which is thought to underlie much of the pathology of cystic fibrosis. The aim of these studies was to characterise the acid/base transporters that are expressed in the apical membrane of Calu-3 cells grown as polarised monolayers on Transwell polyester filters (Corning). Filters were superfused bilaterally with HEPES or HCO_3^- -buffered solutions and maintained at $37^\circ C$ on the stage of an inverted fluorescence microscope. Cells were loaded with the pH-sensitive fluoroprobe BCECF and intracellular pH (pH_i) was recorded by standard microfluorometric techniques. The recovery of pH_i from an acid load induced by brief bilateral exposure to NH_4^+ was entirely dependent on extracellular Na^+ . In HCO_3^- -free conditions there was a significant recovery of pH_i when Na^+ was restored to the apical bath in the absence of basolateral Na^+ . This was blocked by apical EIPA (30 μM) and therefore attributed to apical expression of an Na^+/H^+ exchanger, most probably NHE2 or -3. When repeated in the presence of HCO_3^- , there was no evidence for the involvement of any additional Na^+ -dependent processes at the apical membrane. Substitution of Cl^- by gluconate on the apical side of the Calu-3 cells led to a small increase in pH_i (0.06 ± 0.02 , mean $\pm$ s.e.m., $n = 8$, $P < 0.05$ by paired t test). However, following combined stimulation with forskolin (3 μM) and IBMX (100 μM) to elevate intracellular cAMP, apical Cl^- substitution resulted in a large and rapid increase in pH_i (0.37 ± 0.03 , $n = 9$, $P < 0.001$). Although this alkalization might normally be attributed to Cl^-/HCO_3^- exchange across the apical membrane, it was relatively insensitive to the non-specific anion exchange inhibitor DIDS (28% inhibition

with 100 μM and 57% with 500 μM). In contrast, it could be totally abolished by the specific CFTR channel blocker CFTRinh-172 (79% inhibition with 5 μM and 95% with 10 μM). This suggests either that the alkalinization is due to HCO_3^- entry through the CFTR channel, which would require a reversal of the apical membrane potential, or that the depolarization resulting from Cl^- efflux through CFTR alters the driving forces for other electrogenic acid/base transporters.

Hug MJ et al. (2003). *News Physiol Sci* 18, 38-42

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC207

Time profiles of rebound potentiation and sensitivity to protein kinases in mouse cerebellum

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Rebound potentiation (RP) is a plasticity that occurs at interneuronal-Purkinje cell (PC) synapses in the cerebellum. It is caused by PC depolarisation inducing a Ca^{2+} influx that triggers a long term potentiation of GABAergic transmission. Although protein phosphorylation has been implicated in RP, the precise protein kinases involved are not defined and little is known about the physiological profile of this plasticity. In this study, we have investigated the time profile of RP after PC depolarisation at different time points following the formation of the whole-cell configuration. We have also identified protein kinases that appear to mediate RP.

Day 11-14 postnatal Purkinje cells in parasagittal slices were voltage-clamped at

-70mV. RP was induced after 5 mins by depolarising the PC to 0mV for 100ms duration (8x) at 0.5Hz. The mIPSC amplitudes gradually increased reaching a plateau of $163 \pm 9\%$ (mean $\pm$ sem; $n = 7$) after 10 mins. The RP persisted for up to 30 mins after initiation. However, the amplitude potentiation for RP and its time profile varied with an increase in the latency of PC depolarisation. At 12 mins, a peak RP (short-term RP; $154 \pm 17\%$), occurred rapidly within 1 min after the stimulus followed by a rapid decline to baseline, before the resumption of a slower phase of RP (long-term RP) reaching a potentiation of $152 \pm 14\%$ after 30 mins. With longer stimulus latencies up to 20 mins, short-term RP was abolished leaving only the long-term RP.

To investigate the role of protein kinases in RP, we applied a novel and highly specific CaMKII inhibitor, CaMKIINtide. At 500nM, intracellular dialysis with this peptide completely abolished RP which was activated by PC stimulation after a 5 min latency (mIPSC amplitude = $88 \pm 8\%$ of control at 30 mins post-stimulation). By contrast, the short-term RP observed with 12 min stimulus latency was only reduced to $126 \pm 7\%$ of control at $t = 1$ min post-stimulation). The role of protein tyrosine kinases was assessed using the inhibitor genistein. Using a 12 min stimulus latency, intracellular-application of 100 μM genistein abolished long-term RP ($99 \pm 13\%$ of control), but only reduced the short-term RP to $127 \pm 17\%$ compared to control). Co-application of

CaMKIINtide and genistein did not inhibit short-term RP any further ($92 \pm 8\%$). Finally, the non-specific kinase inhibitor staurosporine (10 μM) also failed to completely block the short-term RP ($120 \pm 4\%$). These results indicate that RP can be separated into both short- and long-term potentiations of inhibitory synaptic inhibition depending on the latency of the stimulus. Furthermore, although the long-term RP is dependent on protein phosphorylation, the short-term RP exhibits a component that is either phosphorylation independent or insensitive serine-threonine and tyrosine kinase inhibition.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC208

A helically structured M1-P1 loop is essential for pH sensitivity of the two-pore domain potassium channel, TASK2

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Two-pore-domain potassium ($\text{K}_{2\text{p}}$) channels underlie background currents that control the resting membrane potential and excitability of many cells. Although classed as "leak" channels, currents through $\text{K}_{2\text{p}}$ channels are tightly regulated. For example, the pH sensitive $\text{K}_{2\text{p}}$ (TASK) channels are inhibited by extracellular acidification with pK_a s of 7.3 for TASK1 and 6.6 for TASK3. Whilst a pore neighbouring histidine is thought to be the pH sensor of TASK3 and TASK1 it is not present in TASK2 and does not account for the differing pK_a s of TASK1 and TASK3. TASK2 was the second member of the TASK channel subfamily to be cloned; however it is now known to have closer amino acid homology to the alkaline sensitive TALK family channels. More like the TALK family, TASK2 is also activated at alkaline pH with a pK_a of around 8. Residues within the large extracellular M1-P1 loop have been implicated as forming the pH sensor of TASK2 (Morton et al., 2005), but this data has recently been refuted and an arginine within the second pore demonstrated to be the real pH sensor (Niemeyer et al., 2007)

In this study we show that the M1-P1 loop is a structured entity that, whilst not being the pH sensor *per se*, contributes to the differential pH sensitivity of TASK channels. Chimaeric TASK channels, constructed by swapping M1-P1 loops between family members, were expressed in *Xenopus* oocytes as previously described (Clarke et al., 2006). Channels were characterised using standard two-electrode voltage-clamp techniques. All data is shown as mean $\pm$ SEM. NMR spectroscopy studies were performed on a synthetic peptide of TASK2 M1-P1 (Auspep).

The pH sensitivity of TASK2 was essentially abolished with the replacement of its M1-P1 loop with that of TASK3 and vice versa (TASK2: $\text{pK}_a = 8.0 \pm 0.04$, $n=8$; T2/T3 M1P1: $\text{pK}_a = 5.3 \pm 0.2$, $n=9$; T3/T2 M1P1: $\text{pK}_a = 4.9 \pm 0.1$, $n=10$).

Intriguingly, replacement of the TASK1 M1-P1 with that of TASK3 led to channels with TASK3 like pH sensitivity (TASK1: $\text{pK}_a = 7.3 \pm 0.07$, $n=7$; T1/T3 M1P1: $\text{pK}_a = 6.7 \pm 0.06$, $n=6$,

TASK-3, $pK_a = 6.6 \pm 0.04$, $n=7$), however TASK3 pH sensitivity was not affected by the replacement of its M1-P1 loop with that of TASK1 (T3/T1 M1P1: $pK_a = 6.6 \pm 0.04$, $n=5$). Our work demonstrates that the M1-P1 is essential for the pH sensitivity of TASK2 and TASK1 channels and the mechanism of channel closure during extracellular acidification appears more complex than simple channel block by protonation of single pore lining residues.

Clarke, C.E. et al. (2006) *J. Physiol.* 573; 291-304.

Morton, M.J. et al. (2005) *PNAS* 102(44); 16102-16106.

Niemeyer, M.I. et al. (2007) *PNAS* 104(2);666-71.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC209

Ser/Thr Protein Phosphatases are involved in the regulation of calcium-activated chloride channels in the cystic fibrosis pancreatic duct cell line, CFPAC-1

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An understanding of the regulation of calcium-activated chloride channels (CaCC) is important in the development of pharmacotherapies for cystic fibrosis. Protein kinase C and Ca^{2+} /Calmodulin protein kinase II have been implicated in the regulation of CaCC in several cell lines (1, 2); however, little is known about the involvement of protein phosphatases in CaCC regulation.

The iodide efflux assay was used to investigate the effect of protein phosphatase inhibitors on the UTP-evoked response in CFPAC-1 monolayers. Initially, okadaic acid (OA), which can inhibit protein phosphatases (PP) 1, 2A and 2B at specific concentrations, was assessed. OA (5nM) reduced the UTP-response by 48%, from $12.6 \pm 1.7\%$ to $6.5 \pm 0.8\%$ ($n=6$, $p<0.001$, 2-way repeated measures ANOVA). OA (50nM) further reduced the UTP-induced efflux by 64%, from $13.7 \pm 2.4\%$ to $5.0 \pm 0.3\%$ ($n=6$, $p<0.001$). However, 100nM OA only decreased the UTP-mediated response by 25% from $10.9 \pm 1.2\%$ to $8.2 \pm 1.3\%$ ($n=6$, $p<0.05$). These data could be indicative of the involvement of PP1 or PP2A in the regulation of CaCC in CFPAC-1 cells. To try to elucidate this further, Endothall, a compound that inhibits PP2A and also weakly inhibits PP1, was used. Endothall (1 μ M) did not have a significant effect on the UTP-induced iodide efflux ($n=6$, $p>0.05$). This would suggest that PP2A is not involved in the regulation of CaCC, as this concentration is around the IC_{50} value for PP2A. Endothall (100 μ M) reduced the UTP-induced efflux by 29%, from $10.0 \pm 1.4\%$ to $7.1 \pm 1.5\%$ ($n=8$, $p<0.001$). Taken together this data indicates that PP1 could be involved in the regulation of CaCC.

The other protein phosphatase that could be implicated from the initial OA results is PP2B, also known as calcineurin (CaN).

The major CaN inhibitors are the immunosuppressant drugs cyclosporin A (CsA) and FK506, which bind specifically to CaN (3). CsA (10nM) reduced the UTP-induced efflux by 64%, from 8.4 ± 0.8 to 3.0 ± 0.4 ($n=4$, $p<0.001$). FK506 (10 μ M) reduced efflux by 43%, from $7.0 \pm 1.0\%$ to $4.1 \pm 1.1\%$ ($n=8$, $p<0.001$). To support the CaN-dependency of the FK506 response, rapamycin was used. Rapamycin complexes with the FKBP12, which FK506 also complexes with, but acts on a pathway independent of CaN by binding the mammalian target for rapamycin (mTOR) (4). 1 μ M Rapamycin did not significantly effect the UTP-induced response ($n=6$, $p>0.05$) suggesting no involvement of mTOR and supporting CaN-dependent FK506 activity.

Taken together these data suggest the involvement of PP1 and PP2B/CaN in the regulation of CaCC in CFPAC-1 cells.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC210

Modulation of TRPC5 but not TRPM2 calcium channels by anaesthetics

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TRPC5, a member of the Transient Receptor Potential (TRP) channel family, is widely expressed with roles in neuro and vascular physiology. We recently discovered the capability of TRPC5 but not TRPM2 to sense important lipid signalling molecules such as lysophosphatidylcholine (LPC) (Flemming et al. 2006). LPC activates TREK1 potassium channels too, an effect which is thought to be through deformation of the lipid bilayer (Maingret et al. 2000). TREK1 is also activated by anaesthetics (Patel et al. 1999). We therefore explored the effect of anaesthetics on TRPC5. Tetracycline inducible TRPC5- or TRPM2-expressing HEK293 cell lines were used. Cells were studied using real-time 96-well plate assays with the fura-2 calcium indicator dye and whole-cell patch clamp. TRPC5 activation by gadolinium, carbachol or LPC was inhibited by halothane or chloroform. TRPC5 was activated by isoflurane, whereas sevoflurane had no effect. Chloroform, halothane and isoflurane were relatively ineffective at the common clinical concentrations (1, 0.2 and 0.27 mM respectively). Propofol inhibited TRPC5 activation by gadolinium or carbachol but not LPC. The effect of propofol was not through its anti-oxidant property because the inhibition was mimicked by di-isopropylbenzene, an analogue that lacks the hydroxyl group. In patch-clamp experiments, propofol evoked large activation followed by sustained inhibition of TRPC5, suggesting it has a dual effect. Activation of TRPM2 by hydrogen

peroxide was not affected by any of the anaesthetics. The data show striking effects of anaesthetics on TRPC5. Effects occurred at relatively high concentrations and may reflect, in part, sensitivity of TRPC5 to biophysical properties of the lipid bilayer.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC211

Modulation of hTREK-1 via gasotransmitters

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Mammalian tandem pore domain K⁺ channels (K2P channels) give rise to background currents and control excitability within various tissues (Mathie, 2007). Within the central nervous system, TREK channels appear to serve important roles, for example in the manifestation of depression (Heurteaux et al., 2006). TREK channels are known to be regulated by a number of specific neurotransmitters (Mathie, 2007; Heurteaux et al., 2006). Here we report the effects of "atypical", gaseous neurotransmitters (Baranano et al., 2001) carbon monoxide (CO) and nitric oxide (NO) on the recombinant human TREK-1 channel stably expressed in HEK 293 cells.

Whole cell patch clamp recordings were made from HEK293 cells expressing the hTREK-1. Bath application of the CO donor CORM2 (30µM) evoked a reversible activation of the channel (e.g. by 24.6 ± 6.1% at +60mV; mean ± s.e.m., P<0.05, Student's paired t test, n=7), but at 100µM led to an inhibition of the channel (by 14.6 ± 3.7%, P<0.05, n=5). The inactive form of CORM2 had no significant effect on the current density of the channel at 100µM (157 ± 6.2 to 153.8 ± 3.9 pA/pF, P>0.05, n=5). To determine the mechanism behind the CORM2 effect we examined the role of cGMP. Pre-incubation (2h) of RP-8-Br-PET-cGMPs (100nM), had no effect on TREK currents but abolished the CORM2 (30µM) induced activation, suggesting the involvement of cGMP. Parallel experiments examined the effect of SIN-1 (a NO donor) on the hTREK-1 channel. Similar to CORM2, SIN-1 activated the channel at 10µM (51.9 ± 3.4% at +60mV, P<0.05, n=5). The NO effect was blocked by pre-incubation of the same cGMP inhibitor, also indicating a role for cGMP.

These data suggest a novel modulation of the TREK-1 channel via CO that is cGMP dependent and may be mechanistically similar to NO-mediated TREK regulation.

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Heurteaux C et al. (2006). *Nat Neurosci* 9, 1134-1141

Mathie A (2007). *J Physiol* 578, 377-385

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PC212

Does Ca²⁺ influx via TRPM2 mediate oxidative-stress induced cell death?

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An accumulating body of evidence implicates activation of TRPM2, a non-selective, Ca²⁺-permeant cation channel, in cell death (reviewed by Miller, 2006). Channel opening is stimulated by oxidative stress, a feature of numerous disease states. The wide expression profile of TRPM2 renders it a potentially significant therapeutic target in a variety of pathological settings including cardiovascular and neurodegenerative diseases.

To examine the role of TRPM2 in cell death, HEK293 cells were stably transfected with a pCIneo/IRES-GFP bicistronic expression vector containing the human TRPM2 gene. Intracellular Ca²⁺ ([Ca²⁺]_i) was measured from fura-2 loaded cells and cell viability was assessed using the MTT assay.

In HEK293/hTRPM2 cells H₂O₂ (10-1000µM) stimulated both Ca²⁺ influx (within 10min) and cell death (over 24h) in a concentration-dependent fashion. HEK293/hTRPM2 cells were more susceptible to H₂O₂-mediated cell death compared to untransfected controls, and the latter did not show a rise of [Ca²⁺]_i in response to H₂O₂.

A number of compounds are reported to inhibit TRPM2 activation, including poly(ADP-ribose) polymerase (PARP) inhibitors, flufenamic acid (FFA) and N-(p-amylicinnamoyl) anthranilic acid (ACA). FFA (200µM) reduced Ca²⁺ influx in response to 100µM H₂O₂ (to 41±8%, n=4), with no effect on viability at the same [H₂O₂] (24h). ACA (20µM) also attenuated Ca²⁺ influx (to 43±24%, n=4), but again had no effect on viability. PARP inhibitors (PJ34 (1µM), DPQ (10µM) and nicotinamide (1mM)) abolished Ca²⁺ influx and mediated some restorative effects on viability of HEK293/hTRPM2 cells. However, PARP inhibition also offered some protection against cell death in untransfected controls (in which the H₂O₂-stimulated Ca²⁺ influx seen in transfected cells was absent).

As a widely expressed channel which permits Ca²⁺ influx in response to oxidative stress, TRPM2 would seem destined for a role in cell death pathways and indeed much evidence points towards this being the case (Miller, 2006). However, our data suggest that although transfection with hTRPM2 sensitises cells to H₂O₂-induced cell death, the underlying mechanism is at least in part Ca²⁺-independent, since blockade of channel opening (as evidenced by suppression of Ca²⁺ influx) did not correlate with protection from cell death. In addition, since PARP inhibition had protective effects in untransfected control cells, part of the mechanism by which PARP inhibitors act to preserve viability of HEK293/hTRPM2 cells may be TRPM2-independent.

Miller BA (2006). The role of TRP channels in oxidative stress-induced cell death. *J Membr Biol* 209 31-41.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC213

Inhibition of inward rectifier potassium channels by a mitochondrial uncoupler is reversible in intact cells but not (despite run-up) in inside-out patches

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Mitochondria and inward rectifier K⁺ channels are both impaired in failing myocardium. We previously showed that K_{ir}2 inward rectifier K⁺ channels are inhibited by agents that affect mitochondria, and that K_{ir}2.2 and K_{ir}2.3 are more sensitive than K_{ir}2.1^{1,2}. The sensitivity of K_{ir}2.1 channels to carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) is increased by mutation of His53 to Gln (the equivalent residue in K_{ir}2.2 and K_{ir}2.3), Ala or Glu, but not Arg or Lys³. Neutralisation of His53 decreases the affinity of K_{ir}2.1 for phosphatidylinositol bisphosphate (PIP₂), a positive regulator of K_{ir}2 channels⁴, raising the possibility that FCCP inhibits K_{ir}2 channels via PIP₂ depletion. Such a mechanism may be reversible in intact cells but not in excised membrane patches in conditions that preclude PIP₂ regeneration. Treatment of *Xenopus* oocytes expressing K_{ir}2.2 with 10 µM FCCP for 105 min inhibited inward rectifier current to 7.2±1.8% (mean±sem) of control (p<.0001; n=5). Removal of FCCP resulted in recovery within 26.5 hr. K_{ir}2.2 current in cell-attached patches was 148±49 pA (n=13) in FCCP-treated oocytes and 1689±495 pA (n=15) in untreated oocytes (p<.01). K_{ir}2.2 current increased upon excision and perfusion of inside-out patches from FCCP-treated oocytes. However, this current run-up did not reflect recovery from inhibition by FCCP because run-up also occurred in inside-out patches from untreated oocytes. The fraction of total current that increased after patch excision was 0.79±0.05 in control and 0.89±0.03 in FCCP-treated oocytes (p=0.1; n=6). These results are consistent with a PIP₂ depletion mechanism of FCCP action. Run-up in inside-out patches suggests a soluble inhibitory factor in *Xenopus* oocytes. This was supported by experiments in which K_{ir}2.2 current was re-inhibited by 'cramming' inside-out patches into the oocyte; p<.05 for cell-attached *vs* maximum inside-out (I_{max}), p>.05 for cell-attached *vs* crammed patch, p<.05 for I_{max} *vs* crammed patch (n=8; repeated measures ANOVA). In summary, (1) FCCP inhibits K_{ir}2.2 by a mechanism that may involve PIP₂ depletion; (2) recovery requires intracellular components that are not membrane-bound; (3) *Xenopus* oocytes possess an endogenous soluble factor that inhibits K_{ir}2.2. Statistics: unpaired t-test unless otherwise stated.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC214

Effect of growth arrest on L-type calcium channel subunit expression in human vascular smooth muscle cellsD. Sykaras¹, S. Wijetunge¹, K.M. Page², A.C. Dolphin² and A.D. Hughes¹¹Clinical Pharmacology, Imperial College London, London, UK and ²Pharmacology, University College London, London, UK

L-type voltage gated calcium channels are responsible for calcium entry into vascular smooth muscle cells (VSMC), which plays a role in excitation-contraction coupling, cell migration and growth. We previously reported that growth arrest by serum deprivation was associated with increased functional importance of L-type calcium channels in human VSMC [1]. In this study we examined if growth arrest was associated with a change in expression of L-type calcium channel subunits, α1, α2δ-1 and β3.

Human VSMC were grown from explant cultures of saphenous vein and confluent cells at passage 3 were growth arrested by serum deprivation for up to 7 days. mRNA expression was measured by real-time PCR with SYBR Green and normalized to 2 reference genes, 18SrRNA and RPLP0. Protein expression was measured by SDS-PAGE and Western blotting using specific antibodies to the different L-type calcium channel subunits, and normalized to β-actin. Data are means ± SEM of 3 - 4 separate experiments.

Serum deprivation for 5 days significantly increased α1 subunit mRNA expression (97±29% increase; p = 0.008). No significant difference was observed in α2δ-1 mRNA (18±10% decrease; p = 0.2), while a small increase was noted in the mRNA for the β3 subunit in growth arrested cells (44±9% increase; p = 0.007). There was also a time-dependent increase in levels of α1 and α2δ-1 protein following serum deprivation for up to 7 days (Table 1). The maximum increases in α1 and α2δ-1 were 40% and 34% respectively at day 7 of growth arrest. There was no significant change in β3 protein expression.

The increase in functional L-type calcium channels following growth arrest of human VSMC is probably largely due to an increased expression of α1 subunit, although increased levels of α2δ-1 protein may also play a role. Changes in L-type calcium channel subunit expression during phenotypic modulation of VSMC may account for the failure of calcium channel antagonists to inhibit atherosclerosis in some studies *in vivo*.

Table 1: L-type calcium channel subunit protein levels normalized to β-actin in human VSMC growth arrested for 1-7 days.

Calcium channel subunit	0 days	1 day	3 days	5 days	7 days	p value
α1	0.30±0.03	0.32±0.02	0.35±0.02	0.39±0.02	0.42±0.03	0.029
α2δ-1	0.74±0.05	0.78±0.04	0.85±0.03	0.92±0.04	0.99±0.06	0.019
β3	0.65±0.05	0.64±0.04	0.63±0.03	0.61±0.04	0.60±0.06	0.36

Patel MK et al. (2005). *Br J Pharmacol* **145**, 811-817.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC215

Delayed-rectifying and inwardly-rectifying K⁺ channels are expressed in rat brain endothelial cellsI.D. Millar¹, S. Wang¹, M.A. Barrand¹, P.D. Brown² and S.B. Hladky¹¹Department of Pharmacology, University of Cambridge, Cambridge, UK and ²Faculty of Life Sciences, University of Manchester, Manchester, UK

Endothelial cells of brain microvessels constitute a tight barrier between the blood and the brain. They also secrete the interstitial fluid of the brain, Na⁺ transport being principally by the serial action of an NKCC cotransporter on the luminal membrane (O'Donnell et al, 2004) and the Na-K-ATPase on the abluminal membrane (Betz, 1983). This model of secretion suggests that K⁺ is recycled on both membranes of the cell. Indeed, brain endothelial cells have a high permeability to radiolabelled K⁺ (Smith & Rapoport, 1986), although little is known about the mechanisms of K⁺ transport that the above model requires. Thus, we have investigated conductive K⁺ transport in brain endothelial cells.

Endothelial cells from brain microvessels were isolated from rats, killed in accordance with UK legislation. Cells were maintained in primary culture for up to 6 passages and were plated onto permeable membrane supports not more than 3 hours prior to experimentation. Channel activity was determined by whole cell patch clamp. The standard bath solution was a high NaCl Ringer. The pipette solution was high KCl plus 4mM ATP. Data are expressed as mean ± SEM. Statistical significance was tested using Student's t-test.

Rat brain endothelial cells showed two current types: a time-dependent delayed-rectifying outward current activated by depolarising voltage shifts (more positive than -40mV) and a time-independent inwardly rectifying current active at hyperpolarising voltages, suggesting the expression of Kv-like and Kir-like channels respectively. The outward current was sensitive to 1mM 4AP, reducing peak current recorded at +40mV from 9.9±1.4pApF⁻¹ to 5.2±1.0pApF⁻¹ (p<0.001, n=10). The voltage-activated current was inhibited by the Kv1.1 and 1.3 specific blockers dendrotoxin-K (100nM) and margatoxin (10nM) by 55±6 and 57±5 % respectively (p<0.01, n=9/11). The inwardly rectifying current was inhibited by 5mM Ba²⁺. In symmetrical K⁺ solutions, the K_d of Ba²⁺ block of the inwardly rectifying current was found to be of high affinity and voltage sensitive, being 1.0±0.3μM at -120mV and 16.1±4.0μM at -40mV (p<0.05, n=5). This pharmacological profile is typical of a Kir2-type channel and real time PCR analysis identified mRNA for Kir2.1, 2.2 and 2.3 in the rat brain endothelial cells.

These data indicate that rat brain endothelial cells express two populations of K⁺ conductance mediated by Kv1.1/1.3 and Kir2 channels respectively. These channels may play an important role in K⁺ recycling necessary in the secretion of brain interstitial fluid.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC216

Role of polar side chains of amino acids in the second transmembrane of rat P2X₂ receptors

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The second transmembrane domain (TM2) of P2X receptor may form part of the ion permeation pathway. Polar amino acid side chains are conserved in TM2. We tested their participation in permeation by single-channel and whole-cell recordings, applying ATP (0.1 - 30 μM) to HEK293 cells expressing wild type (wt) and mutated receptors. Unitary conductance (γ) in outside-out patches of the wt channels was 21.7 ± 0.8 pS (n = 15; -100 mV). T330 is situated near the outer end of TM2; substitution with Ala, Cys, Gly, Ser, Trp and Tyr did not change unitary currents whereas Asp, Glu, Lys and Arg gave no detectable current. N333 could be replaced by Ala, Cys, Asp, Gly, Ser and Thr without changing γ, whereas large (Trp and Tyr) or charged (Asp, Glu, Lys, Arg) side chains reduced γ. T336 may be in the permeation pathway (Rassendren, F. et al. 1997): substitution with Cys, Glu, Asp, Gly, and Ser did not change γ, but γ was significantly reduced with Ala, Asn, Gln or Trp at this position. For T336C, ethyltrimethylammonium methane thiosulphonate reduced the probability of opening of single channels but did not change γ. T339 was less tolerant of substitution: T339S and T339C had γ similar to wt values, but T339A, T339E, T339G, T339N, and T339Q had significantly lower γ. The neighbouring residue S340 was also intolerant of substitution, with only S340E similar to wt. γ for S340A, S340G, S340K, S340N, S340Q, S340T ranged from 14 to 11 pS, whereas S340Y and S340W were both <10 pS. Substitutions at S345 were much better tolerated: S345A, S345C, S345D, S345E, S345G, S345T were similar to wild type γ, but S345K, S345R, S345W and S345Y had much lower values. The conserved Asp at position 349 could be substituted by Glu (γ = 23 pS), less well by Asn (8.4 pS), Ser (8.1 pS) and Thr (9.2 pS), and not at all by Ala, Cys, Gly or Tyr. We observed spontaneous channel openings in the absence of agonist for many substitutions at N333, T336, T339 and S340 but none at T330, S345 or D349; the value of γ was the same as that observed when ATP was added. We quantified the degree of spontaneous opening by measuring the holding current at -60 mV in the absence of ATP (wt value was 1.2 ± 0.3 pA/pF, n = 15). In some cases (N333K, T336D T336N and S340K) the spontaneous current was very large (> 8.6 pA/pF) and in these cells application of ATP (100 μM) evoked no further current. These results are consistent with the view (Egan T.M. et al., 2006) that the region around T339 and S340 may provide the selectivity filter. The spontaneous activity observed indicates that the entire region from N333 to S340 is also important for gating transitions, given that relatively minor disturbances to side chains in this region (eg. T339S) can greatly destabilise channel closed states.

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PC217

Developmental expression pattern of SK3 in monoaminergic systems of rat brain

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Small conductance calcium-activated potassium channels (SK channels) mediate apamin sensitive afterhyperpolarising (AHP) currents in neurons following action potentials, and as such are responsible for controlling neuronal excitability and regulating spike frequency adaptation (Stocker, 2004). Functional SK channels consist of α -subunit tetramers, of which three family members have been cloned SK1, SK2 and SK3. For each α -subunit a differential distribution pattern has been shown in the adult rat brain. SK1 and SK2 display an overlapping distribution pattern with a clear localisation to the cortex and hippocampus. SK3 expression is almost absent from the cortex and the hippocampus, whereas high levels of SK3 expression are observed in sub-cortical regions such as the thalamus and basal ganglia. In certain brain regions the presence of SK channels correlates with the presence of apamin sensitive AHP currents, in other regions where SK channel α -subunits have been found, apamin sensitive calcium-activated currents have yet to be demonstrated.

In this study the distribution of the SK3 α -subunit in rat brain at different developmental stages (postnatal days 1, 3, 6, 12 and 25) is shown using an affinity purified anti-CSK3 antibody. The specificity of the anti-CSK3 antibody was tested by western blot and immunocytochemistry on SK channels heterologously expressed in HEK293 (human embryonic kidney) cells. No cross-reactivity was observed for the SK channel family members. Next, the time course of SK3 expression was investigated by Western blot analysis at different developmental stages. These immunoblots showed a single band, specific for SK3, because it was not detectable in pre-absorption controls and the estimated molecular weight (75 kDa) was in accordance with the predicted molecular weight of 81 kDa. The level of SK3 protein expression appears to remain constant and the molecular weight does not change throughout the analyzed developmental stages. The anti-CSK3 antibody was then used for immunohistochemistry on rat brain sections during development. The distribution and level of SK3 expression remains constant throughout development and is overall in accordance with the pattern previously described for adult rat brain. SK3 is highly expressed in many different neuronal populations already at early postnatal stages. Of particular interest are the high levels of expression seen in monoaminergic nuclei such as the substantia nigra *pars compacta*, locus coeruleus and the dorsal raphe nucleus. The expression of SK3 in the monoaminergic neurons has been shown at postnatal day 25 by double immunostaining with dopaminergic and noradrenergic markers.

Stocker M (2004). *Nat. Rev. Neurosci.* 5: 758-770

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PC218

Characterisation of sustained calcium-gated chloride currents in murine aortic smooth muscle cells

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Calcium-gated chloride currents (IClCa) have been recorded in a number of different in smooth muscle cell types predominantly from non-murine species. As future molecular studies necessitate the development of a murine model the goal of the present study was to characterize the biophysiological and pharmacological properties of sustained IClCa in murine aortic myocytes. These characteristics were then compared to IClCa in the rabbit pulmonary artery (rPA) myocytes, which have been investigated extensively, to determine if there are any differences in the recorded current.

Balb/c mice (6-8 wk old) were killed by cervical dislocation. Thoracic aorta smooth muscle cells were obtained by enzymatic dispersion. Recordings were made using the whole cell voltage-clamp technique with an external solution of (mM): NaCl 126, MgCl₂ 1.2, CaCl₂ 1.5, glucose 11, HEPES 10, TEA-Cl 10, adjusted to pH 7.2 with NaOH. The pipette solution contained (mM): TEA-Cl 20, CsCl 106, MgCl₂ 1.1, HEPES 10, Na₂-GTP 0.2, Na₂-ATP 3, BAPTA 10 and 7.8 mM CaCl₂.

Similar to IClCa in rPA myocytes pipette solutions containing 500 nM free [Ca²⁺]_i activated a sustained inward current at 50 mV that exhibited distinctive, voltage dependent kinetics. This was manifest as an increase in the outward current upon stepping to +70 mV so the mean current increased from 35 ± 1.1 pA to 110 ± 2.6 pA over the 1.5 s step (n = 10). Repolarisation to 80 mV evoked an inward current with a mean amplitude of 175 ± 3.5 pA, which declined exponentially (mean τ = 57 ± 0.5 ms, n = 10). Stationary noise analysis of the current at the end of a test step to different potentials produced a mean single-channel conductance of 1.6 ± 0.15 pS and 1834 ± 498 channels (n = 5). The reversal potential of IClCa was close to the theoretical equilibrium potential ECl of 0 mV and was shifted by replacement of external Cl⁻ by SCN⁻ by 28.8 ± 2.3 mV (n = 3). Niflumic acid (100 μ M) produced a complete but transient inhibition of IClCa, which was followed by a prominent increase to greater than control levels. Anthracene-9-carboxylate (500 μ M) produced inhibition of currents at +70 mV and increased the inward current upon repolarisation to 80 mV by 173.5 ± 74.3 pA (n = 3). These effects were similar to those observed in rPA myocytes. NPPB at 100 μ M had no apparent effect on IClCa in these cells. Rapid rundown of IClCa was not observed in aortic myocytes unlike rPA cells but a slow runup, similar to that found in rPA, occurred in the absence of intracellular ATP.

In conclusion, murine aortic myocytes displayed IClCa with similar pharmacological and biophysical properties to those possessed by rPA myocytes but showed notable differences in terms of regulation.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC219

The macrophage P2X₇ receptor elicits tissue factor de-encryption

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The macrophage adenosine 5' triphosphate (ATP)-gated P2X₇ receptor (P2X₇R) couples to opening of a cation permeable channel, triggers permeation of large dyes into the cell (<900 Da), rapid externalization of phosphatidylserine (PS) and secretion of pro-inflammatory cytokines. All these events can occur in the absence of cell lysis (Moore & MacKenzie, 2006; MacKenzie *et al.* 2001). Changes in lipid topology are reported to play a crucial role in de-encryption of tissue factor (TF); a membrane bound glycoprotein that initiates the extrinsic clotting cascade (Price *et al.* 2004; Wolberg *et al.* 1999). We have investigated whether macrophage P2X₇Rs can interact with pro-thrombotic signalling pathways and de-encrypt cell surface TF.

In the current study we have evaluated the ability of P2X₇Rs to de-encrypt cell surface TF in RAW264.7 macrophages. Active TF complexes with the activated protease, Factor VII (FVIIa), and processes Factor X (FX) into active FX (FXa) (Price *et al.* 2004). De-encryption of TF was monitored in the presence of FX (150 nM) and FVII (5 nM) where generation of FXa was detected using a chromogenic FXa substrate (0.5 mM S-2765TM). All experiments were conducted at time points where no release of lactate dehydrogenase could be detected (Moore & MacKenzie, 2006). Assays were performed at 37 °C in NaCl-based saline containing (in mM) 147 NaCl, 2 KCl, 10 HEPES, 12 Glucose, 1 MgCl₂, 2 CaCl₂ (pH 7.3 with NaOH). Data expressed as mean ± s.e.m. RAW 264.7 macrophages were pulsed with 3 mM ATP for 5 mins and an increase in FXa was detected above background levels (Δ FXa = 249 ± 42 pM, $n = 3$, $p < 0.01$ *versus* control using one-way ANOVA with Dunnetts Post-Hoc Test). Pre-incubation of cells with the P2X₇R antagonist KN-62 (10 μ M, 5 min) abolished this increase ($n = 3$, $p > 0.05$ *versus* control using Students T-test). ATP-induced increases in FXa generation was found to be both PS and TF dependent as incubation of stimulated cells with either 500 nM Annexin-V or 25 μ g ml⁻¹ TF IgG abolished increases in FXa generation ($n = 3$, $p > 0.05$ *versus* control using Students T-test). Increases in FXa generation were not accounted for by an increase in surface expression of tissue factor. FACS analysis using TF IgG, demonstrated that brief 5 min ATP stimulation (3 mM) gave no significant change in percentage of cells expressing TF ($n = 3$, $P > 0.05$) and furthermore led to a reduction in mean fluorescence intensity ($n = 3$, $p < 0.01$).

These results suggest that macrophage P2X₇Rs, classically described as pro-inflammatory receptors, can also engage pro-thrombotic signalling pathways with rapid de-encryption of cell surface TF.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC220

Parallel changes to drug potency and channel inactivation for the hERG K⁺ channel blocker E-4031: findings with a double inactivation mutant

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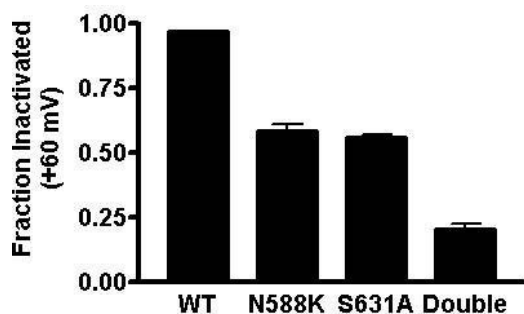
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Drugs that block hERG are proposed as a potential adjunct therapy for the SQT1 form of the arrhythmogenic disorder short QT syndrome. High affinity hERG blockers are known to have their hERG inhibition attenuated by the S631A mutation and by the N588K mutation of hERG, both of which result in attenuated inactivation. Here we use the S631A/N588K double mutant to show that both mutations together are synergistic in both attenuating inactivation and in reducing blockade by E-4031, a high affinity hERG blocker.

Whole-cell patch-clamp of transfected mammalian cells (CHO), using a K⁺ based pipette solution and normal Tyrode's extracellular solution at 37°C, was performed with the wild type (WT) channel and the 3 mutants. To determine potency, concentration response curves were fitted to a Hill equation after measuring fractional blockade of hERG tail currents at -40 mV following a 2 second step from -80 mV to +20 mV. The WT IC₅₀ was 15.96 ± 0.04 nM, those of the single mutants S631A and N588K were almost identically increased to 192 ± 0.03 nM and 183 ± 0.04 nM, respectively; that of the S631A/N588K double mutant was 583 ± 0.02 nM ($n = 5$ for all concentrations).

The fractional inactivated current of each channel was determined by comparing the current when the channel was at a steady state level of inactivation to when the channel was fully recovered from inactivation while remaining at steady state levels of activation. The membrane potential was stepped from the holding potential (-80 mV) to a depolarised potential (+60 mV), inactivation was relieved by a 2 ms step to -100 mV, and then the membrane potential was returned to the depolarising potential. As shown in the figure, fractional inactivation at +60 mV was greatest for WT hERG, attenuated (to a similar extent) by the single N588K or S631A mutants, and greatly attenuated by the N588K/S631A mutant. Thus, the S631A and the N588K mutations are similar in their effects on both blockade by E-4031 and attenuation of inactivation; furthermore, when combined, these mutations are synergistic in both sets of effects.

These results are concordant with a model in which the degree of blockade of the whole-cell hERG current by E-4031 varies with the degree of channel inactivation, and with a model in which inactivation comprises two synergistic processes that are discretely affected by S631A and N588K mutations.



Fractional Inactivation of WT, N588K, S631A & N588K/S631A channels (n = 4-8)

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PC221

The role of the cystic fibrosis transmembrane conductance regulator in HCO_3^- secretion by the ileum of the metatherian mammal, *Trichosurus vulpecula*, the brushtail possum

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The direct involvement of the cystic fibrosis transmembrane conductance regulator (CFTR) in the epithelial secretion of HCO_3^- is contentious (1, 3). However, there are a limited number of epithelia that, like the guinea pig pancreatic duct (2), secrete isosmotic NaHCO_3 . The ileum of the marsupial, *Trichosurus vulpecula*, appears to have a pure HCO_3^- secretory response, as cAMP-dependent secretagogues stimulate a Cl^- independent, HCO_3^- dependent short circuit current, which is inhibited by serosal 4,4'-diisothiocyano-stilbene-2,2'-disulfonate (DIDS), but is insensitive to serosal bumetanide. In view of this we have investigated whether CFTR plays a role in HCO_3^- secretion by this tissue. To do this we have used RT-PCR and *in situ* hybridization studies, plus Western blots, to investigate the expression of CFTR in the possum ileum. These studies were combined with Ussing chamber studies of the intact tissues and characterization of the properties of the cloned possum CFTR (pCFTR) expressed in Fischer rat thyroid (FRT) cells, using both transepithelial measurements of chloride current through the apical membrane of confluent sheets of FRT cells and whole cell patch clamp measurements of single FRT cells. Transcript for pCFTR was expressed in the ileal tissue of the possum and this was located predominantly in the base of the villi, with declining levels of expression along the length of the villi. Western blots of protein extracted from ileal epithelial cells confirmed that mature CFTR was expressed in the ileal epithelial cells. Consistent with these observations the HCO_3^- secretory response of the ileum was inhibited by mucosal (m) 5 nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) ($K_i \approx 29 \mu\text{M}$), but not DIDS (1mM, m) or glibenclamide (250 μM , m). A similar pharmacology was seen for

cloned pCFTR when expressed in FRT cells; NPPB inhibiting pCFTR ($K_i = 3.7 \mu\text{M}$), whereas glibenclamide had little effect ($K_i \approx 500 \mu\text{M}$) and DIDS (1 mM) had no effect. Despite the comparable pharmacology between the cloned channel and the native tissue, pCFTR had a relatively low permeability to HCO_3^- ($P_{\text{HCO}_3^-}:P_{\text{Cl}^-} = 0.22 \pm 0.04$, n=5). The expression of CFTR in the possum ileum combined with the comparable pharmacology of the HCO_3^- secretory response of the intact tissue and the cloned pCFTR, implicate CFTR in the secretion of HCO_3^- by this tissue. However, the relatively low permeability of pCFTR to HCO_3^- questions the role of CFTR in HCO_3^- secretion, unless it is expressed at high levels in the HCO_3^- secretory cells.

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PC222

Isoform-specific inhibition of TRPC4 by phosphatidylinositol 4,5-bisphosphate

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Mammalian TRPC channels are most closely related to the prototypical *Drosophila* dTRPs both structurally and functionally as they are commonly gated by PLC activation (Hardie, 2007). Although a number of second messengers modulating TRPC4,5 activity have been identified, their prime activator in the PLC pathway remains elusive (Plant and Schaefer, 2005). We hypothesised that, similarly to dTRPs, the PLC substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) can inhibit TRPC4. HEK293 cell lines stably expressing two major splice variants of TRPC4, murine TRPC4 α and TRPC4 β , were used for patch-clamp recordings performed in symmetrical 124 mM Cs^+ solutions with internal Ca^{2+} strongly buffered at 100 nM. Infusion of GTP γ S (0.2 mM) via pipette activated cation currents which showed characteristic doubly-rectifying I-V relationships with peak current densities at -50 mV of $-93.1 \pm 34.8 \text{ pA/pF}$ (n=7) and $-28.9 \pm 11.3 \text{ pA/pF}$ (n=11) for the TRPC4 α and TRPC4 β , respectively. These responses were inhibited by the PLC blocker U73122 (2.5 μM) (by $70 \pm 6\%$ and $75 \pm 9\%$ in the case of the α - and β -isoforms, respectively; n=4, $P < 0.02$ by paired *t*-test), but not by its inactive analogue U73343. Cell treatment with pertussis toxin (100 ng/ml, 16-18 hrs) inhibited both TRPC4 α and β currents by about 80% (n=8, $P < 0.01$). Thus, distinct G proteins appear to act in synergy in TRPC4 activation.

The addition of the stable PIP₂ analogue diC8-PIP₂ to the pipette solution (20 µM) suppressed TRPC4α activation in a voltage-independent manner (by 84%, n=7; P<0.05), but had no significant effect on TRPC4β activation. By *in vitro* binding assay, we showed that PIP₂ binds directly to the 84 amino acid C-terminal domain which is only present in TRPC4α and known to be an autoinhibitory domain (Schaefer et al., 2002). As TRPC4 is bound to a PDZ domain-containing protein, NHERF (Tang et al., 2000), which in turn interacts with the actin cytoskeleton via the adaptor ERM proteins regulated by PIP₂, we also analysed the consequences of cytoskeleton disruption. Treatment of HEK293 cells with cytochalasin D (5 µM for 2 hrs) completely prevented the inhibitory effect of diC8-PIP₂ on TRPC4α activation.

Channel release from the inhibition exerted by PIP₂ and actin cytoskeleton is suggested as a novel mechanism of TRPC4 activation, which is specific to its full-length α-isoform.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC223

Mechanosensitive activation of K⁺ channel via phospholipase C-induced depletion of phosphatidylinositol 4,5-bisphosphate in B lymphocytes

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In various types of cells mechanical stimuli on plasma membrane activate phospholipase C (PLCs). However, the regulation of ion channels via mechanosensitive degradation of phosphatidylinositol 4,5-bisphosphate (PIP₂) is not known yet. The mouse B cells express large conductance background K channels (LK_{bg}) that are inhibited by PIP₂. In inside-out patch clamp studies, the application of MgATP (1 mM) also inhibited LK_{bg} due to the generation of PIP₂ by PI-kinases. In the presence of MgATP, membrane stretch induced by negative pipette pressure activated LK_{bg}, which was antagonized by PIP₂ (>1 µM) or higher concentration of MgATP (5 mM). The inhibition by PIP₂ was partially reversible. However, the application of methyl-β-cyclodextrin, a cholesterol scavenger disrupting lipid rafts, induced the full recovery of LK_{bg} activity and facilitated the activation by stretch. In cell-attached patches, LK_{bg} were activated by hypotonic swelling of B cells as well as by negative pressure. The mechanoactivation of LK_{bg} was blocked by U73122, a PLC

inhibitor. Neither actin depolymerization nor the inhibition of lipid phosphatase blocked the mechanical effects. Direct stimulation of PLC by *m*-3M3FBS or by cross-linking IgM-type B cell receptors activated LK_{bg}. Western blot analysis and confocal microscopy showed that the hypotonic swelling of WEHI-231 induces tyrosine

phosphorylation of PLCγ2 and PIP₂ hydrolysis of plasma membrane. The time-dependence of PIP₂ hydrolysis and LK_{bg} activation were similar. The presence of LK_{bg} and their stretch-sensitivity were also proven in fresh isolated mice splenic B cells. From above results, we propose a novel mechanism of stretch-dependent ion channel activation, namely, that the degradation of PIP₂ caused by stretch-activated PLC releases LK_{bg} from the tonic inhibition by PIP₂.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC224

Automated planar patch clamp technology for primary cells

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Patch-clamp is still the gold standard for direct recording of ionic currents through channel proteins. Conventional patch clamp is extremely sensitive and provides a wealth of detailed information, but has limited throughput and is labour intensive. Automated planar patch clamp devices show considerable promise, being easier to operate with higher throughput. Protocols have been developed for cell lines, but we were interested in the potential benefits for studies of endogenous channels of primary cells. Using two planar systems, Patchliner Quattro and Port-a-Patch (Nanion Technologies GmbH), we routinely obtained GΩ seals and high-quality whole-cell patch clamp recordings from human saphenous vein (HSV) smooth muscle cells and rabbit synovocytes. These cells have previously proved quite difficult to patch-clamp by conventional methodology. In this study we used sphingosine-1-phosphate (S1P) a major endogenous signalling phospholipid which plays an important role in the cardiovascular system (Levade *et al.* 2001). We also used gadolinium (Gd³⁺), which is a commonly used blocker of non-selective cation channels, but in the micromolar range stimulates TRPC5 channels (Xu *et al.* 2006). Currents were elicited by voltage ramps from -100 mV to +100 mV for 1 s every 10 s from a holding potential of 0 mV or -60 mV for smooth muscle cells and synovocytes, respectively. Cell membrane capacitance for the HSV cells was 56.2 ± 4.1 pF and access resistance was 5.9 ± 0.4 MΩ (n = 48). Application of Gd³⁺ (100 µM) caused a significant inhibition of the endogenous basal currents (36 ± 0.6 % decrease, n = 7, P<0.01). However, in the presence of S1P (10 µM), Gd³⁺ produced a significant activation of endogenous current (73 ± 4.5% increase, n = 9, P<0.01). Success in producing Patchliner

recordings from HSV cells, maintained for 12–15 min, was surprisingly greater than for conventional patch-clamp methodology (56.2%, $n = 128$ vs $<10\%$ $n = 403$). Success was also greater for synoviocytes (38.2%, $n = 189$ vs $<10\%$ $n = >500$). These results suggest that there is potential for planar patch-clamp devices in the study of ionic currents through endogenous channel proteins of primary cells.

Levade T *et al.* (2001). *Circ Res* **89**, 957–968

Xu S-Z *et al.* (2006). *Circ Res* **98**, 1381–1389

Nanion Technologies for their excellent customer support. Wellcome Trust (CJM, AC, FZ, JL), BHF (AC, KEP), Heart Research UK (KEP), ORSAS (PS).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

50–100 μM and TRPV1 agonist capsaicin 1–3 μM had no effect. Broad spectrum TRP antagonist, ruthenium red 30 μM , had no effect or was slightly excitatory. The TRPM8 antagonist, capsaizepine 100–200 μM , blocked cold evoked responses (15%^{**}). The results are consistent with the involvement of TRPM8 channels mediating the increased spontaneous firing during cooling. BRETAG, A.H. (1969). Synthetic interstitial fluid for isolated mammalian tissue. *Life Sci.*, **8**, 319–329.

IGGO, A. (1969). Cutaneous Thermoreceptors in Primates and Sub-Primates. *Journal Of Physiology-London*, **200**, 403–430.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC225

A pharmacological study of slowly adapting mechanoreceptors responsive to cold stimulation

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Slowly adapting, but not rapidly adapting, mechanoreceptors are particularly sensitive to thermal stimulation (Iggo, 1969). Increased spontaneous activity was observed to thermal cooling in the range 40–17 degrees Celsius, and depressed by warming. In order to study what mechanisms may underlie thermal sensitivity, we tested a range of transient receptor potential (TRP) channel agonists and antagonists. A total of 22 adult albino Wistar-derived rats (mean weight 444 g) were used. Briefly, each animal was deeply anaesthetized with intraperitoneal urethane (2 g/kg), and then killed with urethane injected into the heart. The whisker pads were then removed from each side of the face, and individual sinus hair follicles with hair and nerve attached were microdissected out and placed in carbogenated (with bubbled medical 95% oxygen, 5% carbon dioxide) synthetic interstitial fluid (SIF) (Bretag, 1969). After slitting a follicle lengthways, it was fixed with insect pins onto a silicone elastomer Sylgard platform in a custom-made tissue bath. The follicle was pinned open to allow easy access of drugs to mechanoreceptors within the capsule. Approximately 10 mm length of the deep vibrissal nerve was kept attached, was stripped of its outer sheath, and the nerve bundle repeatedly divided and fine nerve strands teased onto a silver recording wire. Single unit recordings from mechanoreceptors were then made. Drugs were made up in SIF and applied at the rate of 1 ml/min. Drug effects were tested by comparison with control using *t* tests, where ^{**} indicates $P < 0.01$.

Of 43 units studied, 27 responded to cooling by increased spontaneous firing. The thermoTRP agonist icilin 30–100 μM increased spontaneous firing in these units, in a dose-dependent manner ($N = 17$, $r = .738$, $P = .001$). TRPM8 channel agonist, (-) menthol 200 μM , had mixed effects on spontaneous firing but consistently enhanced cold responses (121%^{**} of control). Other TRP agonists, cinnamaldehyde 1–2 mM and camphor 0.5–2 mM, significantly reduced spontaneous activity (45% and 2%^{**} respectively). TRPA1 agonist allyl isothiocyanate (mustard oil)

PC226

Functional cysteine pair in the turret of TRPC5 calcium channel

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Mammalian homologues of *Drosophila* TRP proteins form non-selective cation channels in the lipid bilayer of many cell types. The TRP channel family is divided into 6 subfamilies based upon sequence similarity and comprises 28 members (Nilius *et al* 2007). One member of the canonical subfamily, TRPC5, is expressed in a range of tissues including parts of the brain and blood vessels. It is implicated in hippocampal growth cone extension and vascular smooth muscle injury response. Similar to other members of the TRP family, TRPC5 is presumed to have six membrane spanning domains and the amino acid residues between fifth (S5) and sixth (S6) segments are likely to be involved in the formation of the ion pore. TRPC5 functions as a plasma membrane channel, with mixed cation permeability and forms homo- or hetero-multimers with TRPC1 or TRPC4 subunits (Beech 2007). TRPC5 responds to a multiplicity of activation signals including G protein coupled receptor agonists, lipids and lanthanides (Beech 2007). A glutamate residue in the predicted extracellular region between S5 and S6, i.e. in the turret structure of the channel, is essential for activation by lanthanides (Jung *et al* 2003). Amino acid sequence alignment of TRPC1, 4 and 5 channels revealed the presence of two conserved cysteine residues in close proximity to this glutamate. We hypothesised that these cysteine residues may be functionally important. Such cysteine residues may form a disulphide bridge or be free and thus modified by reducing agents or S-nitrosylation respectively. We tested these possibilities by over-expressing TRPC5 channels in human embryonic kidney cells and using electrophysiological and calcium imaging techniques. Also, we explored the effect of mutating the residues. We find that reducing agents have a marked stimulatory effect on TRPC5 and act from extracellular side of the membrane. Mutation of either cysteine residue (to alanine) led to large constitutive currents, which were not stimulated by

dithiothreitol (a reducing agent) or lanthanides, but were inhibited by 2-aminoethoxydiphenyl borate. Dithiothreitol did not activate TRP channels that lack a cysteine pair in this position (TRPC6 and TRPM2). The nitric oxide donor SNAP had a weak stimulatory effect on TRPC5. The data suggest an inhibitory disulphide bridge can exist in the turret of TRPC5 conferring the possibility of regulation by redox potential.

Nilius B et al (2007). *Physiol Rev* 87(1), 165-217.

Beech DJ (2007). *Handb Exp Pharmacol* 179, 109-23.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC227

Expression and function of TRPM3 calcium channel in synoviocytes

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The Transient Receptor Potential (TRP) gene was originally identified in *Drosophila* photoreceptors. Since then, 28 mammalian homologues have been reported and classified into 6 families of TRPC, TRPV, TRPM, TRPML, TRPA and TRPP (Nilius et al., 2007). They are ion channels with permeability to calcium, sodium and, in some cases, magnesium. Their importance in physiology and pathology is being increasingly appreciated.

Fibroblast-like synoviocytes - the main cell type of the synovial membrane - play important roles in synovial joint homeostasis. For example, they regulate synovial fluid volume and promote lubrication by secreting hyaluronan and lubricin. However, these cells also actively participate in diseases like rheumatoid arthritis, which has hallmarks including synovial hyperplasia and excessive cartilage degradation by secretion of matrix-degrading enzymes. Because calcium signaling is often associated with such processes, we screened for TRP expression in synoviocytes.

Immuno-staining using anti-TRPM3 antibody suggested TRPM3 expression in both rabbit synoviocytes and human synovial tissue sections. We investigated whether there is TRPM3 function in synoviocytes using a TRPM3 activator, sphingosine (Grimm et al., 2005). Sphingosine elicited a biphasic intracellular calcium signal, comprising transient and sustained phases. Sphingosine analogues, dimethylsphingosine and dihydrosphingosine, also elicited calcium responses, consistent with TRPM3 involvement. Calcium-free bath solution significantly reduced the transient sphingosine response, whereas gadolinium, lanthanum, and ruthenium red each abolished both the transient and the sustained phases. These blockers did not affect the ATP response. The sustained response was suppressed but not abolished by a functional anti-TRPM3 blocking antibody, while the transient response was unaffected.

We observed TRPM3 expression and sphingosine-induced calcium responses in synoviocytes. The data are consistent with the existence of multiple components to the sphingosine response, with TRPM3 contributing to the sustained phase.

Grimm et al (2005). *Mol Pharmacol* 67 (1). 798-805

Nilius et al (2007). *Physiol Rev* 87 (1). 165-217

Firestein G S (2006). *N Engl J Med*, 354, 80-2

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PC228

The presence of a functional t-tubule network increases the sensitivity of RyR1 to caffeine in mechanically skinned skeletal fibres

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Previous work on cultured myotubes provided evidence that the presence of functional t-tubules is associated with a decrease in the open probability of associated ryanodine receptors (RyRs), located in the junctional SR (Zhou et al. 2006). It was proposed that the underlying mechanism might involve a tonic inhibitory influence of dihydropyridine receptors (DHPRs) on coupled RyRs.

We have used mechanically skinned fibres as a model to study the influence of functional t-tubules on RyR1 in adult skeletal muscle. Rats (200-250 g) were humanely killed. Single extensor digitorum longus (EDL) fibres were mechanically skinned under oil and then perfused with solutions approximating to the intracellular milieu. The perfusing solution contained fluo-3 acid (10 μ M) to allow detection of cytosolic Ca^{2+} using confocal microscopy. Following skinning, the t-tubules can reseal and repolarise, allowing the physiological SR Ca^{2+} release mechanism to be triggered by field stimulation.

In approximately 25% of fibres, field stimulation resulted in non-uniform ("patchy") Ca^{2+} release. Experiments involving trapping of fluo-5N in the t-tubules revealed that the non-responding areas corresponded to regions in which the t-tubules had failed to re-seal. Fibres exhibiting patchy Ca^{2+} release were exposed to sequentially to 5 and then 20 mM caffeine. Under these conditions, it was found that 5 mM caffeine only induced Ca^{2+} release in regions of each fibre with re-sealed t-tubules (n=8). However, subsequent exposure to 20 mM caffeine induced Ca^{2+} release in both sealed and un-sealed areas (n=8). Following Ca^{2+} reloading of the SR, and readdition of 20 mM caffeine, there was no significant difference between the Ca^{2+} transient amplitude in the sealed and unsealed regions, suggesting a similar SR Ca^{2+} content (n=8, p<0.05). The higher sensitivity to caffeine was unaffected by depolarisation of the re-sealed t-tubule network following substitution of K^{+} with Na^{+} in the perfusing solution. However, fibres washed with Ca^{2+} -free (1 mM EGTA) Tyrode's solution, prior to skinning under oil, did not exhibit

differential sensitivity to caffeine in areas with sealed and unsealed t-tubules.

These data suggest that in adult EDL muscle fibres the presence of functional re-sealed t-tubules increases the sensitivity of RyR1 to activation by caffeine. This effect is apparently not dependent upon polarisation of the membrane per se, but rather the presence of Ca^{2+} in the re-sealed t-tubules. It is possible that Ca^{2+} may influence RyR1 sensitivity (i) by influencing the conformational state of the DHPR or (ii) via a localised efflux from the re-sealed t-tubule.

Zhou et al. (2006). *Am.J.Physiol Cell Physiol* 290 C539-C553.

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PC229

Elucidating the factors regulating contraction of human ureter

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During urinary tract infection there is abnormal contractility of the ureters but little is known about the normal characteristics of ureteric physiology or how bacterial infection modulates smooth muscle contraction. We aimed to characterise the primary mediators controlling normal ureteric physiology using pharmacological tools and biochemical methods.

Phasic contractions and calcium transients evoked by electrical field stimulation (EFS) of rings of human ureter loaded with the ratiometric calcium indicator Indo-1 were studied. Exposure to pharmacological agents which modulate L-type calcium channels (1 μM BayK8644 and 10 μM Nifedipine), SERCA pump (20 μM CPA) and BK_{Ca} channels (1 mM TEA) was performed by perfusing the tissue rings at 35°C with each drug. Further to this we studied the expression and tissue distribution of SERCA 1-3, BK_{Ca} α and β , RyR 1-3, L-type calcium channels and IP_3R in human ureters. Tissues were obtained with informed consent from healthy renal transplant live-donors and studied using western blotting and immunohistochemical tissue microarray techniques with a panel of well characterized isoform specific antibodies.

BayK8644 produced a time-dependent increase in the amplitude and duration of both calcium transients and phasic contractions while nifedipine caused their inhibition. TEA at 1 mM, which blocks mainly BK_{Ca} channels produced an increase in the amplitude and duration of the calcium transients and phasic contractions, implicating the involvement of BK_{Ca} channels in controlling contraction. On increasing the dose to 10 mM it produced a further increase suggesting the involvement of K_v channels. Inhibition of the SERCA with CPA produced only a small increase in the amplitude of calcium transients and phasic contractions.

Biochemical and immunohistochemical studies showed that the human ureter expresses 2 isoforms of SERCA (2 and 3), L type calcium channels and BK_{Ca} α and β in the smooth muscle while RyR3 and IP_3R are expressed in the smooth muscle and urothelium.

These studies show for the first time that it is possible to measure force stimulated by electrical field stimulation (EFS) while simultaneously recording emitted signals from the dual emission calcium indicator Indo-1 AM in cross-sectional rings of human ureter. Studies also show that contraction of the human ureter is regulated in part by L-type calcium channels and BK_{Ca} channels. Compared to other phasic smooth muscles the role sarcoplasmic reticulum appears to be small. Current work is establishing how infection interferes with the Ca signalling.

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PC230

Protein Kinase C dependent modulation of hERG potassium channels: Evidence for direct phosphorylation of the N-terminus

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The Human Ether-a-go-go Related Gene (hERG) encodes a delayed rectifier potassium channel that is widely expressed in neuronal, cancerous and cardiac tissue. In the heart, hERG channels contribute to the repolarisation of the action potential. Attenuation of hERG current, resulting from inherited mutations in the channel, or drug block, causes abnormal action potential repolarisation and cardiac arrhythmias, such as Torsades de Pointes. Arrhythmias are often precipitated by extreme emotion, stress or physical exertion, implying a role for the sympathetic nervous system.

In this study we investigated hERG channel modulation and phosphorylation by protein kinase C (PKC). hERG current stably expressed in HEK cells was attenuated by $44 \pm 3\%$ ($n = 9$) upon direct activation of PKC by 10 μM 1-oleoyl-2-acetyl glycerol (OAG). The response to OAG could be inhibited by the PKC inhibitor bis-1, or by down-regulating PKC by chronic PMA treatment of cells. The hERG channel has 18 putative PKC phosphorylation sites. To investigate if PKC regulation occurs via direct phosphorylation of the channel, we measured phosphorylation of hERG by [³²P]-orthophosphate incorporation. Untreated hERG-HEK cells showed two bands at 155 and 135 kDa, corresponding to surface expressed, fully glycosylated, and core glycosylated forms of hERG respectively, indicating substantial levels of basal phosphorylation. OAG increased phosphorylation of both bands by $18 \pm 2\%$ ($p < 0.001$, $n = 6$). Surface protein experiments revealed equal amounts of hERG protein were immunoprecipitated in OAG-challenged and untreated cells. Currents from ΔPKC -hERG, a channel in which 17 of the 18 putative PKC phosphorylation sites have been mutated to Ala

(Thomas *et al.*, 2003) were also modulated with OAG, suggesting phosphorylation occurs at the 18th site, T74 on the amino terminus, or at an atypical site. Δ PKC-hERG channels were not recognised by hERG antibodies and T74 mutants (T74A, V, D or E) were non-functional. Nevertheless, deletion of the N-terminus generated functional channels, but prevented modulation of channel activity and the increase in phosphorylation.

Our results suggest PKC can modulate hERG currents through a mechanism that induces direct phosphorylation of the N-terminus. Further work will investigate if α_1 adrenergic receptor stimulation regulates hERG by a mechanism involving PKC-dependent phosphorylation.

Thomas D *et al.* (2003) *Cardiovasc Res* **59**(1), 14-26.

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PC231

Make no bones about TRP channels

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Bone is not the inorganic, inanimate scaffolding it may so often unfairly be considered, but makes up an important and complex living organic network. It supports, interacts, responds and develops with the rest of the body over time according to both intrinsic (including hormonal) and environmental factors. Like all other organs, the skeleton is vulnerable to disorders and diseases, e.g. osteoporosis and cancers. Transient receptor potential (TRP) ion channels are currently receiving much interest, and may be linked to store-operated calcium entry (SOCE). TRP channels are not particularly selective, and are generally quite widely expressed with increased expression in some malignancies¹, and TRPV1 and TRPM8 ligands may have potential uses as anti-cancer agents². We aim to improve on current knowledge and further characterise TRP channels in bone and hormone-sensitive tissues, and the role they play in calcium storage and release, and subsequently in bone disorders.

RT-PCR was carried out using primer pairs for TRPM7, TRPM8, TRPV1, TRPV5 and TRPV6 subunits in MG63 osteoblast-like cells, and LNCaP and DU145 prostate cancer cells. Cell growth assays were performed to test the effects of TRPV1 and TRPM8 agonists, modulators and blockers on cell number, using trypan blue exclusion testing and MTS-conversion absorbance measurement.

TRPV1 was shown to be expressed in MG63, DU145 and LNCaP cell lines by RT-PCR. TRPM8 was also shown to be clearly present in LNCaP cells, but present only in low numbers in DU145s and MG63s. RT-PCR also shows that TRPM7, TRPV5 and TRPV6 are also expressed in MG63 cells. In the presence of ≥ 10 μ M capsaicin or capsazepine, MG63 cell number was significantly reduced ($P < 0.05$). Resiniferatoxin (TRPV1 agonist),

SB366791 (TRPV1 blocker) and anandamide (cannabinoid and TRPV1 modulator) all decreased MG63 cell numbers.

Single channel electrophysiology has shown channel openings characteristic of TRPM8 and TRPV1, but menthol (100 μ M) and capsaicin (1 - 10 μ M) did not increase activity. As these TRP channel subunits are clearly expressed in MG63, DU145 and LNCaP cell lines, further work is required to locate and also investigate their role in bone and hormone-sensitive cancers.

Tsavalier L *et al.* (2001). *Cancer Res* **61**(9):3760-9

Athanasίου A *et al.* (2007). *BBRC* **354**:50-55

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC232

A possible role for transient receptor potential (TRP) channels in mechanically evoked responses from slowly adapting type II mechanoreceptors

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Slowly adapting type II mechanoreceptors exhibit a highly regular pattern of interspike intervals when responding to a mechanical stimulus, and often maintain a background discharge of spontaneous firing (Gottschaldt *et al.*, 1973). In order to study the possible contribution of transient receptor potential (TRP) channels in mechanotransduction at the nerve terminals, a range of agonists and antagonists were studied on spontaneous and mechanically evoked responses.

Seven adult Wistar-derived rats were used, with a mean weight of 502g. Briefly, each animal was deeply anaesthetized with urethane (2 g/kg) I.P., and then killed. Whisker pads were removed, and individual sinus hair follicles with hair and nerve attached were microdissected out and placed in carbogenated (with bubbled medical 95% oxygen, 5% carbon dioxide) synthetic interstitial fluid (SIF). After slitting a follicle lengthways, it was fixed with insect pins onto a silicone elastomer Sylgard platform in a custom-made tissue bath. The follicle was pinned open to allow easy access of drugs to mechanoreceptors within the capsule. Drugs were made up in SIF and applied at the rate of 1 ml/min. Statistical analyses included t tests comparing effects with control.

A total of 17 St II units were recorded. The results are summarized in the table. The broad spectrum TRP channel antagonist ruthenium red slightly increased activity in 2 units, and had no effect on the other 3. Capsazepine, the TRPM8 and TRPA1 channel antagonist, had powerful depressant effects on static and dynamic responses, and less effect on spontaneous firing, so that in some units there was only firing between stimuli. Cinnamaldehyde had its greatest effect on static, and less effect on dynamic and spontaneous. Camphor reduced spontaneous firing most, and dynamic responses least. Icilin increased spontaneous firing, while menthol had inconsistent effects but did significantly enhance cold evoked responses ($121 \pm 7.2\%$, $t(4) = 6.62$, $P = .003$), Cahusac & Noyce, this meeting.

These data suggest that TRP channels play a role in the mechanotransduction processes of slowly adapting type II units.

Compound	Spont	Dynamic	Static	Latency Change ms	N
icilin 50 - 100 μ M	292*	115	94	-81 $\pm$ 13**	8
menthol 200 μ M	87	93	95	-73 $\pm$ 96	6
cinnamaldehyde 1 - 2 mM	44	50**	22**	+1125 $\pm$ 644	9
allyl isothiocyanate 50 - 100 μ M	78	96	99	+31 $\pm$ 25	6
camphor 0.5 - 2 mM	2**	51	33*	+594 $\pm$ 294*	10
capsaicin 1 - 3 μ M	138	96	101	-3 $\pm$ 7	6
ruthenium red 30 μ M	163	110	115	-27 $\pm$ 18	5
capsazepine 100 - 200 μ M	53**	23**	19**	+3077 $\pm$ 581**	11
tetraethylammonium 3 mM	905**	126	115	-198 $\pm$ 23**	4

Spike activities (spontaneous, dynamic and static) are shown as % of control. * indicate $P < .05$, ** indicate $P < .01$. Latency changes given $\pm$ SEM. Gottschaldt K-M, Iggo A, & Young DW (1973) J Physiol (Lond) 235, 287-315.

Stirling University Psychology Department Research Committee.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC233

KCNQ currents in interstitial cells isolated from the guinea-pig bladder

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Interstitial cells (IC) isolated from the guinea-pig bladder possess several potassium currents including BK and voltage-dependent delayed rectifier currents(1). The aim of the present investigation was to investigate whether KCNQ channels could be contributing to the total outward potassium current in guinea-pig bladder interstitial cells. Such channels have been reported in other smooth muscle cells including portal vein(2).

Bladders were removed from guinea-pigs, killed by cervical dislocation. Bladders were opened longitudinally, mucosa removed and underlying detrusor was chopped into mm3 pieces. Cells were isolated enzymatically as previously described 1 and studied using the whole-cell or amphotericin B perforated patch clamp technique. IC were depolarized from -60mV to +40mV to elicit outward currents. This current was reduced from 814 $\pm$ 79pA to 709 $\pm$ 71pA ($n=23$, $p<0.001$) by the KCNQ channel blocker, XE991 (10 μ M). In the presence of penitrem A (100nM) to block BK channels, XE991 significantly reduced the current at +40mV from 375 $\pm$ 69pA to 259 $\pm$ 56pA, $n=4$, $p=0.014$. To address the concern that penitrem A could be blocking KCNQ channels, experiments were carried out with EGTA (5mM) and ATP (3mM) in the pipette solution to remove BK and K-ATP currents; an approach used by Evans et al(3). Under these conditions, XE991 reduced the outward current from 542 $\pm$ 45pA to 472 $\pm$ 40pA ($n=12$ cells, $p=0.001$). Increasing the concentration of XE991 to 30 μ M further decreased the current to 419 $\pm$ 39pA, $n=12$, $p=0.007$. Similar effects were found using another KCNQ blocker, linopirdine, although this drug was less effective than XE991 (690 $\pm$ 149pA reduced to 549 $\pm$ 144pA, $n=4$, $p=0.065$). The KCNQ channel opener, flupirtine, increased outward currents across the voltage range from -60mV to 0mV ($n=7$, $p<0.05$). This effect was not significant at more positive potentials. Cells were held at -20 mV and stepped to hyperpolarizing potentials from

-90mV to -30mV to evoke deactivation tail currents. In 60mM external potassium solution to enhance current amplitude and 5mM 4AP to block delayed rectifier current, XE991 (10 μ M) significantly reduced currents when stepping to potentials ranging from -90mV to -50mV. At -90mV, the current of -143 $\pm$ 22pA was reduced to -89 $\pm$ 19pA, $n=7$, $p=0.0015$. These data suggest that KCNQ channels are responsible for a proportion of the total outward current in guinea-pig bladder interstitial cells. Functional experiments on strips of guinea-pig bladder in our laboratory (see presentation by Carson and McCloskey) show that these channels are involved in the regulation of spontaneous contractile activity in this tissue.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC234

A dual tracer method to quantify glucose metabolism in anaesthetized obese Zucker rats *in vivo*

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Obesity and insulin resistance (IR) are both part of the Metabolic Syndrome and have developed into world-wide epidemics. The demand for drugs that improve glycemic control after a meal is high and will increase in future years. Many of the current drugs are being tested for their potential metabolic action in rodents, both in healthy controls and in models of obesity and IR. However, the existent methodology to quantitate glucose and fat metabolism in animal models of IR is limited.

The aim of this study was to quantify glucose and fat metabolism in obese Zucker rats, a model of IR, using a downscaled version of a dual stable isotope tracer method that is currently used in man.

Glucose and fat metabolism were measured in obese and lean Zucker rats after an overnight fast and under general anaesthesia. Animals were placed in an open-flow indirect calorimeter chamber to determine oxidation rates of glucose and fat. Two catheters were in place for intravenous infusion of [6,6-2H2]glucose and anaesthetic. A glucose solution enriched with [U-13C]glucose was administered into the stomach by gavage. Blood was sampled from the left carotid artery. Whole-body insulin sensitivity was calculated from glucose and insulin levels during a 2 hour oral glucose tolerance test (OGTT) as described in [1]. Non-steady state glucose kinetics were calculated using Steele's single pool model and the calculations described in [2] during the OGTT. Table 1 shows that obese Zucker rats were highly insulin resistant compared with lean controls. Area under the curve (AUC) of plasma insulin after glucose ingestion was higher in obese rats, but no difference in glucose-AUC was measured. Total and gut

glucose rate of appearance were higher in the obese animals in the fasted state and at most times during the OGTT (Fig 1). However, no difference in suppression of hepatic glucose output was found ($-27.8\% \pm 3.3$ v $-20.6\% \pm 1.1$) ($P=0.07$). The contribution of fat oxidation to total energy production was lower in obese rats in both the fasting state ($60.8\% \pm 4.4$ v $78.1\% \pm 1.7$)* and during the OGTT ($46.3\% \pm 3.2$ v $66.1\% \pm 2.1$)*#. No difference in the suppression of fat oxidation was measured ($P=0.13$). (* $P<0.05$ compared to lean; # $P<0.05$ compared to basal).

We conclude that glucose and fat metabolism could successfully be quantified in anaesthetized obese Zucker rats using a downscaled version of a dual stable isotope tracer method. Future work will focus on the effect of known and novel hyperglycemic drugs. By using the same methodology as in man, compound efficacy with respect to glucose clearance and hepatic glucose production can now be investigated in animal models.

	Insulin AUC ($\mu\text{g}^{-1} \cdot 120\text{min}^{-1}$)	Glucose AUC ($\text{mM} \cdot 120\text{min}^{-1}$)	IS ($\text{mg}^{-1} \cdot \text{dl} \cdot \mu\text{U}^{-1} \cdot \text{ml} \cdot 10^{-4}$)
Obese	$467.5 \pm 98.9^*$	286.6 ± 65.2	$0.49 \pm 0.07^*$
Lean	41.2 ± 7.0	266.1 ± 24.7	11.17 ± 0.96

Results expressed as mean $\pm$ SEM; * $P<0.05$ compared to lean

Table 1

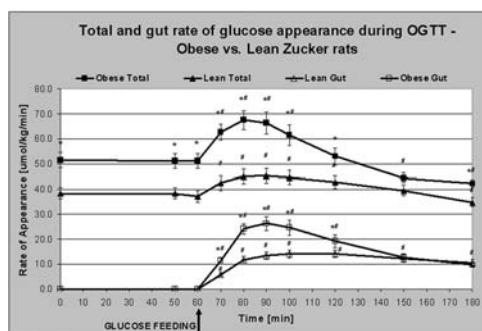


Figure 1

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC235

Insulin sensitivity is improved by the prior ingestion of sodium acetate during an oral glucose tolerance test

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In the original description of the glucose-fatty acid cycle Randle and co-workers (1963) identified that a high circulating concentration of free fatty acids (FFA) was associated with a reduction in insulin sensitivity, primarily due to long chain fatty acids inhibiting muscle glucose uptake. Whether all lengths of fatty acids exert the same metabolic effect is, however, unclear. Acetic acid, a short chain fatty acid, has been shown to decrease glucose appearance in the blood in rats when ingested immediately prior to a glucose load (Ebihara and Nakajima 1988). Additionally sodium acetate administration has been found to

enhance insulin secretion in the rat model when glucose was co-infused, resulting in a quicker rate of glucose disappearance (Shah *et al.* 1977). The current study examined whether sodium acetate ingestion prior to the consumption of a glucose load affects insulin sensitivity and/or the glycaemic and insulinaemic response in humans. Six healthy male subjects volunteered to ingest an equimolar dose (4 mmol/kg body mass) of sodium acetate (NaAc) or sodium bicarbonate (NaHCO₃) over a 60 min period followed by the consumption of 75 g of glucose. Trials were completed on two separate occasions 7 days apart. Arterialised-venous blood samples were taken prior to sodium salt ingestion, immediately before consuming the glucose solution and at 15, 30, 60, 90 and 120 min after ingestion of the glucose load. Data were analysed using a 2-way repeated measures ANOVA and where appropriate by post-hoc Wilcoxon matched pairs test. Both sodium salts induced a mild metabolic alkalosis (blood pH ~ 7.42 vs. 7.48 pre- and post-ingestion in both trials). During the first 15 min of the oral glucose tolerance test there were greater increases in blood glucose ($P=0.03$) and plasma insulin ($P=0.03$) in the NaAc compared with the NaHCO₃ trial. Although there was no difference in the glycaemic response between trials thereafter, plasma insulin was found to be significantly lower at the end of the NaAc trial (10.6 ± 2.9 $\mu\text{U/ml}$ NaAc; 19.3 ± 3.2 $\mu\text{U/ml}$ NaHCO₃, $P=0.046$). Insulin sensitivity was significantly improved ($P < 0.05$) following the consumption of sodium acetate when compared with the bicarbonate trial, as determined using the insulin sensitivity index (ISI_{0,120}). In conclusion the ingestion of sodium acetate 60 min prior to a 75 g oral glucose load was found to significantly improve insulin sensitivity when compared to the insulinaemic response following sodium bicarbonate ingestion.

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PC236

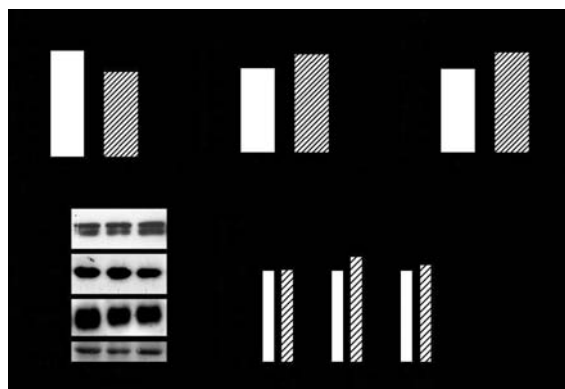
Does Pink1 RNAi silencing in primary brown adipocytes trigger a change in mitochondrial gene expression?

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Parkinson's disease and type II diabetes are both associated to mitochondrial dysfunction. PTEN induced putative kinase 1 (PINK1), a serine threonine kinase, has recently been linked to familial parkinsonism and to mitochondrial dysfunction in general. Basal and over-expressed PINK1 has been shown to locate to the inner membrane of the mitochondria. A loss of this gene in *Drosophila* muscle cells causes depletion in mitochondrial architecture and an increase in reactive oxygen species (ROS) which is suggested to be rescued by expression of human PINK1. The loss of pink1 in *Drosophila*, gives a similar phenotype as loss of parkin and is rescued by the over-expression of parkin. Parkin

is a gene that has been associated to interactions with mitochondrial transcription factor A (TFAM), a gene shown to be involved in mitochondrial DNA (mtDNA) metabolism. The main approach in this study was to silence Pink1 with RNAi technology in a mitochondrial abundant mammalian cell system and investigate whether the expression of Tfam, parkin and oxidative phosphorylation (OXPHOS) genes (nd5 and citrate synthase) would be affected. Primary brown adipocyte cells were dissected out from 3-4 weeks old male NMRI mice and optimized siRNA and lipofectamine concentrations and conditions were used. The cells were transfected 24 h post-plating followed by harvesting after 72 h. 80% knockdown of Pink1 was achieved and expression of the putative genes were analysed by quantitative Real-Time PCR and Western blot. The mRNA expression of nd5 and citrate synthase (cs) was unchanged which was consistent with protein expression of tfam and parkin. In conclusion, in our model, ablation of pink1 had no effect on mitochondrial gene expression and did not appear to be involved in tfam-OXPHOS gene regulation.



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This work was funded by Swedish Diabetes Association.

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PC237

Competent non-adrenergic recruitment of brown adipocytes by PPARgamma agonist is associated with dominant conversion of PPARgamma to a novel isoform

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Most of physiologically induced examples of recruitment of brown adipose tissue (BAT) occur as a consequence of chronic

sympathetic stimulation (norepinephrine release within the tissue). However, in some physiological contexts (e.g. prenatal and prehibernation recruitment), this pathway is contra-indicated. Thus, a non-sympathetically mediated mechanism of BAT recruitment must exist. Here we have tested whether a PPARγ pathway could recruit BAT fully independently of sympathetic stimulation by continuously treating brown (pre)adipocytes in culture with the potent PPARγ agonist rosiglitazone. We found that this drug accelerated differentiation of brown adipocytes in culture (judged by lipid accumulation and expression levels of general adipogenic and BAT-related genes). Brown adipocytes in which PPARγ was constantly in an activated state expressed UCP1 maximally even in the absence of norepinephrine. In cultures chronically treated with rosiglitazone, the expression of PPARγ was markedly increased but this could not explain the brown-fat promoting effects of rosiglitazone, as these effects were still evident in PPARγ-null cells. Strikingly, the isoform profile of the PPARγ proteins was markedly altered in the presence of rosiglitazone: the PPARγ1 protein became barely detectable, while the amount of PPARγ2 protein became much higher than in control cells; the PPARγ2 protein consisted mostly of novel PPARγ2 protein isoforms formed by post-translational modification, including phosphorylation (at a site different from Ser-112) and with predominant nuclear localization. Thus, ligand-bound and presumably transcriptionally active PPARγ2 apparently appears in rosiglitazone-treated brown adipocytes as a novel isoform with potent effects on brown adipogenic genes such as UCP1.

Swedish Medical Research Council and Swedish Diabetes Association

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC238

Pro-inflammatory cytokines increase glucose, alanine and triacylglycerol utilization, but inhibit insulin secretion in a clonal pancreatic beta-cell line

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We have investigated the effects of prolonged exposure (24 hr) to pro-inflammatory cytokines on β cell metabolism and insulin secretion using clonal BRIN-BD11 beta cells. Expression of genes related to nutrient metabolism in the pancreatic beta-cell have been reported to be significantly altered in the presence of pro-inflammatory cytokines. We now report that addition of IL-1β, TNFα and IFNγ (at concentrations that did not result in a significant increase in cell death - IL-1β 0.3125U/ml, TNFα 31.25U/ml, IFNγ 15.625U/ml) inhibited chronic (24hr) and acute (20 min) levels of insulin release (2-fold and 5-fold), increased glucose and alanine consumption (2-fold and 2.5-fold) and also elevated lactate and glutamate release (3.7-fold and 1.5-fold). However, cellular triacylglycerol and ATP levels were decreased (2-fold and 1.2-fold) while glutathione was increased (1.5-fold). Pro-inflammatory cytokines appear to shift β-cell metabolism away from the primary role of stimulus-secretion coupling and

towards a catabolic state which may be related to cell defence. This work is of importance to determining the beta cell response to inflammation, which is normally associated with the pathogenesis of type-1 diabetes.

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Health Research Board of Ireland (HRB)

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC239

Analysis of free coded amino acids by capillary electrophoresis

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Most of modern popular methods of amino acid analysis involve expensive and laborious derivatization stage and require complicated equipment [1,2]. Development of new cheaper, more selective and express methods for amino acid composition determination of biologically active peptides is an important problem [3]. Moreover, free amino acids, contained in physiological fluids, might be the molecular markers of the number of diseases. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) were used for coded free amino acids separation. Direct UV-photometric method of detection was applied for amino acid identification. Influence of various factors to the efficiency of separation was investigated: the background electrolytes (BGE) pH value, voltage, inner diameter and effective length of capillary. Influence of organic solvents (2-propanol, methanol, acetonitrile) and of various anionic surfactants was studied. The mixture consisting of 16 free coded amino acids was completely separated by CZE using water-organic BGE with highly acidic pH value (1.9-2.1). Analysis of 14 free amino acids in alkaline pH ranging from 10.2 up to 11.0 based on application of micellar electrokinetic chromatography with direct UV-photometric detection was developed. Time of analysis was less than 30 min, and R.S.D. value was 0.06-0.08. Developed methods were applied to amino acid composition determination of some natural and synthetic biologically active peptides.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC240

Does experimental diabetes affect the immunoreactivity of aromatase in hippocampal and gonadal tissues?

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Aromatase (cytochrome P450 19A1) is responsible for catalyzing the conversion of androgens to estrogens and is expressed in a variety of tissues including ovary, brain, placenta and adipose tissues. Strong biological evidence supports the benefits of estrogen on brain functioning, morphology and also on ovarian function. Although insulin has been shown to stimulate ovarian steroidogenesis and could alter aromatase activity there are few data about the effects of diabetes on aromatase enzyme of brain and gonadal tissues. In this study we investigated the effects of experimental diabetes on aromatase expression in hippocampus, ovary and uterus. Female Sprague-Dawley rats were injected with streptozotocin (45 mg/kg) to induce diabetes. Age-matched non-diabetic rats were used as control. At the end of 8-weeks diethylstilbestrol (0.1 mg/kg, s.c.) was injected to rats 24 hours before exsanguination for initiating oestrus cycle. After the isolation of hippocampus, ovary and uterus, tissue homogenates were prepared and evaluated for aromatase proteins by western blot. Responses of unilateral uterus tissues of rats to carbachol (10⁻⁸-10⁻⁴ M) and oxytocin (10⁻⁸-10⁻⁶ M) were investigated via organ chamber experiments. Aromatase levels were not altered in hippocampal brain extracts but attenuated in ovary and uterus tissue homogenates of diabetic rats compared to control. Contraction responses induced by both carbachol and oxytocin were decreased in uterus tissues of diabetic rats when studied in vitro. In conclusion, these results indicated for the first time that, aromatase levels were not altered in hippocampus but attenuated in uterus and ovary of 8-weeks experimentally diabetic rats. Uterus contraction responses were also impaired in diabetic rats.

* Approved by Hacettepe University Animal Ethics Committee (2006/8-6).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC241

Maternal undernutrition and offspring birth weight, growth and adult body composition in sheep

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Maternal energy and/or protein malnourishment has been shown to predispose adult offspring to increased risk of metabolic disorders. In this study significant protein or energy restriction during early or late gestation, alongside control-fed animals was

used to investigate the effect of prenatal nutritional experience on birth weight, postnatal growth and body composition of the adult offspring as assessed with Dual-Energy X-ray Absorptiometry (DXA).

39 Scottish Blackface sheep were either fed an adequate diet for protein and energy requirements from day 0 (mating date) to term (control, n=12) or a diet deficient in energy (65% requirement; LE) or protein only (50% control intake; LP). The period of nutritional restriction was also varied within dietary groups, thus; LE was imposed during early (0-65 days, LEE; n=7) or late (65-125 days, LEL; n=7) gestation and also LP during early (0-65 days, LPE; n=7) or late gestation (65-145 days, LPL; n=6). Lambs delivered naturally and were ewe-reared to weaning at 10 weeks and then fed grass thereafter. Weights were recorded periodically to 2 years of age. The Lunar Hologic DPX-L fast-detail smartscan recorded whole body fat, lean mass and bone mineral density (BMD).

There were no significant effects of diet or treatment on birth weight, but a significant effect of diet ($P=0.01$) was observed on growth to weaning. In effect, LP exposed lambs grew more slowly (234 ± 10 g/day) and LE grew faster (270 ± 10 g/day) than controls (251 ± 10 g/day). By 2 years of age, male sheep were larger than female sheep in all groups (REML estimate $+5.15 \pm 1.5$ kg males > females) and LP exposed sheep remained lighter than both controls and LE exposed sheep (Control, 50 ± 1 ; LP, 46 ± 1 ; LE, 51 ± 1). DXA examination indicated no effect of prenatal diet on % fat mass, lean mass or BMD. There was no effect of offspring sex on % fat mass or BMD but lean mass was, as expected lower in female (40 ± 1 kg) vs. (45 ± 1 kg) male offspring.

Prenatal protein vs. energy undernutrition in sheep has contrasting effects on postnatal growth. Low protein significantly reduced growth, while low energy significantly increased growth relative to control. The effect of a maternal low protein diet on offspring growth is greater when fed over late gestation whereas the reverse is true for a low-energy diet; growth being greater when the period of energy restriction was confined to early gestation. In contrast to many studies in laboratory species, prenatal undernutrition (of protein or energy) has no effect on adult body composition as assessed by DXA in this study.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC242

Kidney function analyses in genetically modified hypercholesterolemic mice fed bitter yam (*Dioscorea polygonoides*) proprietary preparation

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Proprietary preparations of Bitter yam (*Dioscorea polygonoides*) are implicated in lowering cholesterol in rats (McAnuff, 2005).

Recent studies have shown that hypercholesterolemia is a growing problem worldwide. Efforts to control or minimize the incidence of cardiovascular diseases often seek to maintain the normal range for cholesterol in the blood.

The main objective of this study is to assess the effect of the consumption of bitter yam proprietary preparation on kidneys of genetically modified hypercholesterolemic mice.

Genetically modified male mice were fed the following diets: normal rat chow, normal rat chow supplemented with the bitter yam proprietary preparation, a high cholesterol rat chow and a high cholesterol rat chow supplemented with the bitter yam proprietary preparation. At the end of feeding period, the mice were killed and the kidneys collected. Lipid peroxidation analysis was carried out using the method of Okhawa *et al* 1978. Blood urea nitrogen, creatinine and electrolytes levels were determined at Index Laboratories Inc., USA.

A significant increase ($p < 0.05$) in lipid peroxidation was observed in the kidneys of normal and hypercholesterolemic mice fed diets supplemented with bitter yam proprietary preparation (397.33 ± 54.21 to 1522.77 ± 89.07 and 2032.26 ± 12.39 nmol MDA/g wet weight, respectively). Blood Urea Nitrogen level also increased in hypercholesterolemic mice fed a diet supplemented with bitter yam (25.00 ± 1.00 to 32.50 ± 0.50 g/dL). Significant increases were observed in the levels of serum sodium in hypercholesterolemic mice fed a high cholesterol diet and hypercholesterolemic mice fed diet supplemented with bitter yam proprietary preparation (149.5 ± 0.5 U/L to 154.0 ± 1.0 U/L and 152.5 ± 0.5 U/L, respectively). There was also a significant increase in the level of serum magnesium of the normal mice fed diet supplemented with bitter yam proprietary preparation (2.20 ± 0.00 U/L to 4.65 ± 0.35 U/L).

It would appear that supplementation of the diet with 5% bitter yam proprietary preparation may cause damage to the kidneys. Lower doses of this preparation should be analysed for efficacy and safety. McAnuff MA, Omoruyi FO, Morrison EY and Asemota HN (2005) Changes in some liver enzymes in streptozotocin-induced diabetic rats fed sapogenin extract from bitter yam (*Dioscorea polygonoides*) or commercial diosgenin. *West Indian med. Journal*. vol.54 no.2 pp 97-101.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC243

Methionine concentration triggers switching between parallel tracks in liver sulfur metabolism

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Methionine (Met) is an essential amino acid and the only substrate for synthesis of S-adenosylmethionine (AdoMet), an important biological methyl donor. In liver Met consumed in AdoMet synthesis can be replenished via remethylation (RM) of homocysteine or cleared via transsulfuration (TS) pathway. Our previous modeling studies [1] predicted the existence of two

modes of Met metabolism in liver characterized by low metabolic rates and metabolite levels at [Met] equal or below its normal physiological value ($\sim 50 \mu\text{M}$) and by high metabolic rates and metabolite concentrations at [Met] above this value. According to the model Met metabolism sharply switches from one mode to another and the switch is triggered by [Met]. The switch from “low” to “high” mode is associated with sharp increase in steady-state [AdoMet]. In the present work we experimentally analyzed the dependence of [AdoMet] and Met consumption rate on [Met] in suspension of freshly isolated mouse hepatocytes. Hepatocytes were isolated by the method of Berry et al. [2]. [AdoMet] and [Met] were measured by HPLC as described in [3, 4]. For analysis of experimental data we constructed an extended mathematical model of liver Met metabolism, including detailed description of kinetics of all enzymes involved in Met metabolism and simplified description of folate metabolism.

It was found that Met added to hepatocyte suspension is uniformly distributed between the cells and the medium. Thus extracellular and intracellular [Met] are equal. Initial [Met] in hepatocyte suspension was varied from 40 to 400 μM . In this range [AdoMet] increased from $79 \pm 37 \mu\text{mol/l}$ cells ($n=24$) to $930 \pm 350 \mu\text{mol/l}$ cells ($n=12$). Corresponding Met consumption rate increased from $0.86 \pm 0.40 \text{ mmol/h} \times \text{l cells}$ ($n=15$) to $6.0 \pm 2.4 \text{ mmol/h} \times \text{l cells}$ ($n=12$). A sharp increase in the steady-state [AdoMet] and Met consumption rate was found within a narrow range of [Met] from 50 to 100 μM (Fig. 1A,B). The dependence of [AdoMet] and Met consumption rate on [Met] below and above this range was weak. Similar results were obtained in rat hepatocytes. Analysis of the model shows that the increase in [Met] above normal physiological value switches Met metabolism from conservation to a disposal mode and is associated with a redistribution of metabolic flux between RM and TS. To our knowledge liver Met metabolism demonstrates the new mechanism of trigger behavior that is based on allosterically regulated switch between two sets of enzymes catalyzing parallel metabolic fluxes.

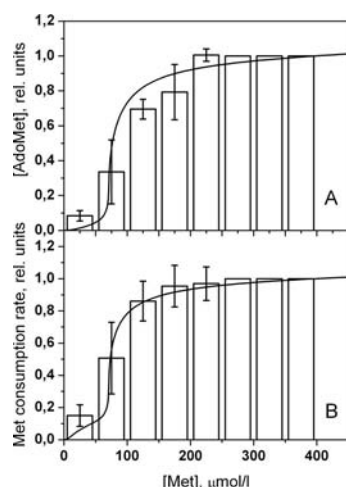


Fig. 1. The dependence of steady-state values of (A) [AdoMet] and (B) the rate of Met consumption on [Met] in mouse hepatocytes. Bars indicate experimental data ($n=12$) averaged (mean $\pm$ SD) over 50 μM [Met] intervals. Solid lines show the results of modeling. Data in each experiment is normalized to values obtained at an initial [Met] 400 μM . The theoretical data is normalized appropriately.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC244

Human resistin induces cholesterol esterification and lipid accumulation in macrophages

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Adipocytes secrete bioactive molecules called adipokines which exert both local and systemic effects. Resistin, a novel cysteine-rich secretory protein, was originally identified as a murine adipokine, and proposed to link obesity, insulin resistance and cardiovascular disease. However, the (patho)physiological role of human resistin, which exhibits significant sequence divergence from its murine counterpart, and is secreted predominantly from macrophages, rather than adipocytes, remains controversial. Here, we hypothesized that resistin may regulate macrophage cholesterol homeostasis, and lipid accumulation, possibly via regulation of 5'-AMP activated protein kinase (AMPK).

The effect of human recombinant resistin on lipid metabolism was studied using human THP-1 macrophages, which do not express or secrete endogenous resistin. Treatment (24h) with resistin (0-100 ng ml⁻¹) significantly increased cholesteryl ester mass by up to 172% and 229%, in the absence or presence of oxidized LDL (50 $\mu\text{g ml}^{-1}$), respectively, and increased incorporation of [³H]cholesterol (140.1%) and [³H]oleate (163.4%) into the cholesteryl ester pool, compared with control untreated cells. Incorporation of [³H]oleate into the cholesteryl ester pool was blocked by an ACAT inhibitor (10 μM Sandoz 58-035; $p < 0.01$); however, expression of ACAT-1 mRNA or protein were not affected by treatment with resistin. The observed increases in the cholesteryl ester pool could not be explained by changes in macrophage cholesterol efflux mechanisms, as resistin did not impact upon apoAI/apoE dependent cholesterol efflux, or expression of ABCA1. Resistin did, however, significantly increase triacylglycerol accumulation in THP-1 macrophages, by 148% of untreated control cells ($p < 0.01$). Intriguingly, the 'anti-obesity' agent, C75, which inhibits fatty acid synthesis and promotes fatty acid oxidation, effectively inhibited resistin-induced cholesteryl ester and triacylglycerol mass accumulation, suggesting that altered fatty acid metabolism might explain these increases in macrophage neutral lipid mass. Uptake of [³H]oleate was significantly increased (119% of control; $p < 0.05$) by preincubation with resistin (50 ng ml⁻¹), while oxidation of [¹⁴C]oleate was reduced by nearly 30% after exposure to resistin ($p < 0.05$). These effects were accompanied by a significant reduction in AMPK activity, as judged by phosphorylation of acetyl CoA carboxylase (ACC).

Thus, treatment with resistin causes alterations in macrophage fatty acid metabolism, contributing to triglyceride and cholesterol ester deposition, which may predispose 'foam cell' formation and atheroma progression.

This work was supported by Diabetes UK

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC245

Insulin controls the location of GLUT2 in the brush border membrane of enterocytes

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Prolonged fructose consumption in rodents stimulates intestinal expression of transporters and enhances intestinal sugar absorption. These dietary conditions also induce peripheral insulin resistance. Intestinal sugar transport into enterocytes is controlled by transient expression of GLUT2 in the brush border membrane (BBM) after a sugar load. Sugars target GLUT2 to the BBM (reviewed in Kellett & Brot-Laroche, Diabetes, 2005; 54:3056-3062). Sugar ingestion increases plasma insulin and might regulate BBM GLUT2 location. The aim of this study was to investigate the potential role of insulin on GLUT2 trafficking in enterocytes in health and disease.

In glucose-fed Caco2/TC7 enterocytes, GLUT2 was found in the BBM. Insulin addition to culture media inhibited BBM GLUT2 location as shown by surface biotinylation and immunofluorescence labelling. Therefore insulin signalling is perceived by enterocytes in the absence of other changes in cell environment. Insulin decreased BBM GLUT2 location and fructose transport in the jejunum of hyperinsulinemic-euglycaemic (HIEG) clamped mice. Decreased BBM GLUT2 lowered sugar uptake. Moreover, mice fed fructose for more than 3 weeks exhibited systemic insulin resistance and high BBM GLUT2 that could not be removed in HIEG clamps. Thus insulin resistance produces insulin insensitive BBM GLUT2 trafficking. Alike diabetic patients who perform insulin injections prior to meals, insulin injection to mice prior an oral fructose load prevented BBM GLUT2 translocation into the BBM.

These results obtained in vivo and in vitro indicate that GLUT2-mediated sugar absorption in the intestine is controlled by insulin. We thus propose that insulin exerts, part of its hypoglycaemic effect by slowing down dietary sugar absorption, a mechanism that would limit glucose excursions in the blood stream.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC246

Tumour necrosis factor- α signals via a β -catenin/TCF4 (TCF7L2)-dependent pathway to inhibit adipogenesis

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Tumour necrosis factor- α (TNF- α), a pro-inflammatory cytokine, is a potent inhibitor of adipocyte differentiation. However, the mechanism of TNF- α -mediated anti-adipogenesis remains incompletely understood. In this study, we first confirm that TNF- α inhibits adipogenesis of 3T3-L1 preadipocytes by preventing the early induction of the adipogenic transcription factors peroxisome proliferator-activated receptor- γ (PPAR γ) and CCAAT/enhancer binding protein- α (C/EBP α). This suppression coincides with enhanced expression of several reported mediators of anti-adipogenesis that are also targets of the Wnt/ β -catenin/TCF4 pathway. Indeed, we found that TNF- α enhanced TCF4-dependent transcriptional activity during early anti-adipogenesis, and promoted the stabilisation of β -catenin throughout anti-adipogenesis. We investigated the effect of TNF- α on adipogenesis in 3T3-L1 cells in which β -catenin/TCF signalling was impaired, either via stable knockdown of β -catenin, or by overexpression of dominant-negative TCF4 (dnTCF4). The knockdown of β -catenin enhanced the adipogenic potential of 3T3-L1 preadipocytes and attenuated TNF- α -induced anti-adipogenesis. However, β -catenin knockdown also promoted TNF- α -induced apoptosis in these cells. Similarly, overexpression of dnTCF4 prevented TNF- α -induced anti-adipogenesis but showed no apparent effect on cell survival. Finally, we show that TNF- α -induced anti-adipogenesis and stabilisation of β -catenin requires a functional death domain of TNF- α receptor 1 (TNFR1). Taken together these data suggest that TNFR1-mediated death domain signals can inhibit adipogenesis via a β -catenin/TCF4-dependent pathway.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC247

The effects of glucose and insulin on contractility and Ca signalling in myometrium from diabetic and non-diabetic pregnant women

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Diabetic women are known to encounter more problems with pregnancy and labour than non-diabetic women. In particular they are more likely to require an emergency Caesarean delivery, even when their blood glucose is well controlled and their babies are of normal weight. This suggests that the myometrium of diabetics may be less able to contract well or over a prolonged time period compared to non-diabetics. However, very little is known about the intrinsic properties of myometrium from diabetic

women, or the response to changes in glucose. We have therefore begun to compare in vitro contractility and Ca signalling between the two groups and examine the effects of glucose deprivation or hyperinsulinaemia.

Uterine biopsies were obtained at term with informed, ethical consent from diabetic and non-diabetic women have elective Caesareans, myometrial strips dissected, loaded with Indo-1 and force and Ca signalling simultaneously measured, at 37 °C.

Under control conditions the frequency of Ca transients and contractions were less in diabetics, being 5.3 ± 1.0 and in controls, 9.2 ± 0.5 contractions/hour, $n = 13$ and 68 respectively. Contraction amplitude, relative to high-K contractions was also lower in the diabetics; 73% compared to non-diabetic controls.

When the perfusate was changed from control (8 mM) to 0 glucose there was a marked variation in how long contractions were produced for between tissues in both groups, however those from diabetics were abolished faster (7.7 ± 2.1 hours, $n = 6$) than non-diabetics (11.9 ± 2.7 hours, $n = 27$). Only 1 diabetic sample continued to contract after 12 hours in 0-glucose, whereas 10 controls did so, two of which continued to contract for over 2 days. When hyperinsulinaemia was simulated by adding 700 pM insulin after control contractions had been established, little change occurred in diabetic samples ($n = 3$) but a profound decrement in force amplitude (from 100 to 19 ± 13 %, $n = 8$) occurred in the non-diabetic samples and their frequency was also significantly reduced. Similar changes in Ca occurred.

These preliminary findings suggest that there are differences in uterine contractility associated with diabetes, with them perhaps being less able to withstand the metabolic demands of labour, as blood flow and hence glucose falls with contraction. This may relate to differences in glycogen utilization. Insulin resistance in diabetic women may account for the reduced effects of hyperinsulinaemia.

supported by Diabetes UK

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC248

Translational signalling in human muscle after hyperinsulinaemic hyperaminoacidaemic clamp in young and old men

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Previously, we have shown that in young and old men essential amino acids (EAA) stimulate anabolic signalling pathways associated with the control of muscle protein synthesis when insulin is clamped at fasting plasma concentrations. However, the degree of activation of some signalling enzymes is reduced in older men in concert with a diminished protein synthetic response, possibly contributing to ageing sarcopenia. We have now investigated whether the reduced signalling observed in the presence of AA is apparent even under hyperinsulinaemic conditions. We therefore administered intravenous mixed AA (102 mg/kg/h) with plasma insulin maintained at 150 μ U/ml in 8 young and 8 older men (mean 24 and 67 y, respectively). Quadriceps muscle was

sampled using local anaesthesia at baseline and after 2.5 h. Phosphorylation was assessed by Western blotting for protein kinase B (PKB) thr308, glycogen synthase kinase 3 β (GSK3 β) ser9, eukaryotic initiation factor 2 α (eIF2 α) ser51, mammalian target of rapamycin (mTOR) ser2448, p70s6K1 thr389, 4E-binding protein-1 (4E-BP1) thr37/46. Eukaryotic initiation factor 4E (eIF4E) proteins were extracted for analyses of eIF4E associated with eIF4G using m7-GTP sepharose. Phosphorylation of PKB and GSK3 β increased in young and old men (40-fold; $P < 0.01$ vs 10-fold; $P < 0.05$ and 3.9-fold; $P < 0.05$ vs 2.1-fold; $P < 0.05$, respectively) although significantly more in younger men ($P < 0.05$). Phosphorylation of eIF2 α decreased significantly only in younger men, 57 % ($P < 0.05$) compared with 24 % in older men. Phosphorylation of mTOR did not change in either group, nor did amounts of eIF4E associated with eIF4G. The phosphorylation of p70s6K1 was increased in both groups (24-fold; $P < 0.01$ vs 7-fold; $P < 0.05$) although to a greater extent in younger men ($P < 0.05$). The phosphorylation of 4E-BP1 was increased to a similar extent in both young and old men (2.8-fold vs 3-fold; both $P < 0.05$, respectively). The reduction in eIF2 α phosphorylation in the younger men suggests that the exchange of GTP on eIF2B is modulated by feeding, an affect diminished with age. Coupled with the reduced GSK3 β phosphorylation in older men, this might reduce cellular eIF2B-GTP concentrations and thus Meti tRNA recycling. The observed differences in phosphorylation of signalling molecules in older men (possibly related to dysregulated insulin receptor signalling) might explain age-related differences in the normal food stimulated increase in protein synthesis and conceivably even the blunting of protein breakdown observed in skeletal muscle. We provide new information concerning AA and insulin-induced signalling events in human skeletal muscle and show that hyperinsulinaemia does not rejuvenate the blunted anabolic signalling observed in older men.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC249

Smoking suppresses muscle protein synthesis

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Smokers are more likely to have sarcopenia than non-smokers. However, the mechanism by which smoking decreases skeletal muscle maintenance, including possible effects on muscle protein synthesis and breakdown is unknown. We therefore tested the hypothesis that the rate of muscle protein synthesis is lower in smokers than in non-smokers. We measured the fractional rate of mixed muscle protein synthesis and whole-body leucine rate of appearance in plasma (Ra, an index of whole-body proteolysis) by using stable isotope-labeled tracer techniques in eight habitual smokers (≥ 20 cigarettes per day for ≥ 20 years) and eight non-smokers matched for sex (4 men and 4 women per group),

age (65 ± 3 y and 63 ± 3 y; means $\pm$ SEM) and BMI (25.9 ± 0.9 and 25.1 ± 1.2 kg/m²). Furthermore we measured the expression of genes in muscle known to be related to the regulation of muscle mass, namely those for the proteolytic associated enzymes MaFBx and MuRF-1 as well as that for the cytokines TNF α and myostatin. We also measured the concentration of a variety of inflammatory markers in plasma (C-reactive protein, tumor necrosis factor- α , interleukin 6) since they have been shown to be negatively correlated with muscle protein synthesis rates in other conditions. There were no differences between non-smokers and smokers in plasma leucine concentration (109 ± 7 μ M vs. 104 ± 6 μ M; $P = 0.59$), leucine Ra (1.31 ± 0.09 μ mol/kg BWmin vs. 1.23 ± 0.03 μ mol/kg BW.min; $P = 0.42$) or in plasma concentrations of inflammatory markers. However, mixed muscle protein synthesis rate was markedly less in smokers compared with non-smokers (0.037 ± 0.005 vs. 0.059 ± 0.005 %/h, respectively; $P = 0.004$). Furthermore the expression of MaFBx and myostatin mRNA in the muscle of the smokers was markedly elevated compared to that in the non-smokers (4.5 ± 1.6 & 11 ± 6.6 -fold respectively). We conclude that habitual smoking inhibits muscle protein synthesis and increases the expression of genes associated with increased muscle proteolysis and inhibition of muscle maintenance. These may be mechanisms by which smoking increases the risk of age-associated loss of muscle mass.

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PC250

Serum levels of leptin and adiponectin in old chinese men with visceral obesity or metabolic syndrome

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BACKGROUND

Leptin and adiponectin are adipose tissue derived hormones that are related to visceral obesity. Visceral obesity and insulin resistance have been identified as independent risk factors for metabolic syndrome (MS). Metabolic syndrome subjects exhibited low levels of adiponectin and high levels of leptin. Recent study shows adiponectin-leptin (A/L) ratio was more efficacious as a parameter of insulin resistance than adiponectin or leptin alone. (1)

METHODS

206 old chinese men (over 60 years, 73.42 ± 6.41 years) were recruited in this study. Serum levels of leptin and adiponectin were detected using RIA kit (Linco). Visceral fat area (VAF) was measured using computed tomography scanning. Subjects were divided into four groups: control group (n=50) ie: non-MS subjects (according to metabolic syndrome diagnostic criteria of

China(2)) with normal weight (BMI <25kg/m²) and without visceral obesity (VAF < 100cm²); 2nd group (n=49) ie: non-MS subjects with normal weight (BMI <25kg/m²) and visceral obesity (VAF $\geq$ 100cm²); 3rd group (n=47) ie: overweight (BMI $\geq$ 25kg/m²) non-MS subjects and with visceral obesity (VAF $\geq$ 100cm²); 4th group (n=60) ie: MS subjects.

RESULTS

1) The visceral fat area (cm²), the serum levels of leptin (ng/dl), the serum levels of adiponectin (mg/ml), the adiponectin-leptin ratio of the four groups are 68.53 ± 22.56 , 134.90 ± 32.18 , 155.53 ± 52.12 , 151.77 ± 51.09 ; 4.61 ± 2.89 , 5.51 ± 3.43 , 9.96 ± 5.97 , 10.18 ± 6.65 ; 11.74 ± 7.98 , 9.48 ± 5.98 , 9.25 ± 6.27 , 7.5 ± 5.3 ; 4.63 ± 6.96 , 2.25 ± 1.76 , 1.24 ± 1.04 , 0.91 ± 0.77 respectively. Comparing to the control group, the 2nd, 3rd, 4th groups have significantly larger visceral area ($p = 0.000 \sim 0.032$) and lower A/L ratio ($p = 0.000 \sim 0.031$); the 3rd, 4th group have significantly higher serum levels of leptin and lower serum levels of adiponectin ($p = 0.000 \sim 0.034$). As to the above four parameters, there is no significant difference between the 3rd and 4th group. 2) The serum levels of leptin ($r = 0.551$ $p = 0.000$) and the adiponectin-leptin ratio ($r = -0.342$ $p = 0.000$) were significantly correlated with visceral fat area in old chinese men.

DISCUSSION

From the result we can see that: 1) The subjects of the 3rd and 4th group have the same level of serum leptin and adiponectin and we shall pay equal attention as to MS subjects to those overweight and metabolically obese although non MS subjects. 2) The serum level of leptin and the adiponectin-leptin ratio were significantly correlated with visceral fat area in old chinese men which indicate that those two parameters might help to evaluate visceral obesity in old chinese men more conveniently and cheaper than CT.

CONCLUSIONS
The results suggested that serum level of leptin and the A/L ratio are effective in evaluating visceral obesity than adiponectin alone in old chinese men.

Inoue M, Yano M, Yamakado M, Maehata E, Suzuki S.

Relationship between the adiponectin-leptin ratio and parameters of insulin resistance in subjects without hyperglycemia.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC251

The role of the aspartate-glutamate exchanger Aralar1 in insulin secretion, glucose and amino acid metabolism in the BRIN- BD11 beta cell line

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In the beta cell, mitochondrial Redox- NADH- shuttles are important for coupling glycolysis to mitochondrial energy

metabolism. Aralar1, a mitochondrial Aspartate-Glutamate exchanger in the Malate-Aspartate shuttle, has been found to be important for regulation of insulin secretion and glucose metabolism in the INS- 1E cell line as well as in the clonal BRIN- BD11 cell line.

Certain amino acids, in particular L- alanine, L-Leucine and L- glutamine, are known to stimulate insulin secretion and glucose metabolism in primary islets and cell lines including BRIN- BD11 either on their own or synergistically in combination with glucose. We have now over- expressed Aralar1 by the means of the recombinant Adenovirus AdCA- Aralar1 and have subsequently investigated the impact of Aralar1 on insulin secretion and metabolism, using glucose, pyruvate and various amino acids as fuel stimuli. We have also investigated the effects of an inhibitor of aminotransferase reactions, important for amino acid metabolism (Aminooxyacetate), and a calcium chelator (BAPTA-AM). Experimental parameters measured were insulin secretion, glucose and glutamine consumption, lactate and glutamate generation, mitochondrial membrane potential, NADH generation, triglyceride and glycogen content.

Generally, Aralar1 over- expression in BRIN-BD11 cells resulted in a potentiation of insulin secretion, glucose consumption, mitochondrial membrane hyperpolarization and NADH generation ($p < 0.05$ for all parameters). All the latter effects were further enhanced by the presence of 10 mM L- alanine. However, insulin secretion and glucose consumption were reduced by the presence of 10mM L- aspartate. Addition of L- glutamine did not have a significant impact on any of the latter parameters.

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Prof. Pierre Maechler, Geneva, for collaboration and donation of the Adenoviri constructs.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

effects. Therefore changes in the levels of NEFA are likely to be important for the regulation of β -cell function and viability under physiologic conditions. In addition, the switching between endogenous FA synthesis or oxidation in the β -cell, together with alterations in neutral lipid accumulation, may have critical implications for β -cell function and integrity. Long Chain Acyl CoA controls several aspects of β -cell function including activation of specific isozymes of PKC, modulation of ion channels, protein acylation, ceramide formation and/or NO-mediated apoptosis, and transcription factor activity. Both the level of saturation and chain length appear to be critical in determining signaling and functional outcome (1). In the present study, the effects of chronic exposure (24hr) to the polyunsaturated fatty acid, Arachidonic acid (AA) were studied using the clonal BRIN BD11 β -cell line. Gene expression profiling of BRIN BD11 cells exposed for 24 hours to 100 μ M AA was conducted using microarray analysis. Culture for 24 hours in 100uM AA compared to control resulted in significant (using the Bioconductor software package) changes in 3 genes. Stearoyl-CoA dehydrogenase and ATP-binding cassette, sub-family G (WHITE) member 1 expression were downregulated 3.87 and 1.7 fold respectively. On the other hand Enoyl Coenzyme A hydratase 1, expression was upregulated 1.73 fold relative to control. Subsequent experiments were carried out to establish if the effects were long chain polyunsaturated fatty acid specific. ROS generation which we have previously demonstrated to be NADPH oxidase dependent in the BRIN BD11 cell line (2), was determined after incubation for 24hour in the presence of 100 μ M palmitic acid (PA) or 100 μ M AA. We have determined that β -cell ROS production was regulated by type and degree of saturation of the fatty acids tested.

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PC252

Analysis of arachidonic acid-induced gene expression changes in the BRIN BD11 rat clonal pancreatic β -cell line

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The result of acute exposure of the pancreatic β -cell to high glucose concentrations and/or saturated non-esterified fatty acids NEFA is a substantial increase in insulin release, while chronic exposure leads to desensitization, a reduction in secretion followed by apoptosis. Acute stimulation by some unsaturated NEFA can promote insulin release but they are less toxic to β -cells following chronic exposure and can even exert protective

PC253

Low glycemic index foods: A nutritional approach for management of hyperglycemia and cardiovascular disease in type 2 diabetics

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Background

The global epidemic rise in type 2 diabetes (Barceló *et al.* 2003) has prompted the use of the glycemic index (GI) as a possible approach for optimizing the nutritional management of individuals diagnosed with type 2 diabetes (Brand-Miller *et al.* 2003). Although there is some level of debate surrounding the use of GI in diabetes management, diabetes prevention trials have shown that nutrition and lifestyle approaches with low GI diets may significantly improve the presence of an abnormally high concentration of glucose in the blood (Jarvi *et al.* 1999) and aid in the prevention of cardiovascular diseases (Ebbeling *et al.* 2005)

Objective

To investigate the effect of the consumption diets of low GI indigenous Caribbean carbohydrate-rich foods on A1c, lipoprotein profile, homocysteine and C-reactive protein in type 2 diabetics.

Research Design and Methods

A randomized control parallel trial compared two dietary treatments in adult type 2 diabetics (n = 65) over six months. The experimental treatment group (n = 32) emphasized the consumption of low glycemic index foods, while subjects in the control group (n = 33) were not so advised. Attempts were made to ensure that both groups were isocaloric so as to avoid the confounding effect of the intervention group being relatively hypocaloric with 45-50% of energy from carbohydrates. Changes in study outcomes by multivariate and regression analysis were carried out and the results expressed as mean percentage change.

Results

After the 24 weeks study period subjects in the intervention group showed a significantly greater mean decrease in A1c concentration than subjects in the control group (-9.03% and -4.03% respectively). Mean homocysteine levels decreased significantly in the intervention and control groups (-17.53% and -6.98% respectively). A similar decrease in C-reactive protein concentration (-38.24% and -15.18 respectively) was also observed. The experimental diet group showed a sharper decline in plasma triglycerides than did the control diet group (-16.13% and -5.93% respectively; $p < 0.05$). There were also significant changes in the HDL and LDL levels in both groups. Changes in total cholesterol concentrations, blood pressure, body weight and BMI did not differ significantly between the groups.

Conclusion

The results from this study indicate that the consumption of low glycemic index indigenous carbohydrate-rich Caribbean foods may have a positive effect in glycaemic control and in the prevention of diabetes and its associated complications. This is seen in the improvement in glycaemic control and a decline in cardiovascular inflammatory biochemical markers. Consumption of low GI complex carbohydrates should prove useful and be promoted by health care professionals and dieticians in diabetes education.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC254

The correlation between granular properties and the *in vitro* digestion of native starches extracted from yams (*Dioscorea* spp.)

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Yam (*Dioscorea* spp.) is a monocotyledous tuber bearing plant belonging to the family Dioscoreaceae within the genus *Dioscorea*. Yams are consumed in the tropical and subtropical regions as a primary source of digestible carbohydrate. The effect of starchy foods on blood glucose and insulin responses may vary significantly, which could be attributed to the physiochemical properties of the starches. The degradability of starch when consumed is important especially among diabetics and hyperlipidemic individuals, as starches that are easily degraded tend to have a higher insulin demand than slower degrading starches. This can significantly affect insulin sensitivity, and influence the development of type II diabetes. The diet of diabetics is of concern; especially in the Caribbean where in Jamaica alone over 300,000 cases of diabetes is reported in a population of just over 2.7 million. Understanding the properties of starches from carbohydrate rich foods consumed and how it relates to digestibility may impact the level of control over blood glucose responses.

This study was designed to investigate the relationship between granule properties and the *in vitro* digestibility of starches extracted from *Dioscorea cayenensis* cultivar (cv) Round leaf yellow yam, *Dioscorea polygonoides* (cv) Bitter yam and *Dioscorea esculenta* (cv) Chinese yam.

Significant variations were observed in the particle properties of the different yam starches studied ($p < 0.05$). The projected mean granular diameter ranged from 5.4 μm (Chinese yam) to 34.5 μm (Round leaf yellow yam). The variations observed in the granular size may have influenced the surface properties of the starches, as Chinese yam and Bitter yam were found to have the largest specific surface area (625.91 m²/kg and 258.76 m²/kg respectively) while Round leaf yellow yam (117.4 m²/kg). The results also showed a direct correlation between the particle size and specific surface area on the digestibility of the various starches. Chinese yam and Bitter yam starches, with the smallest mean granule diameters and highest specific surface areas, were found to be the most digestible (21.27 ± 0.01 and 18.11 ± 0.02 % respectively), while Round leaf yellow yam with the largest mean granule diameter and lowest specific surface area was the least digestible (13.74 ± 0.03 %) at 37 °C under *in vitro* conditions. Starches with small granules and large specific surface areas may be more digestible than those with larger granules. This suggests that these starches may be digested and absorbed at different rates when consumed, which could be of importance to diabetic and hyperlipidemic individuals as the differences in digestibility of the different yam starches could affect post-prandial blood glucose levels.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC255

Elucidating a role for beta-secretase in energy homeostasis

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β -secretase (Beta-site APP Cleaving Enzyme 1 – BACE1) is a key player in the development and progression of Alzheimer's disease. Amyloid Precursor Protein (APP) is sequentially cleaved by β - then γ -secretase resulting in the production of the β -amyloid ($A\beta$) peptide, which aggregates and forms the major component of senile plaques found in the brains of all Alzheimer's Disease (AD) patients. BACE1 has been validated as a therapeutic target for AD as BACE1 knockout (KO) mice have reduced $A\beta$ levels while being viable and fertile. Recent reports have demonstrated altered body weights in young BACE1 KO mice. Here we measured body weight of BACE1 KO mice over a period of several months. The results show that the BACE1 KO mice were leaner than their wildtype (wt) littermate controls over this period. The significant differences observed led us to challenge the phenotype with a high fat diet (58% fat). The BACE1 KO mice gained significantly less weight than wt controls. We measured Peri-genital fat (PGF) weight, food intake and insulin sensitivity to help determine the source of the decreased weight gain. Our results suggest an interesting link between BACE1 and body weight.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC256

Fatty acid sensing of hypothalamic arcuate neurons and their link to glucose-sensing

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It is well established that obesity and diabetes may be influenced by defects in hypothalamic signalling, which are associated with high levels of free fatty acids (FFA) in the circulation. Hypothalamic arcuate (ARC) neurons are important in linking nutrient sensing with metabolism and feeding. Intracerebroventricular (icv) administration of the long-chain fatty acid, oleic acid (OA) and pharmacological manipulation of fatty acid metabolism alter energy balance and glucose homeostasis. The present study aims to elucidate the cellular mechanisms of OA on ARC neuron excitability of C57BL/6 mice and to determine whether this correlates with glucose-sensitivity. Whole-cell current-clamp

recordings in the presence of 10 mM glucose revealed acute effects of OA (100 μ M in the presence of 0.05% BSA) in 69% of ARC neurons of which 58% hyperpolarized and 11% depolarized. Similar outcomes were observed in perforated-patch recordings in the presence of 2 mM glucose with 32% hyperpolarizing and 26% depolarizing in response to OA. Our results show that the inhibitory effects of OA are exerted via activation of ATP-sensitive K (K_{ATP}) channels. Challenging perforated-patch recorded ARC neurons with a reduction in glucose from 2 mM to 0.1 mM showed that the majority of ARC neurons (92%; $n = 23/25$) displayed sensitivity to glucose, with 56% hyperpolarizing (glucose-excited (GE) neurons) and 36% depolarizing (glucose-inhibited (GI) neurons). Examination of identified glucose-sensing ARC neurons showed that GE neurons responded by activation of K_{ATP} and hyperpolarization, and GI neurons by depolarization respectively, in response to challenge with OA. Thus, our data indicate that the actions of OA on ARC neurons are glucose-independent as similar responses occur in different external glucose concentrations (10 and 2 mM) but that the response induced by OA correlates strongly with their glucose-sensing phenotype. Further studies are underway to determine whether glucose and oleic acid metabolism utilize similar intracellular mediators to elicit changes in ARC neuron excitability.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC257

Design of high-producing, soluble and bioactive globular adiponectin variants

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Globular adiponectin (gAd) is a promising candidate for protein replacement therapy of metabolic syndrome. Recombinant native human gAd is a poorly behaved molecule with low solubility in physiological solutions and relatively poor expression. Therefore, evaluation of gAd as a potential biotherapeutic requires a molecule with improved biochemical properties relative to native. At Xencor we have used our proprietary protein design methods to create gAd variants with improved expression and solubility. Our lead compound XENP3962 has ~100-fold increased expression and > 100-fold increased solubility relative to native gAd. XENP3962 is biologically active in multiple cell based assays; for example, XENP3962 treatment of cultured myotubes promoted AMPK and ACC phosphorylation, AMP Kinase activation, fatty acid oxidation, and glucose uptake. Studies using primary human adipocytes demonstrated a dose-dependent effect of XENP3962 on lipolytic activity and glucose uptake. Additional experiments showed that XENP3962 induces AMPK phosphorylation in mouse macrophage-like cells as well as in both bovine and human aortic endothelial cells. Pilot pharmacological studies in mice showed that XENP3962 was (1) effective at controlling hyperglycemia and (2) demonstrated activity

in cardiovascular tissues. In conclusion, we have created gAd variants with significantly improved protein expression and solubility, and our lead compound is biologically active in multiple in vitro and in vivo pharmacology models. With success in engineering and producing large quantities of bioactive gAd, it is now possible to conduct the first rigorous pharmacological assessment of gAd as a potential therapy for metabolic disorders.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC258

Red wine polyphenols, Provinols™, improve endothelial function via an increase of nitric oxide production and a reduced oxidative stress in Zucker fatty rats (Fa/Fa)

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The prevalence of obesity is rapidly rising to epidemic proportions around the world at an alarming rate. Obesity is associated with numerous complications including significantly increased risks of diabetes and cardiovascular diseases. Epidemiological studies report an inverse association between dietary flavonoid consumption and mortality from cardiovascular diseases.

The aim of this work was to study the vascular effects of dietary supplementation of red wine polyphenols extract, Provinols™, in the Zucker fatty rats (ZF).

Rats received normal diet or supplemented with Provinols™ (20 mg/kg/day) for 8 weeks. Vascular reactivity was assessed and tissular content of NO and superoxide anion (O₂⁻) production were measured. Provinols™ reduced the change in body weight. Systolic blood pressure was not different among the groups and was neither affected by Provinols™. In the aorta, Provinols™ improved endothelium-dependent relaxation to acetylcholine (ACh) without affecting that of the NO donor, sodium nitroprusside. Provinols™ did not modify contraction induced by phenylephrine (Phe). The NO synthase inhibitor, L-NAME, increased to the same extent contraction to Phe in vessels from the two groups. The non-selective cyclooxygenase (COX) inhibitor, indomethacin, and the COX-2 selective inhibitor, NS398, reduced the response to Phe in aorta from both groups. However, the contraction to Phe was greater in Provinols™ in presence of indomethacin. In small mesenteric arteries, Provinols™ did not affect both the relaxation to ACh and the contraction to Phe. Provinols™ increased vascular (aorta, carotid and small mesenteric arteries) NO release that was accompanied with a decrease of O₂⁻ production. The later occurred via reduction of NF-κB and Nox-1 stainings in the aorta. In summary, Provinols™ increase NO production, reduce both oxidative stress and inflammatory process both in conductance and resistance arteries from ZF rats. This effect is associated with improved endothelial function and reduction of the participation of vasoconstrictor metabolites from COX within the aorta.

Altogether, these results point out beneficial effects of plant-derived polyphenols on endothelial function and vascular reactivity in obese rats.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC259

Increased procoagulant, endothelial, erythrocytes and platelet microparticles account for endothelial dysfunction in patients with metabolic syndrome

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Microparticles are membrane vesicles with procoagulant and proinflammatory properties released during cell activation. Elevated levels of microparticles have been reported in many cardiovascular diseases. Here, we have characterized the cellular origins and studied the effects on endothelial cells of circulating microparticles from healthy subjects and patients with metabolic syndrome (MS), which is referring to the clustering of several cardiometabolic risk factors, including abdominal obesity, hyperglycaemia, dyslipidaemia and elevated blood pressure.

MPs were obtained from whole blood of healthy and MS subjects by serial centrifugations. Then, their counts and cellular origins were determined by flow cytometry in platelet-free plasma. Human endothelial cell line Eahy 926 was treated for 24h with circulating concentration of MPs and subjected to measurement of both nitric oxide (NO) and superoxide anion (O₂⁻) by electronic paramagnetic resonance. Enzymes linked to NO pathway were also analyzed by Western Blotting.

Patients with MS displayed increased circulating levels of microparticles compared to healthy subjects (14718.4±4095.5 vs. 5770.2±2029.9 events/μL of plasma). Levels of platelet-derived (CD41+), endothelial-derived (CD144+ and CD146+), erythrocytes-derived (CD235+) and procoagulant (Annexin V+) microparticles were also increased in MS patients versus healthy subjects. However, microparticles derived from granulocytes (CD66b+) and leukocytes (CD45+) were not significantly different between MS and healthy subjects. In vitro treatment of Eahy 926 cells with microparticles from MS patients but not from healthy subjects reduced both NO and O₂⁻ productions by 51% and 42%, respectively. Microparticles from healthy subjects did not affect neither endothelial NO-synthase (eNOS) expression nor enhanced its phosphorylation at both activator and inhibitor sites (Ser 1177 and Thr495, respectively) and did not modify caveolin-1 expression. Microparticles from MS also did not change eNOS expression or its phosphorylation at the activator site S1177 but they markedly enhanced its phosphorylation at the inhibitor site Thr495. Finally, MS microparticles did not modify caveolin expression.

In conclusion, we demonstrate that patients with MS displayed increased circulating microparticles especially those originated from platelet, endothelial cells and erythrocytes as well as procoagulant microparticles. Interestingly, microparticles from MS

reduce NO bioavailability rather by a mechanism independent to an increase of oxidative stress in endothelial cells via reduction of eNOS activity that probably accounts for endothelial dysfunction. Taken together these results highlight a relationship between increased circulating of procoagulant and platelet microparticles with endothelial dysfunction in MS.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC260

The digestion enzyme used in isolation procedures influences metabolic capacity of hepatocytes

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Isolated hepatocytes provide a useful whole cell *in vitro* system for the evaluation of xenobiotic metabolism and toxicity. Several modifications of the two-stage collagenase perfusion technique, originally described by Seglen¹, have been reported for the preparation of hepatocytes. This study seeks to investigate the effect of digestion enzymes on the drug-metabolising activity of isolated rat hepatocytes in suspension.

Hepatocytes were isolated from male Sprague Dawley rats (180-220 g) using collagenase type II (C II) as described previously². Modifications of this technique using collagenase A/trypsin inhibitor (C A/TI) and collagenase/dispase (C/D) were also investigated. Hepatocytes prepared using the three different digestion enzymes were characterised based on cell yield, % viability (LDH leakage), cytochrome P450 and reduced glutathione (GSH) content, phase 1 metabolism (7-ethoxycoumarin o-deethylase activity (ECOD)) and phase 2 metabolism (glucuronidation and sulfation of 7-hydroxycoumarin (7HC)). For metabolic studies, incubations were performed over 120 min with a cell density of 2×10^6 viable cells/ml in Krebs Hepes buffer, pH 7.4, in rotating round bottomed flasks at 37°C under an atmosphere of 5% CO₂/95% O₂.

Table 1 shows the parameters measured on initial isolation. There were no statistical differences in these parameters. However, at a substrate concentration of 100 µM the use of C A/TI results in a significant ($p < 0.05$) increase in the capacity to form 7HC (3.32 ± 0.25 nmol/ 10^6 cells) compared with C II (1.30 ± 0.23 nmol/ 10^6 cells) and C/D (1.79 ± 0.55 nmol/ 10^6 cells). C A/TI also results in a significant ($p < 0.05$) increase in the yield of 7HC glucuronide (94.0 ± 9.5 nmol/ 10^6 cells) compared with C/D (59.4 ± 5.2 nmol/ 10^6 cells) at a substrate concentration of 500 µM. These initial observations suggest that the use of trypsin inhibitor in perfusion solutions may be beneficial for the metabolic capacity of isolated rat hepatocytes.

Table 1: Parameters measured on initial isolation (mean $\pm$ SEM, n=5)

Digestion Enzyme	P450 Content (nmol/ 10^6 cells)	Viability* (%)	Yield (10^8 cells/liver)	GSH Content (nmol/mg protein)
C II C A/TI C/D	0.10 $\pm$ 0.02 0.07 $\pm$ 0.02 0.10 $\pm$ 0.02	70.90 $\pm$ 0.97 72.18 $\pm$ 2.17 70.90 $\pm$ 1.73	7.10 $\pm$ 0.96 5.70 $\pm$ 0.78 6.02 $\pm$ 0.78	8.32 $\pm$ 1.50 12.28 $\pm$ 1.98 13.36 $\pm$ 1.78

* % viability of cells with no clean up procedure measured by LDH leakage.

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PC261

The antabuse effect of hydrogen sulfide

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Disulfiram (DS) has been clinically used to treat recovering alcoholics for over 50 years. It exerts its pharmacologic effect by inhibiting hepatic aldehyde dehydrogenase (ALDH). It has been proposed that DS inhibits ALDH by forming an inter-molecular mixed disulfide, presumably with the active site thiols. It has also been shown that reactive sulfur species are produced during DS and H₂S metabolism. Here, we have shown that H₂S, at or above 50 µM inhibits yeast ALDH, in an *in vitro* system. There was a dose-response relationship for this effect as increasing H₂S concentration further decreased ALDH activity. The decrease in ALDH activity is enhanced if H₂S is incubated with ALDH for 30 minutes. Cytochrome C, a hemoprotein and/or some metal cations increased ALDH inactivation by H₂S. H₂S is, therefore, a weak inhibitor of ALDH and requires a catalyst to convert it into a more active species. It is postulated that heme or metal cations catalyse the oxidation of H₂S to reactive sulfur species which can form persulfides with the ALDH cysteine at the catalytic site and inhibit its activity. ALDH activity was restored by adding reducing agents such as dithiothreitol and glutathione to reduce the cysteine persulfide moiety of the enzyme. H₂S also increased the cytotoxicity of acetaldehyde and/or 2-chloroacetaldehyde in the presence of the alcohol dehydrogenase inhibitor 4-methylpyrazole towards freshly isolated rat hepatocytes. ALDH inhibition by H₂S indicates that the H₂S must have an "antabuse" effect which would suggest unpleasant reactions to alcohol in the people exposed to H₂S gas.

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PC262

Constitutive expression of PAX4 enhances differentiation of human embryonic stem cells to insulin-producing cells

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The success of pancreatic islet-transplantation therapy to treat diabetes is limited by a lack of sufficient human donor material. Guiding human embryonic stem cells (hESC) to differentiate into functional insulin-secreting β -cells *in vitro* offers an attractive alternative. Since the transcription factor PAX4 initiates terminal β -cell differentiation during murine pancreatic development, we investigated whether constitutive expression of PAX4 in hESCs could promote progression towards a functional β -cell phenotype *in vitro*.

H7 hESC stably-transfected with PAX4 (H7.Px4) and untransfected H7 controls (H7) were allowed to spontaneously differentiate over a 3-week period following embryoid body (EB) formation. Expression of key genes important during hESC maintenance (*OCT4*), pancreatic development (*PDX1*, *ISL1*, *NEUROD1*, *KRT19*) and for mature β -cell function (*SLC2A1*, *GCK*, *ABCC8*, *KCNJ11* and *INS*) were assessed using standard RT-PCR and SYBR-green quantitative PCR (Q-PCR) at days 0, 7, 14 and 21 following initiation of differentiation. After 21 days differentiation, EBs were re-plated, enzymatically dissociated and subjected to fluorescence-activated cell-sorting using Newport Green to isolate a Zn²⁺ positive population of cells. The Zn²⁺ positive cells were subsequently assayed for c-peptide secretion using an ultrasensitive c-peptide ELISA kit.

OCT4, *ISL1*, *NEUROD1*, *KRT19*, *SLC2A1*, *GCK*, *ABCC8* and *KCNJ11* transcripts were expressed at all time points in both control and H7.Px4 EBs (n=3 for each). Interestingly, Q-PCR revealed substantially higher levels of expression of *PDX1* and *INS* mRNA in H7.Px4 EBs than H7 control EBs (n=3) at the mid- to late- stages of differentiation. Following FACS, the Zn²⁺ positive cells were found to be positive for *INS* expression by RT-PCR and Q-PCR, they also contained c-peptide protein (77 $\pm$ 7 pg/10⁴ cells; n=3) and secreted c-peptide in response to stimulation with the insulin-secretagogue, tolbutamide (100 μ M; basal 23 $\pm$ 5 pg/10⁴ cells/15 min vs tolbutamide 68 $\pm$ 4 pg/10⁴ cells/15 min; n=3; Student's t-test p<0.001).

These studies describe for the first time the enhancement of β -cell differentiation in hESC by constitutive expression of PAX4, and also a novel method to separate differentiating insulin-secreting cells from undifferentiated precursors.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC263

11 β -Hydroxysteroid dehydrogenase type1 (11 β HSD1) - key regulator in oxysterol metabolism?

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7-Ketocholesterol (7KC) and 7 β -hydroxycholesterol (7 β OH) are oxygenated metabolites of cholesterol. They are implicated in manipulation of lipid transport, development of oxidative stress & apoptosis in the vascular wall. Recent studies have demonstrated that oxysterols are inter-converted by 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1), better known for its role in amplifying local glucocorticoid concentrations. Inhibition of 11 β HSD1 protects against atherosclerosis, but it is uncertain whether this reflects altered glucocorticoid, or oxysterol metabolism. Our study aimed to compare 11 β HSD1 metabolism of 7KC & 7 β OH with that of corticosterone. Kinetics of oxysterol and glucocorticoid metabolism were determined using mouse recombinant 11 β HSD1 protein, liver microsomes and kidney homogenates from C57Bl/6 and 11 β HSD1^{-/-} mice (male, 9wks, n=6). Oxysterols and glucocorticoids were quantified by HPLC-UV/radiodetection and GCMS.

In microsomes, 11 β HSD1 catalysed dehydrogenation of 7 β OH to 7KC (Km = 3.5 μ M; Vmax = 32.6 pmol/min/mg); the affinity for 7 β OH was 10 fold less than that for corticosterone (B) (Km = 0.314 μ M Vmax = 4.654 nmol/min/mg). Dixon plots and global fit analysis have shown these substrates to be mutual competitive inhibitors (R²=0.79), and this confirmed previous Lineweaver-Burk transformation data of IC₅₀ for 7 β OH inhibitor of 2.20 $\pm$ 9.853 μ M with B range [10⁻⁹-2M]. 11 β HSD1 specificity for this reaction was confirmed by lack of similar metabolism in microsomes from 11 β HSD1^{-/-} mice. Furthermore, the inability of 11 β HSD2 to catalyse this reaction was established in renal homogenates. Recombinant 11 β HSD1 was also able to catalyse NADPH dependent reduction of 7KC to 7 β OH, although dehydrogenation was the preferred direction (Km 7 β OH = 327.5 μ M; Km 7KC = 1.2mM). Similarly, the catalytic constants for substrates suggested higher turnover of 7KC than 7 β OH substrate (Kcat 7KC = 1.4 pmol/sec, Kcat 7 β OH=0.114pmol/sec). The 7 α -epimer (7 α OH), was not a substrate for dehydrogenation, nor was it generated by reduction of 7KC. *In silico* modelling of enzyme:substrate interactions, based on published 11 β HSD1-crystallography data, suggested more favourable binding between Y183-S170-NADP⁺ catalytic triad of 11 β HSD1 and the 7 β OH-epimer as opposed to the 7 α OH. These data confirm that murine 11 β HSD1 catalyses the metabolism of specific 7-oxysterols as well as glucocorticoids.

This suggests that elevated circulating concentrations of oxysterols in dyslipidaemia, may impair glucocorticoid action, while varying glucocorticoid levels may influence oxysterol action. This interaction may be crucial in understanding the influence of corticosteroids and oxysterols in the pathophysiology of cardiovascular disease.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC264

Constitutive expression of *PAX4* in human embryonic stem cells enhances voltage-dependent Ca^{2+} channel expression and function during differentiation

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Voltage-dependent Ca^{2+} channels (VDCC) play a vital role in cell control mechanisms. In this study we profiled the expression of VDCC genes, and their function, in human embryonic stem cells (hESC) during *PAX4*-directed differentiation toward the pancreatic β -cell lineage.

H7 hESC clones stably-transfected with *PAX4* (H7.Px4) and untransfected H7 controls (H7) were allowed to spontaneously differentiate over a 3-week period following embryoid body (EB) formation. Gene expression was assessed using standard RT-PCR and SYBR-green quantitative PCR (Q-PCR) at days 0, 7, 14 and 21 following initiation of differentiation. Physiological function was assessed using Ca^{2+} microfluorimetry of Fura2-loaded EBs challenged with depolarising concentrations of KCl (40 mM) at days 7, 14 and 21 of differentiation.

RT-PCR revealed the expression of transcripts for *CACNA1S*, *CACNA1C*, *CACNA1D*, *CACNA1A*, *CACNA1B* and *CACNA1E*, and *CACNB1* to 4 in EBs from H7 and H7.Px4 and also in human foetal pancreas, although neither *CACNA1S* or *CACNA1E* were expressed in human neonatal pancreas. Interestingly, Q-PCR demonstrated significant upregulation of *CACNA1A* and *CACNA1C* expression at all time points in the H7.Px4 EBs whereas *CACNA1D* expression was only transiently upregulated in these EBs relative to H7 control EBs.

The increase in VDCC gene expression over time correlated with an enhanced responsiveness of EBs to 40 mM KCl-induced Ca^{2+} signalling in the H7.Px4 EBs relative to H7 control EBs. In control EBs, intracellular Ca^{2+} responses ($[\text{Ca}^{2+}]_i$) to KCl stimulation were poor between days 7 and 21 of differentiation (e.g. 9/31 EBs responded at day 21) although the size of responses increased over time (16 ± 2 nM; n=11 at day 7 vs 39 ± 15 nM; n=9 at day 21). In comparison, the numbers of H7.Px4 EBs responding to KCl stimulation were significantly higher (e.g. 34/34 EBs responded at day 21) with large changes in $[\text{Ca}^{2+}]_i$ observed from 7 days onwards (e.g. 36 ± 4 nM at day 7, n=13/27).

In summary, *PAX4* overexpression in hESC EBs led to significant changes in intracellular Ca^{2+} signaling. These changes could be explained by upregulated expression of VDCC subunit transcripts. Since constitutive *PAX4* expression also leads to expression of transcriptional and functional markers of insulin-secreting cells, these data provide additional support for the use of *PAX4* as a lineage determinant of β -cells in hESC.

SJB was funded by a MRC DTA. KEC was funded by a MRC Stem Cell Fellowship and a Novo Nordisk Research Foundation Award. We thank Juvenile Diabetes Research Foundation for supporting this work (MJD, PWA, HDM, KEC).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC265

Effects of a three-day high-fat diet on gastrointestinal transit and satiety in men

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Rapid gastric emptying and hence a shortened satiety period may contribute to the development of obesity (1). Feeding a high-fat diet for 14 days has been found to accelerate gastric emptying (GE) and mouth to caecum transit time (MCTT) (2). The aim of the current study was to examine the effect of a three-day high-fat diet on gastrointestinal transit and satiety.

Eleven healthy male volunteers were recruited (age 24.7 ± 3.1 yrs; height 181.5 ± 7.9 cm; weight 81.8 ± 9.1 kg; all data mean $\pm$ S.D.) and undertook three stages: (i) they recorded their diet for a three-day period (PRE), (ii) they repeated their diet plus either a yoghurt supplement (LFY), or a yoghurt and oil supplement (HFY), given in randomised order, and (iii) they repeated their diet plus the alternate supplement. Each three days diet was followed by a test day. On each test day volunteers arrived in the laboratory following a 12-hour overnight fast. They consumed a high-fat pancake breakfast (61% energy from fat). Breath samples were collected for the following six hours and volunteers were asked to rate their satiety using a visual analogue scale (VAS) every 30-minutes. GE was measured using the ^{13}C -octanoic acid breath test (3), and MCTT using the breath H_2 technique (4). Volunteers were allowed to eat ad libitum for the rest of the day weighing and recording the food consumed. Data were tested for significance ($p < 0.05$) using repeated measures analysis of variance (ANOVA).

During the intervention volunteers' daily average fat intake increased from 89.5 ± 14.7 g (PRE) to 97.5 ± 15.6 g (LFY) to 187.3 ± 15.4 g (HFY), and their energy intake from 11.06 ± 1.73 MJ (PRE) to 11.99 ± 1.96 MJ (LFY) to 15.45 ± 1.74 MJ (HFY) ($p < 0.05$ for all comparisons) (n=11). MCTT decreased between PRE and HFY (226 ± 84 min and 280 ± 60 min respectively, $p = 0.025$). No differences existed in MCTT between LFY (291 ± 47 min) and either PRE or HFY. Preliminary results show no differences in GE half times between the three interventions (PRE 160 ± 19 min; LFY 180 ± 32 min; HFY 175 ± 47 min; $p > 0.05$) (n=5). Following all meals volunteers became less satiated over the six-hour period; hunger ($p = 0.005$), desire to eat ($p = 0.003$), and fullness ($p = 0.002$) (n=11). However, results from the VAS showed no difference between the three diets for any of the satiety parameters. There were no significant differences in energy or macronutrient dietary intakes on the day of each test session (n=11).

Results suggest that alteration in gastrointestinal transit following a high-fat diet may begin in the intestine, and may develop

after a longer intervention such as two weeks to accelerating stomach emptying as reported by Cunningham et al. (1991). The lack of changes in GE rates after a three-day high-fat diet may explain why satiety is not altered by the high-fat diet.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC266

Plasma membrane Ca^{2+} ATPases at synapses in the cerebellum

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The plasma membrane calcium ATPases (PMCAs) are a family of proteins that actively control the level of cytosolic Ca^{2+} by pumping Ca^{2+} out of the cell. The four PMCA isoforms (PMCA1-4) show differential patterns of expression with PMCAs 1 and 4 being ubiquitously expressed and PMCAs 2 and 3 being expressed almost exclusively in brain tissue. We propose that PMCAs are important for synapse function, firstly by virtue of their Ca^{2+} extrusion properties but secondly by virtue of molecular interactions with synapse associated proteins.

Immunohistochemistry of rat and mouse cerebellar slices revealed extensive expression of PMCA2 and 3 at Purkinje cell membranes and throughout the molecular layer and granule cell layers. PMCA2 was most evident in the molecular layer whilst PMCA3 showed greatest expression in the granule cell layer. Both PMCAs co-localised with the pre-synaptic proteins synaptophysin and syntaxin and the post-synaptic density protein PSD95 in the molecular and granule cell layers. In addition, both PMCAs were enriched in synaptosome preparations, with some of each found in the pre-synaptic and post-synaptic fractions. Furthermore, the analysis of proteins that co-immunoprecipitate with PMCAs by mass spectrometry and Western Blotting revealed *in vivo* interactions between PMCAs and synaptic proteins.

Our results provide support for a role for PMCAs at cerebellar synapses at both pre- and post-synaptic locations. We conclude that PMCAs are enriched at synapses within the cerebellum. Furthermore, the ability of PMCAs to associate with synaptic protein complexes supports the idea that this important Ca^{2+} extrusion mechanism contributes to synapse function by being in the right place at the right time.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC267

17 β -estradiol, through elevation of cGMP, stimulates plasma membrane Ca^{2+} efflux from rat hepatocytes

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17 β -estradiol has been shown to protect both liver [1] and isolated hepatocytes [2] under pathophysiological conditions that have been associated with a rise in intracellular Ca^{2+} concentration. The classical mode of action of 17 β -estradiol involves its translocation to the nucleus and regulation of transcription. However, numerous rapid non-genomic actions of 17 β -estradiol have been demonstrated in several cell types, including stimulation of plasma membrane Ca^{2+} -ATPase activity [3] and elevation of cGMP [4]. We have previously shown that another circulating hormone, atrial natriuretic peptide (ANP), stimulates hepatocyte plasma membrane Ca^{2+} efflux through elevation of cGMP and activation of protein kinase G (PKG), and have proposed that it may thus protect against Ca^{2+} -dependent injury [5]. The aim of this study was to investigate the effects of 17 β -estradiol on cGMP levels and plasma membrane Ca^{2+} efflux from rat hepatocytes. Hepatocytes were isolated from 150-250g male Wistar rats by collagenase digestion. cGMP was measured by enzymeimmunoassay. Ca^{2+} efflux from hepatocytes in suspension was measured spectrophotometrically, using membrane-impermeant fura-2 as extracellular Ca^{2+} reporter. Data are presented as mean $\pm$ SEM and are from at least 5 independent hepatocyte preparations. Significance of differences between means was assessed by Student's *t* test (*significantly different from control at $P < 0.05$). We show that 10 nM 17 β -estradiol rapidly elevates hepatocyte cGMP, which reaches peak values 5 min following addition of 17 β -estradiol ($266.2 \pm 24.6^* \text{ fmol}/10^6$ cells, $n = 12$, compared with $183.8 \pm 13.7 \text{ fmol}/10^6$ cells, $n = 7$, in control, untreated hepatocytes). An optical isomer, 17 α -estradiol, did not elevate cGMP ($n = 11$). However, the stimulatory effects of 17 β -estradiol on cGMP production were mimicked by 17 β -estradiol rendered membrane-impermeant by linkage to BSA (E2-BSA; $n = 10$). A basal rate of Ca^{2+} efflux was recorded from hepatocytes ($99.9 \pm 3.4 \text{ pmol}/\text{min}/10^6$ cells, $n = 117$). Addition of 10 nM 17 β -estradiol rapidly stimulated an approximate 1.5 fold increase in Ca^{2+} efflux ($150 \pm 9.6^* \text{ pmol}/\text{min}/10^6$ cells, $n = 34$). The stimulation of Ca^{2+} efflux was found to be dependent on the activation of PKG; in the presence of the PKG inhibitor, Rp-8-pCPT-cGMPS, there was no stimulation of Ca^{2+} -efflux upon addition of 10 nM 17 β -estradiol ($n = 9$). 17 α -estradiol did not stimulate Ca^{2+} efflux ($n = 10$). However, the stimulatory effects of 17 β -estradiol on Ca^{2+} efflux were mimicked by E2-BSA ($n = 28$). Taken together, these data suggest that 17 β -estradiol has a rapid and specific effect on hepatocytes, acting at the plasma membrane to stimulate Ca^{2+} efflux through the generation of cGMP and activation of PKG.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC268

The plasma membrane Ca^{2+} ATPase, PMCA2, contributes to pre-synaptic Ca^{2+} dynamics and short term plasticity at the cerebellar parallel fibre to Purkinje neurone synapse

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PMCA2 is a fast, highly effective mechanism to control (by extrusion) resting cytosolic Ca^{2+} and Ca^{2+} excursions in neurones and other excitable cells. The strong expression of PMCA2 in the cerebellum and the cerebellar behavioural deficits presented by PMCA2^{-/-} knockout mice all point to its importance for cerebellar circuit dynamics.

AIM: to provide direct functional evidence for the influence of pre-synaptic PMCA2 mediated Ca^{2+} extrusion for short term plasticity at cerebellar parallel fibre to Purkinje neurone synapses. Whole cell patch clamp recordings from Purkinje neurones (PNs) within cerebellar slices from wild type (wt) and PMCA2^{-/-} mice revealed enhanced paired pulse facilitation (PPF) of parallel fibre (PF) evoked EPSCs from

PMCA2^{-/-} PNs, that also took longer to return to baseline. Similar results were obtained in the presence of the PMCA inhibitor, carboxyeosin, but eosin was ineffective in PMCA2^{-/-} cells. Additional results from intracellular Ca^{2+} measurements from bundles of pre-synaptic PFs showed that the decay of the PF Ca^{2+} transient was also enhanced in slices from PMCA2^{-/-} slices compared with wt.

Our results provide strong functional evidence for a contribution by PMCA2 to the clearance of Ca^{2+} from the PF pre-synaptic compartment to influence short term plasticity at the PF-PN synapse.

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PC269

The translocon and the aspecific transport of small molecules in the endoplasmic reticulum

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Several enzyme activities localized in the luminal compartment of the endoplasmic reticulum require the transmembrane fluxes

of their substrates, products and/or cofactors. Accordingly variety of structurally unrelated compounds is (or should be) able to cross the ER membrane, but only a few transporters have been reported so far. It has been repeatedly observed that microsomal vesicles derived from the endoplasmic reticulum exhibit a basal permeability towards various compounds – including xenogenous molecules – which presumably do not have strictly specific transporters. Nevertheless, it might have a physiological function as a low affinity, low capacity transporter with low selectivity.

The pore of the translocon complex in the endoplasmic reticulum is large enough to be permeated by small molecules and there are more and more data about the permeation of small compounds through the translocon.

Previous works on cell experimental systems indicated that a small, neutral molecule and Ca^{2+} permeate the peptide channel. Our experiments on rat liver microsomes confirmed the role of translocons as Ca^{2+} leak channels. In addition we proved the role of translocons in the non-specific transport of a small, neutral molecule (sucrose) and anions (UDP-glucuronic acid, hexose phosphates). The translocon mediated transport could be increased by puromycin, which inhibits elongation and causes premature releasing of the nascent peptide from the ribosome. The effect of puromycin could be prevented by addition of puromycin antagonist and an antibody against the translocon complex. The non-specific transport - due to the occurrence of translocons - was more explicit in the rough microsomal subfraction.

Our results show that the translocon peptide channel contributes to the non-specific Ca^{2+} leak. The translocon mediated transport can ensure the substrate supply of intraluminal ER enzymes, and can provide counterligands for known ER specific antiporters.

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PC270

The brominated flame retardant, Tetrabromobisphenol A, inhibits SERCA Ca^{2+} pumps and sensitizes the Ryanodine receptor

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Tetrabromobisphenol A (TBBPA) is currently the most commonly employed type of the brominated flame retardant (BFR), used to treat plastics and furnishings in order to reduce their flammability. BFRs including TBBPA and their metabolites have been shown to bio-accumulate to relatively high levels within humans as result of repeated environmental exposure [1]. Recent studies have also shown that low concentrations of TBBPA can modulate a number of cell signalling processes [2].

Using Ca^{2+} ATPase activity measurements as described in [3], the data in table 1 shows that TBBPA is a very potent inhibitor of both SERCA1a and SERCA2b Ca^{2+} ATPases (IC_{50} values of 1.7 and 2.3 μM , respectively). Subsequent studies have indicated that Ca^{2+} pump inhibition is due to TBBPA decreasing its affinity for Ca^{2+} . Thus TBBPA could potentially affect Ca^{2+} homeostasis processes leading to cellular dysfunction.

In order to assess whether other commonly found intracellular Ca^{2+} transporters were also affected by TBBPA, its effects on both the inositol-1,4,5-trisphosphate receptor (IP_3R) and Ryanodine receptor (RyR) was also investigated.

The effects of TBBPA on the function of the RyR was explored by assessing whether TBBPA could affect the opening of the channel by 2-Hydroxycarbazole (2HC), a potent caffeine-like agonist [4]. Ca^{2+} release via RyR was observed from muscle SR vesicles using suboptimal concentrations of 2HC (400 μM). In Table 1 the data shows that pre-incubation of the vesicles with TBBPA at concentrations between 1–4 μM greatly enhanced 2HC-induced Ca^{2+} release through the RyR. This result therefore indicates that TBBPA sensitizes the RyR to release more Ca^{2+} upon stimulation with 2HC. It is yet to be determined whether this increased Ca^{2+} release is due to an increase in the duration of the open state of the channel or due to a higher conductance state. The activation of the IP_3R was monitored by the addition of IP_3 to cerebellar microsomes pre-accumulated with Ca^{2+} and the changes in calcium measured using Fluo-3 [5]. As shown in the table the extent of Ca^{2+} release in cerebella microsomes induced by IP_3 was not significantly affected, in the presence of up to 4 μM TBBPA. The results presented here would indicate that low concentrations of TBBPA could have dramatic affects upon Ca^{2+} signalling processes within cells by abnormally elevating intracellular Ca^{2+} levels, which could ultimately lead to cell dysregulation and death. Our studies would indicate that TBBPA should be treated as a potential environmental 'endocrine disrupter'.

Table 1

[TBBPA] (μM)	Activity of SERCA1a Ca^{2+} ATPase ($\mu\text{moles/min/mg}$ protein)	Activity of SERCA2b Ca^{2+} ATPase (nmoles/min/ mg protein)	% Ca^{2+} release by 2HC (400 μM)	% Ca^{2+} release by IP_3 (0.4 μM)
0	2.3 $\pm$ 0.0	54.8 $\pm$ 0.9	34.7 $\pm$ 0.3	11.9 $\pm$ 0.9
1.0	1.3 $\pm$ 0.0	34.7 $\pm$ 0.6	72.7 $\pm$ 4.2	12.8 $\pm$ 0.8
2.0	1.0 $\pm$ 0.0	30.0 $\pm$ 1.0	69.0 $\pm$ 12.5	13.5 $\pm$ 0.2
4.0	0.6 $\pm$ 0.0	20.5 $\pm$ 0.6	70.6 $\pm$ 5.2	12.9 $\pm$ 1.6

% Ca^{2+} release, related to that released by A23187

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PC271

The effect of tetrabromobisphenol-A, a brominated flame retardant, on Ca^{2+} signalling and cell viability in Sertoli cells

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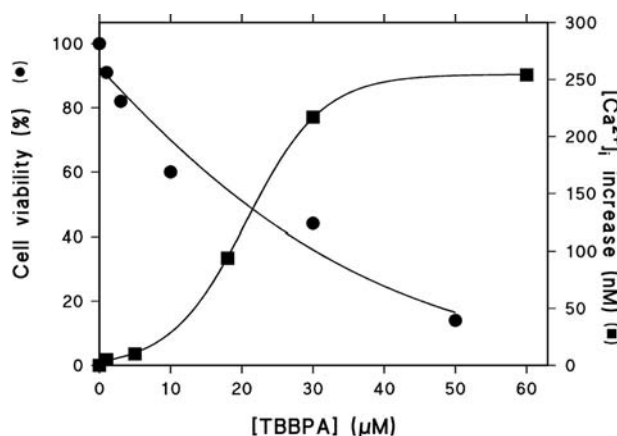
Brominated flame retardants (BFRs) are a family of compounds used at high levels in the modern world. Their purpose is to slow

down the spread of fire in materials in which they are incorporated. One-third of all BFRs produced worldwide consist of tetrabromobisphenol-A (TBBPA) [1]. Popularity in the use of TBBPA in recent years has led to its high level of disposal in the environment. It is now a pollutant commonly found in sewage and air [2]. BFRs are capable of accumulating in living organisms, with some having been detected in human blood at levels of 160 ng/g lipid serum [3]. Recent reports have identified TBBPA to act as an 'endocrine disruptor' by perturbing the activities of thyroid hormones and estrogen [4]. Our previous studies have linked some endocrine disrupting pollutants with dysregulation of Ca^{2+} signalling in testicular Sertoli cells, which are involved in sperm maturation and development. We and others have speculated that exposure to these pollutants, may in part, contribute to the increased incidence of male infertility currently being reported [5]. Here we present data to show that TBBPA can affect Ca^{2+} homeostasis and induce cell death in Sertoli cells.

The TM4 mice Sertoli cell line was used in these experiments. Using Fura-2-based fluorimetry as described in [5], TBBPA was shown to cause a dose-dependent increase in intracellular $[\text{Ca}^{2+}]_i$ in TM4 cells (EC_{50} , 22 μM), with 60 μM TBBPA increasing the $[\text{Ca}^{2+}]_i$ by 250 nM from 100 nM to 350 nM (fig.1). As TBBPA also increased $[\text{Ca}^{2+}]_i$ in cells incubated in buffer containing low $[\text{Ca}^{2+}]$, this rise is likely due to mobilization from intracellular Ca^{2+} stores. From related work with other endocrine disruptors, we believe that this rise is due to TBBPA inhibiting SERCA Ca^{2+} pumps [5]. Cell viability as assessed using the MTT assay, described in [5], showed that treatment of TM4 cells with TBBPA (0–50 μM) for 18 hours, caused a dramatic increase in the degree of cell death (LC_{50} , 20 μM) (fig.1).

Measurements of mitochondrial membrane potential of TBBPA-treated TM4 cells, using rhodamine-123, also showed that TBBPA at concentrations as low as 5 μM could depolarise the mitochondrial membrane, by opening the permeability transient pore (PTP).

Our data indicate that TBBPA can cause abnormally high levels of intracellular $[\text{Ca}^{2+}]$ within Sertoli cells, which could induce 'Ca²⁺ overload' of the mitochondria, leading to the opening of the PTP. This in turn could cause the release of pro-apoptotic factors from the mitochondria leading to the observed decrease in cell viability.



The effects of TBBPA on cell viability (●) and increases in intracellular Ca^{2+} levels (■) within TM4 Sertoli cells

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 PC272

Inositol 1,4,5-trisphosphate (InsP3)-induced calcium release controls cardiac myocyte hypertrophy

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As a result of increased haemodynamic load, genetic factors or disease, the heart undergoes a hypertrophic response. Genetic manipulation of components of various Ca²⁺ regulated gene transcription pathways has revealed that this ion has a fundamental role in stimulating cardiac hypertrophy. Previously, we have shown that Endothelin (ET-1) stimulates InsP₃-dependent Ca²⁺ release in atrial and ventricular myocytes. ET-1 is also a potent inducer of cardiac hypertrophy. Here we investigated whether InsP₃-induced Ca²⁺ release (IICR) contributes to the pro-hypertrophic action of ET-1.

Experiments were performed using spontaneously contracting monolayers of primary cultures of neonatal ventricular rat myocytes (NRVM). Quantitation of atrial natriuretic factor (ANF) by immunofluorescence and real-time PCR, together with cell surface area were used as measures of hypertrophy.

To investigate the mechanism underlying ET-1 induced hypertrophy, 2-APB or adenoviral mediated expression of InsP₃-5'-phosphatase were used to inhibit IICR. Both 2-APB and expression of InsP₃-5'-phosphatase significantly decreased the percentage of myocytes expressing ANF following ET-1 stimulation.

To determine whether excitation-contraction (EC) coupling was required for ET-1-induced hypertrophy, ET-1 was applied to NRVMs in which voltage gated channels were blocked with nifedipine (L-type Ca²⁺ channel antagonist) and mibefradil (T-type Ca²⁺ channel antagonist). Under these conditions, ET-1 significantly increased the percentage of ANF expressing cells in an InsP₃-dependent manner, demonstrating that IICR can induce hypertrophy independently of EC-coupling. However, the percentage of cells expressing ANF was significantly lower in these inhibited cells in comparison to controls. To determine whether hypertrophy induced by increased EC-coupling required IICR, BayK 8644 (L-type Ca²⁺ channel agonist) or isoproterenol (IsoP, β -adrenergic agonist) were used. Both agonists significantly increased the percentage of cells expressing ANF in an InsP₃-dependent manner.

To investigate the mechanism by which increased EC-coupling/workload induces hypertrophy, antagonists of ET-1 binding, production and secretion were used - BQ123, Phosphoramidon and Primaquine, respectively. All three antagonists significantly decreased the percentage of NRVMs expressing ANF

following BayK 8644 or IsoP stimulation, suggesting that increased workload increases ET-1 production and secretion from myocytes. The resultant paracrine/autocrine effect of ET-1 leads to IICR and the induction of hypertrophy.

Together, these data demonstrate that IICR is required for the induction of hypertrophy as a result of Gq stimulation or increased workload. Furthermore, this provides a mechanism by which Ca²⁺ regulated gene transcription can be isolated from the increases in cytosolic Ca²⁺ that occur during every heart-beat.

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 PC273

Investigations into the mechanisms of amyloid beta-peptide induced toxicity

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The amyloid β -peptide (A β) is produced by proteolytic processing of the amyloid precursor protein and it has been suggested, both on genetic grounds and on experimental evidence, that A β is central to the pathogenesis of Alzheimer's disease. Controversy remains regarding the primary pathogenic structure of A β , the cell type it targets and the molecular events underlying its toxicity. While the original formulation of the amyloid hypothesis implicates fibrils in neurodegeneration, more recent evidence suggests that soluble oligomers are the primary neurotoxic agents. Studies have shown that soluble oligomers, but not monomers or fibrils, elicit rapid Ca²⁺ signals in SH-SY5Y cells. A β has also been reported to elicit Ca²⁺ responses and the generation of reactive oxygen species in astrocytes, though cell death was confined to neurons due to a suggested lack of antioxidant support. We aim to identify the primary pathogenic structure of A β , its primary cellular target and its pathological mechanism. Initial experiments will be conducted in the SH-SY5Y neuroblastoma cell line and ultimately in primary hippocampal cultures.

After in vitro incubation, we have shown a time-dependent increase in the mass of the A β oligomeric species. Exposure of cells to A β 42 for 48hr induced death in 50% of cells as determined by the release of lactate dehydrogenase. Furthermore, a MTT assay measured that 30% of cells remained viable following a 24hr exposure to A β 42. To determine whether dysregulation of intracellular Ca²⁺ homeostasis contributed to the toxic effects of A β 42, we investigated whether exposure of cells to peptide induced elevations in intracellular [Ca²⁺]: Fura-2 loaded SH-SY5Y cells were imaged for a 30min period following exposure to A β 42 that had been pre-aggregated (1hr, 2hr or 24hr). A saturating dose of carbachol (100 μ M) was applied thereafter to demonstrate cell viability and that the Ca²⁺ stores were intact. Application of peptide that had been pre-aggregated for 1hr or 2hr induced Ca²⁺ transients in less than 2% of cells. Peptide that had been pre-aggregated for 24hr induced Ca²⁺ transients in a

greater number of cells (<5 %). The amplitude of the carbachol-induced Ca^{2+} transient was unaffected by A β 42 application, irrespective of the pre-aggregation time. The Ca^{2+} responses recorded exhibited no trend in their time of onset, frequency, amplitude or duration, suggesting a non-regulated process. A large degree of variability was observed both within and between experiments. No Ca^{2+} responses were recorded in any of the control experiments.

In summary, we have shown that A β 42 induces cell death and Ca^{2+} increase in SH-SY5Y cells.

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PC274

Ca^{2+} dysregulation as a myogenic basis of overactive bladders

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Increased spontaneous contractile activity in detrusor muscle may contribute to overactive bladder contractions and increased bladder tone associated with urinary incontinence¹. The underlying mechanisms for enhanced contractile function in overactive bladders have never been examined, in particular, the role of intracellular Ca^{2+} , which critically determines contractile function. The objective of this investigation was to test the hypothesis that enhanced spontaneous Ca^{2+} oscillations may be associated with detrusor overactivity.

Human detrusor samples were obtained with ethical approval and patient consent. Dissociated detrusor myocytes were loaded with Fura-2 or Fluo-4; whole cell and local Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured using either conventional epifluorescence microscopy or a laser-scanning confocal system. The magnitudes of whole cell spontaneous $[\text{Ca}^{2+}]_i$ rises were quantified as the time-averaged area under the curve for the duration of observation. Parameters for quantifying Ca^{2+} sparks were amplitude (F/F₀), full duration at half-maximum (FDHM, ms) and full-width at half-maximum (FWHM, μm). Data are mean $\pm$ s.e.m. Student's t-test examined differences between data sets ($p < 0.05$).

Spontaneous transient global rises of $[\text{Ca}^{2+}]_i$ were observed of varying amplitude, duration and frequency and associated with cell shortening. They were Ca^{2+} -dependent and sensitive to the L-type Ca^{2+} -channel antagonist verapamil. The mean increases of $[\text{Ca}^{2+}]_i$ were 5.7 ± 1.2 nM and 12.8 ± 2.3 nM in cells from stable ($n=24$) and overactive bladders respectively ($n=26$, $p < 0.05$). Localised rises of $[\text{Ca}^{2+}]_i$, as discrete, fast sparks and larger cluster-like local activities were also observed in unstimulated

myocytes. These localised sparks either remained local or spread to nearby areas. Repetitive firing of Ca^{2+} sparks and their fusion usually progressed into Ca^{2+} waves, leading to whole cell $[\text{Ca}^{2+}]_i$ transients. Spark activity was inhibited by 10-20 μM ryanodine and facilitated by 1 mM caffeine. The intensity, duration and width of local Ca^{2+} activity were increased in overactive bladders (F/F₀ 1.53 ± 0.05 vs 1.32 ± 0.02 , FDHM 776 ± 153 vs 358 ± 83 ms, FWHM 4.46 ± 0.33 vs 2.55 ± 0.17 μm ; $n=53$ sparks, 4 overactive bladders; $n=103$ sparks, 5 stable bladders, $p < 0.01$).

These observations show that global and localised Ca^{2+} rises can occur spontaneously in resting human detrusor myocytes. Whilst whole cell Ca^{2+} rises generate spontaneous contractile activity, the local Ca^{2+} oscillating events, via ryanodine receptors, contribute to a global Ca^{2+} rise. The upregulation of both global and local Ca^{2+} activities in detrusor myocytes from overactive bladders will contribute to increased contractile activity in overactive bladders.

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PC275

Functional and structural microdomains role in the regulation of inactivation of CRAC channels in Jurkat T cells

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A biphasic increment of intracellular free Ca^{2+} levels is the primary signal of T lymphocyte activation after antigen recognition, as consequence of Ca^{2+} emptying of InsP_3 -sensitive stores and the Ca^{2+} entry through a store-operated Ca^{2+} channel type known as CRAC (Ca^{2+} -release activated Ca^{2+}). As occurs with other Ca^{2+} channels, CRAC inactivates in a Ca^{2+} -dependent manner so that any mechanism that regulates cytoplasmic Ca^{2+} concentration could also modulate CRAC channels activity. In this context, by serving as intracellular Ca^{2+} buffers, mitochondria have been proposed to control CRAC current (I_{CRAC}). However, mitochondria can also regulate I_{CRAC} by a distinct mechanism from direct buffering of Ca^{2+} , such as the release of a soluble factor that we have identified as ATP. Using the whole cell configuration of patch-clamp technique, which permits the control of the intracellular environment, we have investigated the involvement of subplasmalemmal mitochondria in the regulation of Ca^{2+} microdomains and hence, I_{CRAC} characteristics.

Our results indicate that energized mitochondria regulate slow Ca^{2+} -dependent inactivation of I_{CRAC} by increasing subplasmalemmal Ca^{2+} buffering capacity mainly through a localized ATP production and support the idea of a functional relationship between CRAC channels and peripheral mitochondria.

We also will discuss preliminary results about the role played by the composition of plasma membrane in the activity of CRAC channels and the role played by another elements to exert a local control in the activity of CRAC channels.

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PC277

Investigating subcellular calcium signals in cardiac myocytes

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Cardiac myocytes contract in situ in response to electrical stimuli from the sinoatrial (SA) node. Contraction is facilitated by Ca²⁺-induced Ca²⁺ release (CICR) through ryanodine receptors (RyRs) on the surface of the sarcoplasmic reticulum (SR). Cardiac myocytes also express inositol 1, 4, 5-trisphosphate receptors (InsP₃Rs), albeit at a 100-fold lower abundance than RyRs. In response to stimulation of PLC-coupled receptors such as the endothelin-1 receptor (ET-1R), or InsP₃ itself, we have previously observed Ca²⁺ release in adult rat ventricular and atrial myocytes. Unlike the global Ca²⁺ changes observed during excitation-contraction coupling through RyRs, Ca²⁺ liberated via InsP₃Rs is more restricted in its spatial distribution. As well as inducing an inotropic effect, however, InsP₃-induced Ca²⁺ release can promote arrhythmogenic Ca²⁺ signals in cardiac myocytes. A role for InsP₃-induced Ca²⁺ release in hypertrophic signalling has also recently been suggested. Since rat ventricular neonatal myocytes are a widely used and accepted model for analysis of cardiac myocyte signalling pathways, we have now investigated the mechanisms involved in generating Ca²⁺ signals in these cells. To this end, neonatal ventricular myocytes were isolated from 1–2 day old Wistar rats and cultured to form a spontaneously contracting monolayer. Confocal Ca²⁺ imaging of these myocytes demonstrated small Ca²⁺ release events that were primarily perinuclear, which occurred independent of whole cell Ca²⁺ signals controlling contraction. These Ca²⁺ release events were sensitive to inhibition by the RyR antagonist ryanodine but not the InsP₃R antagonist 2-aminoethoxy diphenylborate (2-APB). In spontaneously contracting cells, application of ET-1 induced an increase in frequency but not amplitude of the myocyte Ca²⁺ signals. Application of ET-1 in cells in which CICR was abolished stimulated calcium release, which was concentrated in the perinuclear region. This perinuclear Ca²⁺ transient was also observed in cells exposed to a membrane permeable IP₃ ester. This ET-1-induced Ca²⁺ signal was abolished following pre-treatment with 2-APB. Together, these data indicate that neonatal myocytes express both functional InsP₃Rs and RyRs, which can act independently to generate spatially distinct Ca²⁺ release events.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC278

Disruption of calcium signaling in rat ventricular myocytes from infarcted heart

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There are several lines of evidence indicating disruption of calcium signaling in myocytes of infarcted myocardium. To understand its basic mechanism we have studied effect of experimentally induced infarction in rats by characterizing changes in kinetics of calcium spikes. Myocardial infarction was induced by a single intraperitoneal injection of isoproterenol (150 mg/kg⁻¹). On day 15 after ISO application, cardiac myocytes were isolated and studied by combination of patch clamp and confocal microscopy. The efficiency of the excitation-contraction coupling was assessed by recording calcium spikes in response to membrane depolarization. We have analyzed amplitude and kinetic properties of calcium spikes, evoked by calcium current activated by step depolarizations to 0 mV. Ca spikes were recorded by Leica TCS-SP2 confocal system, using Oregon Green BAPTA-5N (1 mM) as calcium indicator and EGTA (4 mM) in the patch pipette. Temporal fluorescence profiles were fitted by a parametrical model.

We have observed substantial and significant changes in the latency and synchronization of Ca spikes relative to the stimulus onset. The amplitude of Ca spikes was slightly but significantly increased. Also, the rate of rise and the duration of Ca spikes were significantly prolonged. These findings characterize changes in calcium signaling in myocytes from infarcted hearts that are consistent with delayed and less synchronous calcium release observed by others. We conclude that individual dyads may have been modified due to the remodeling of infarcted myocardium.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC279

A caged inhibitor of mitochondrial function that when photolysed in sub-cellular regions influences inositol trisphosphate receptor calcium release in smooth muscle

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The activity of mitochondria impacts upon many aspects of cellular physiology, including ATP production and Ca²⁺ regulation.

We have shown previously that mitochondria regulate InsP_3 -sensitive Ca^{2+} release channels (InsP_3R) on the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), in freshly isolated smooth muscle cells. Depolarisation of the electrical potential across the inner mitochondrial membrane (Ψ_m) inhibited mitochondrial Ca^{2+} accumulation, which in turn inhibited Ca^{2+} release via InsP_3R . This result suggested a close interaction between mitochondria and the microdomain of Ca^{2+} released by InsP_3R ; localised mitochondrial Ca^{2+} uptake may prevent a negative feedback of Ca^{2+} upon InsP_3R activity.

Until now, the only method of investigating the influence that mitochondria exert over cellular processes such as Ca^{2+} signalling has been pharmacological depolarisation of all mitochondria within a cell. We have developed a caged mitochondrial inhibitor that may be released in subcellular regions of interest, by selective exposure UV light to enable depolarisation of mitochondria in subcellular regions to occur. The compound AG10 (also known as tyrphostin 8), depolarises Ψ_m by increasing the proton conductance of the inner mitochondrial membrane. AG10 was caged chemically with a 4, 5-dimethoxy-2-nitrobenzyl alcohol group attached to the 6' hydroxyl of AG10. The caged form of AG10 did not depolarise Ψ_m when introduced into the cytosol of single smooth muscle cells freshly isolated from guinea pig colon, via a whole-cell patch pipette. When AG10 was uncaged in a restricted region of the cell, Ψ_m depolarised, as determined by a loss of fluorescence of the Ψ_m -sensitive dye tetramethylrhodamine ethyl ester (TMRE), in this region only. This depolarisation did not spread to neighbouring mitochondria over the next 20 min but remained restricted to the region selectively illuminated by UV light.

The InsP_3 -generating agonist carbachol (CCh) evoked cell-wide increases in $[\text{Ca}^{2+}]_i$ that were inhibited by localised Ψ_m depolarisation, both at the site of Ψ_m depolarisation and at distant sites where Ψ_m was unaffected. These results suggest that, in smooth muscle, the localised action of mitochondria can affect Ca^{2+} signalling, not only local to the individual mitochondria but also throughout the cell.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC280

The neuroprotective drug, Valproic acid, reduces arachidonic acid release through phospholipase A2 inhibition

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PLA2 enzymes catalyze the hydrolysis of membrane phospholipids at the sn-2 position to release lysophospholipids and free fatty acids including the essential fatty acid arachidonic acid (AA). The biomedical role of PLA2 enzymes may be attributed to AA production playing an essential role in inflammatory signalling, or through other cell signalling events controlled by the enzyme. AA plays a vital role in normal cellular homeostasis and

in a range of signalling and developmental functions including monoaminergic neurotransmission, brain development and synaptic plasticity, and its metabolites are also important in inflammation responses. Biomedical conditions which have been associated with altered PLA2 activity include cancer, ischemia, trauma and psychiatric disorders such as schizophrenia and bipolar disorder (manic depression). PLA2-catalysed overproduction of AA has also been shown in the brain following seizures, resulting in over-activated neurotransmission. The role of the PLA2 signalling pathway in these disorders remains unclear.

A currently used treatment for epilepsy and bipolar disorder, the neuroprotective valproic acid (VPA), reduces the activity of the PLA2 signalling pathway in the brain by an unknown means. To further understand the role of this drug, we have investigated the cellular targets of VPA using the simple model system *Dictyostelium*. We have screened a mutant library for VPA-resistant colonies, and identified one ablated protein homologous to a poorly characterised human iPLA2 enzyme. Ablation of this protein gives rise to partial VPA resistance in *Dictyostelium*. We have also developed an in vivo assay for PLA2, and have used this to show that VPA inhibits PLA2-catalysed AA release in *Dictyostelium*. Using this assay, we have identified the well-defined structural requirements for PLA2 inhibition. These studies suggest that this VPA-catalysed inhibition may be related to the neuroprotective role of the drug.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC281

The reactivity of caveolin-1b in cells transfected with E-ras

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Our previous studies indicated that the expression of caveolin-1 is suppressed and molecular reaction with caveolin-2 is aberrantly disturbed upon transformation with oncogenic S-ras(Q61K). In the present study, we attempted to investigate the reaction of caveolins in cells transfected with embryonic ras, E-ras. The expression of caveolin-1 and caveolin-2 were less suppressed in E-ras transfected cells as compared to cells transfected with S-ras(Q61K). When cellular plasma membranes were isolated and subjected to fractionation with phase-separating agent, Triton X-114, caveolin-2 was totally retained in hydrophobic phase from cells with transfection or not. Similarly, most of caveolin-1 was retained as caveolin-2 except caveolin-1b. An additional fraction of caveolin-1b was found in peripheral membrane fraction of E-ras transfected cells. This extraordinary presence of caveolin-1b and suppressed expression of caveolin-1 resulted in depleted cholesterol recruitment at caveolae. As shown by ultracentrifugation with sucrose gradients, most of caveolae from E-ras transfected cells appeared in 39-43% of sucrose in contrast with 31-35% of sucrose in BALB/3T3 cells without transfections. The reactivity of kinase molecules at caveolae upon depletion of caveolin-1b was investigated in E-ras transfected cells and embryo. Huang J-W and N-N Chuang. 2006. Shift syndecan-2 from RACK1 to caveolin-2 upon transformation with oncogenic ras. *Biochem Biophys Res Commun* 350:227-232.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC282

Neuron-specific and non-specific pro-death signalling from the NMDA receptor

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The "source specificity" hypothesis of NMDA receptor excitotoxicity states that calcium entering specifically through the NMDAR is toxic, due to coupling of the receptor to calcium dependent neuron-specific death effectors associated with the NMDAR. The alternative "calcium load" hypothesis contends that NMDARs promote death pathways solely due to the amount of calcium entering the cell. Analysis of NMDA receptor-dependent cell death revealed that it relies on both JNK and p38 pathways in neurons. Moreover, disruption of p38 signalling could be achieved by uncoupling the NR2B PDZ-binding domain from downstream proteins by using intracellular peptides mimicking the NR2B PDZ-binding motif. This peptide was found to exert its neuroprotective effects without affecting signalling to synaptic plasticity. However, the peptide had no effect on signalling to JNK, suggesting that NMDAR coupling to JNK-dependent death may be independent of the NMDAR signalling complex. Reconstitution of NR1/NR2B NMDARs in AtT20 cells revealed that dose dependent NMDAR-dependent cell death can be recapitulated in these cells. Unlike in neurons, this cell death was not inhibited by p38 inhibitors or indeed the NR2B PDZ ligand peptide, and was reliant solely on JNK signalling. Thus, pro-death signalling from the NMDAR comprises pathways that rely on the NMDAR signalling complex, and others that are triggered simply by calcium influx, encompassing elements of both hypotheses.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC283

Antinociceptive effects of levetiracetam: results from behavioural and electrophysiological assessments in diabetic mice and cultured rat sensory neurones

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The aim of this study was to assess the possible antinociceptive effects of the novel antiepileptic agent levetiracetam (LEV, ucb L059) using both in vivo behavioural and cellular electrophysiological testings. Antinociceptive efficacy of levetiracetam was assessed in a mice model for painful diabetic neuropathy using

the in vivo nociceptive behavioural "hot-plate test". Subsequent to behavioural testing, electrophysiological measurements were performed on cultured neurones of the rat dorsal root ganglia (DRG) using the whole cell patch-clamp technique.

The hot plate test consisted of placing individual mice (adult male Balb/C) on the hot-plate at 50±0.1 degrees and timing the delay for the first hind paw lift (nociceptive threshold). After obtaining control values, diabetes was induced by injection of streptozocin (200 mg/kg i.p.) and 2 weeks after induction of diabetes (serum glucose ≥400 mg/dL) levetiracetam was administered intraperitoneally and hot-plate tests were repeated. Pain threshold values were determined and analyzed by Kruskal-Wallis One-way Analysis of Variance (ANOVA) followed by a pairwise comparison between vehicle and each LEV treated group using a Dunnett's t-test on the ranked data. LEV (60, 300 and 900 mg/kg) had no significant effect on the nociceptive threshold in normal mouse (n=8 for each dose, P>0.05). There were significant decreases in pain threshold latency in diabetic mice compared to normal healthy group and these were significantly and dose-dependently restored by much lower doses of LEV (20, 100 and 200 mg/kg) in a reversible manner.

We next evaluated the effects of levetiracetam on isolated neonatal rat DRG neurones with the aim of assessing cellular mechanism of its antinociceptive action. Effects of LEV (30, 100 and 300 uM) on membrane potential and action potential (AP) parameters (thresholds, peak amplitude, duration, after potentials, decay time) and input resistance were investigated. The effects of LEV on these parameters was dose dependent; 100 and 300 uM LEV caused hyperpolarization of the resting membrane potential, and increased the mean amplitude of after hyperpolarisations and input resistance (n=7, P<0.05) without significantly effecting other parameters of action potentials. Estimated reversal potentials of LEV-induced hyperpolarizing currents were close to the E(K).

In conclusion; results obtained by cellular biophysical procedures in in vitro experiments and in vivo behavioural tests with agreement lend support to the validation of the promising therapeutic potential of the novel antiepileptic agent LEV in treatment of neuropathic pain.

Levetiracetam was provided by UCB Pharma (Belgium)

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC284

FABPs deliver FAEs signalling to PPARs

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The fatty acid amide family (FAEs), including arachidonylethanolamide (anandamide, AEA), 2-arachidonylethanolamide (2-AG), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are a group of lipids which possess anti-inflammatory and anti-nociceptive properties. Many of the physiological responses to FAEs are thought to be due to their action at the cannabinoid receptors (CBs). Recent data suggest that FAEs can bind to and activate the PPAR (peroxisome-proliferator-activated receptor) family of nuclear receptor transcription factors in vitro and in vivo. Although the effects of FAEs

on PPARs have been partly established, the mechanism by which FAEs are transported within the cells to their molecular targets at both the plasma membrane and within the nucleus remains unclear. Intracellular fatty acid binding proteins (FABPs) serve as intracellular acceptors of fatty acids; FABP3 and FABP7 are the major FABPs located in the brain, one of the major sites of synthesis and physiological action of FAEs. To assess the effects of FABP3 and FABP7 on PPAR-mediated transcriptional activation, CHO cells were transiently transfected with a luciferase reporter construct containing three PPRE (Peroxisome Proliferator Response Element) in combination with FABP3 or 7 and PPAR α , β or γ . FABP3 was found selectively increase (2.2 folds, $P < 0.001$) PPAR α -mediated transcriptional activation while FABP7 was found selectively increase (3.2 folds, $P < 0.001$) the transcriptional activation of PPAR γ . No effects of FABP3 or 7 were observed upon the basal transcription activation of PPAR β . The FAEs, OEA and AEA, were shown to activate PPAR α and PPAR γ respectively. Treatment of cells transfected with PPAR α and FABP3 with 10 μ M OEA caused a significant elevation of reporter gene over and above that obtained with either FABP3 or OEA alone. A similar result was obtained with PPAR γ and FABP7 treated with 10 μ M AEA. The existence of FABP7 not only accelerated but also prolonged the effects of AEA on PPAR γ in a time course assay while FABP3 didn't significantly change the time pattern of OEA's effects on PPAR α . These studies suggest FABP3 and FABP7 may play important but diversified roles in intake, transport and degradation of FAEs in the brain. Further experiments are required to reveal the molecular basis and physiological relevance of the specificity of individual FABPs for different PPARs in the central nervous system.

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PC285

Differential G-protein coupling of relaxin family peptide receptors, RXFP1 and RXFP2, is directed by the final 10 residues of the RXFP1 C-terminus

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Relaxin is a two-chain hormone, structurally similar to insulin, that mediates pleiotropic effects in various physiological systems. The recent discovery of the human gene 2 (H2) relaxin receptor, RXFP1 (Hsu et al., 2002), and the related insulin-like peptide 3 (INSL3) receptor, RXFP2 (Kumagai et al., 2002), identified two G-protein-coupled receptors that cause cAMP accumulation. We recently identified differential G-protein coupling of these receptors to cAMP: both receptors couple to G α s and G α oB, which increase and decrease cAMP accumulation respectively (Halls et al., 2006); but only RXFP1 recruits G α i3 with time to further increase cAMP via a G β γ -PI3K-PKC ζ pathway (Halls et al., 2006; Nguyen et al., 2005). This study examined the mechanism of differential G-protein coupling using an α -screen cAMP accumulation assay. C-terminal tail truncates

were generated for both RXFP1 (tRXFP1-703) and RXFP2 (tRXFP2-712). cAMP accumulation characteristics of tRXFP2-712 did not differ from RXFP2 (30min INSL3 (30nM) response as % forskolin (100 μ M) (n): RXFP2 5.2 $\pm$ 1.9(4), pertussis toxin (PTX) 11.2 $\pm$ 2.3(4), $P < 0.01$; tRXFP2-712 4.5 $\pm$ 0.8(6), PTX 9.8 $\pm$ 0.5(5), $P < 0.001$). However, tRXFP1-703 displayed lost coupling to G α i3 and the G β γ -PI3K-PKC ζ pathway, instead becoming 'RXFP2-like' (30min H2 relaxin (30nM) response as % forskolin (100 μ M) (n): RXFP1 20.5 $\pm$ 2.8(5), PTX 10.9 $\pm$ 1.7(6), $P < 0.001$; tRXFP1-703 6.4 $\pm$ 1.6(9), PTX 10.8 $\pm$ 0.4(8), $P < 0.001$). Similarly, truncation of the final 10 C-terminal amino acids of RXFP1 (tRXFP1-747) also switched behaviour (tRXFP1-747 9.9 $\pm$ 3.3(5), PTX 20.6 $\pm$ 4.8(5), $P < 0.01$). Finally, the production of RXFP1/RXFP2 chimeras with exchanged receptor transmembrane regions caused a loss of the delayed pathway signalling for RXFP1 (RXFP1/2 6.8 $\pm$ 1.8(5), PTX 13.2 $\pm$ 2.8(6), $P < 0.01$), but a gain of this pathway for RXFP2 (RXFP2/1 17.6 $\pm$ 5.3(7), PTX 9.0 $\pm$ 1.6(7), $P < 0.01$). Thus the final 10 residues of the C-terminal tail of RXFP1 enable coupling to the G β γ -PI3K-PKC ζ pathway to cause the delayed phase of cAMP accumulation.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC286

Pasteurella multocida toxin (PMT) modifies G-protein coupled receptor evoked calcium signalling in NG108-15 cells

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PMT has been shown to mediate activation followed by inhibition of the phospholipase C coupled G-proteins Gq (not G11), G12 and G13 (Orth et al, 2005). This study investigated PMTs effects on the resting [Ca]²⁺_i, P2Y₂R and M1 mAChR mobilisation of [Ca]²⁺_i in NG108-15 cells.

Differentiated cells were loaded with Indo-1 AM (5 μ M, 1 h), the dye was excited at 360 nm and emission wavelengths recorded at 405 and 488 nm by photomultiplier tubes (Robbins et al, 1992), calibration was performed as detail earlier (Hayat et al, 2003). P2Y₂R mediate calcium increases were evoked by applying uridine triphosphate (UTP, 1-100 μ M) in the presence of CdCl₂ (100 μ M) to block voltage gated calcium entry. The concentration-dependence (10-500 ng ml⁻¹ for 18-24 h) of PMT was complex. A significant increase in resting [Ca]²⁺_i was seen at 100 and 500 ng ml⁻¹ PMT, however the amplification of the UTP evoked

responses required 500 ng ml⁻¹. PMT (500 ng ml⁻¹) was time dependent in its actions. The first effect (3-4 h) was a significant increase in resting [Ca]_i from 158 ± 6 nM to 315 ± 22 nM, (n=5). Followed (12-18 h) by an amplification of the UTP evoked [Ca]_i increase from 365 ± 8 nM to 1504 ± 37 nM (n=5). Finally (42-48 h) a complete block of UTP mediated responses. The EC₅₀ of the UTP concentration response curve was not significantly altered at any time point.

As the change in resting [Ca]_i preceded the amplification of the UTP responses, both in terms of concentration and time, we investigated whether the increase in resting [Ca]_i was responsible for the amplification of the UTP evoke responses. Resting [Ca]_i was significantly lower in cells preincubated in BAPTA-AM (250 μM, 30 min). The resting [Ca]_i was reduced in control cells from 158 ± 6 nM to 45 ± 5 nM (n=5) similarly in PMT treated cells the resting [Ca]_i decreased from 316 ± 21 to 39 ± 10 nM (n=5). UTP responses were much reduced in the BAPTA-AM treated cells however the amplification of the calcium signal in the PMT treated cells was still evident.

In NG108-15 cells transformed to express M1 mAChRs (Robbins et al, 1993) PMT amplified oxotremorine-M (0.1-10 μM) evoked [Ca]_i signals without significantly altering the EC₅₀, similar to that seen for P2Y₂ receptors. This amplification was not seen when cells were pretreated with the PMT^(C1165S) mutant which has lost its toxicity and mitogenicity (Baldwin et al, 2003). These results suggest that P2Y₂ and M1 mACh receptors in NG108-15 cells mediate PI turnover and intracellular calcium release by coupling to Gq rather than G11. PMT increases the efficacy of the signalling pathway without a change in the affinity not requiring an increase in the resting [Ca]_i.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC287

Mitochondria and reactive oxygen species are the main players in heavy metal action on hepatoma AS-30D cells

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The mechanisms of toxic effects of divalent cations of three heavy metals Hg, Cu and Cd in rat ascites hepatoma AS-30D cells cultivated in vitro were studied. In a recent publication (Belyaeva

EA et al (2006). Biochim Biophys Acta 1757, 1568-1574), we have shown that in these cells, Cd2+ induces cell death, both necrotic and apoptotic, that is accompanied by increased generation of reactive oxygen species (ROS), most likely at the level of complex III of the respiratory chain, and opening of mitochondrial permeability transition pore. In the present investigation, we compared the effects of Cd2+ with those of Hg2+ and Cu2+ using the same cells. Changes in intracellular ROS formation and mitochondrial dysfunction were found to be the key events in heavy metal action on AS-30D cells. An increase of intracellular ROS production was an early and sustained phenomenon observed after Cu2+ treatment that was not followed by the dissipation of mitochondrial transmembrane potential. In the case of Hg2+ and Cd2+, ROS generation changes (an increase or decrease compared to control) were dose- and time-dependent as well; however, they were accompanied by the loss of the mitochondrial transmembrane potential. Among the metals tested, the most toxic was Hg2+, which induced both necrosis and apoptosis in a time- and dose-dependent fashion, whereas Cu2+ was weakly toxic and under the experimental conditions did not induce apoptotic death. We showed that cellular respiratory capacity was disturbed by all these heavy metals. In particular, Cu2+ induced stimulation of steady-state respiration rate, whereas Cd2+ decreased steady-state and CCCP-uncoupled cell respiration. High Hg2+ produced practically complete inhibition of the cellular respiration already at early stage of incubation. The data agree well with our observations on isolated mitochondria, where all these heavy metal ions produced severe changes of mitochondrial function manifested, though in different ways, in respiratory disturbance, pyridine nucleotide oxidation, changes of mitochondrial transmembrane potential and ROS production, mitochondrial swelling and K⁺ or/and Ca2⁺ release.

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PC288

Light-dependent mitochondrial activation independent of TRP and TRPL channels in Drosophila photoreceptors

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The fly phototransduction cascade is the archetype of a G-protein coupled signalling pathway. It is also the fastest known biochemical transduction cascade linking rhodopsin, a heterotrimeric G-protein, and the PLCβ with TRP and TRPL channels. In addition to many molecular and structural features,

which allow these rates in the first place, there is also a very important issue of metabolic support and its activation, which allows the photoreceptors to function at such high rates. Although insect photoreceptors are a good model for studying stimulus-dependent mitochondrial activation, the actual intracellular signal involved remained elusive. In a typical cell this normally occurs by activation of mitochondrial F_0F_1 ATP-ase following an increase in $[ADP]_i$. It has however previously been shown that, at least in one insect species – the honeybee, mitochondrial activation precedes the activity of the most obvious ADP source – the Na^+/K^+ ATP-ase (1). By monitoring the flavoprotein redox state time course in *Drosophila* photoreceptors using the flavoprotein autofluorescence we found evidence that mitochondrial activation can occur following activation of PLC β alone without any membrane depolarisation. The mitochondrial activation, detected by transient increase in flavoprotein fluorescence, occurred in the wild type strain as well as in blind *trp;trpl* flies with no functional light activated channels. It was absent in *norpA^{P24}* strain flies with no functional PLC β . This result shows that the mitochondria can be activated downstream of PLC β , without the involvement of light activated channels. Whereas the physiological significance of this finding in *Drosophila* still remains to be elucidated, it has important implications for understanding many other G-protein coupled receptor signalling systems involving PLC β , TRP type channels and mitochondria.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC289

Functional expression of the adenosine A_1 and κ -opioid receptor in myocardial H9c2 cells

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The rat cardiomyoblast cell line H9c2 is widely used as an in-vitro model of cardiac muscle for studies investigating cardioprotection (Kakinuma et al. 2005). G-protein coupled receptors including Gi/o-protein linked adenosine A_1/A_3 and δ/κ -opioid receptors play a major role in cardioprotection (Gross, 2003; Mubagwa & Flameng, 2000). However, the functional expression of adenosine A_1/A_3 and δ/κ -opioid receptors on H9c2 cells has not been reported. The aim of this study was to determine whether these receptors are expressed in these cells. Functional coupling to Gi/o-protein was assessed by monitoring the ability of selective agonists to inhibit forskolin-stimulated accumulation of $[^3H]$ -cAMP in cells pre-labelled with $[^3H]$ -adenine (Scrivens & Dickenson, 2005). The selective adenosine A_1 receptor agonist CPA induced a modest inhibition of 10 μ M forskolin-stimulated $[^3H]$ -cAMP accumulation in H9c2 cells. Interestingly, the concentration-response curve for CPA-induced inhibition of forskolin-stimulated $[^3H]$ -cAMP accumulation was biphasic. Lower concentrations of CPA (maximal inhibition occurring at 100 nM; $22 \pm 4\%$; $n=5$) inhibited cAMP accumulation induced

by 10 μ M forskolin, with an approximate pIC₅₀ of 8.8 ± 0.7 (2 nM; $n=5$). At 1 μ M CPA no inhibition of forskolin-stimulated $[^3H]$ -cAMP accumulation was observed ($114 \pm 11\%$ of the forskolin alone response = 100%) which may suggest coupling of the adenosine A_1 receptor to Gs-protein (Baker & Hill, 2007). Pre-treatment with pertussis toxin (Gi/o-protein blocker; 100 ng/ml for 16 h) completely abolished 100 nM CPA-mediated inhibition of forskolin-stimulated $[^3H]$ -cAMP accumulation ($119 \pm 4\%$ of forskolin response in presence of pertussis toxin = 100%; $n=6$). These data suggest functional expression of the adenosine A_1 receptor to Gi/o-protein (and possibly Gs protein) in H9c2 cells. In contrast the adenosine A_3 receptor agonist Cl-IB-MECA (up to 1 μ M) had no significant effect on forskolin-stimulated $[^3H]$ -cAMP accumulation. However, 10 μ M Cl-IB-MECA induced a significant augmentation of forskolin-stimulated $[^3H]$ -cAMP accumulation ($176 \pm 10\%$ compared to forskolin response alone = 100%; $n=8$). Interestingly, 10 μ M Cl-IB-MECA-mediated augmentation of forskolin-stimulated $[^3H]$ -cAMP accumulation was reduced by the adenosine A_1 receptor antagonist DPCPX (10 μ M; $108 \pm 7\%$ compared to forskolin alone = 100%). The selective κ -opioid receptor agonist (-)-U-50488 (100 nM) induced a robust inhibition of 1 μ M forskolin-stimulated $[^3H]$ -cAMP accumulation ($61 \pm 16\%$ inhibition; $n=3$), whereas the δ -opioid receptor agonist SNC 80 (100 nM) had no significant effect. In summary, these data have shown that H9c2 cells express functional adenosine A_1 and κ -opioid receptors.

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PC290

Stimulation of ionotropic GABA_C receptors activate cAMP-dependent protein kinase via A-kinase anchoring protein 220

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Recent studies suggest that stimulation of ionotropic receptors regulate kinase activity (Yang et al. 2004; Dajas-Bailador et al. 2002). We previously reported that stimulation of GABA_C receptors suppressed neuronal apoptosis through activation of cAMP-dependent protein kinase A (PKA) (Yang et al. 2003). PKA is a broad specificity serine/threonine kinase whose fidelity in signaling is maintained through interactions with A kinase anchoring proteins (AKAPs) (Michel & Scott, 2002). To address the mechanisms underlying such signal transduction from ionotropic GABA_C receptor stimulation to PKA activation, the

potential involvement of AKAPs was examined. Specific antibodies raised against GABA_C receptor ρ_{1-3} subunits precipitated the appropriate ρ subunit, together with AKAP220, and PKA regulatory and catalytic subunits from rat hippocampal tissue lysates. The GABA_C receptor agonist, cis-4-aminocrotonic acid (CACA), activated PKA, as detected by an increased level of phospho-PKA substrate at about 135 kDa in cultured rat hippocampal neurons. This increase was blocked by the specific GABA_C receptor antagonist, (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA) and the PKA inhibitors, KT5720 and H-89. Antisense oligonucleotides for AKAP220 suppressed the GABA_C-induced increase in phospho-PKA substrate 135 kDa. In contrast to the GABA_C-induced selective phosphorylation, a membrane-permeable cAMP, pCPT-cAMP, caused phosphorylation of PKA substrates at 135 kDa, 100 kDa and 75 kDa. These findings suggest that GABA_C receptor stimulation activates PKA to phosphorylate specific substrate(s) via AKAP220.

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PC291

A rapid and non radioactive method for quantification of PtdIns(3,4,5)P₃ levels in cell extracts

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Phosphoinositides (PIs) play a fundamental role as signalling molecules in various cellular processes. PtdIns(3,4,5)P₃, in particular, is a starting point of important signalling cascades such as PKB/Akt, PKC and PLC γ pathways. Increased PtdIns(3,4,5)P₃ levels are observed in various pathophysiological conditions such as cancer, chronic inflammation and allergy. Inhibition of PI3K activity is thus a promising target for pharmacotherapy. Direct analysis of PIs is traditionally accomplished by labelling with ³H myo-inositol or ³²P_i, and subsequent chromatographic separation and scintillation. These assays are time consuming and expensive, and thus not suited for the fast analysis of large sample numbers.

To overcome these limitations we established a rapid LC-MS assay for the separation and quantitative analysis of PtdIns(3,4,5)P₃. The separation of PtdIns(3,4,5)P₃, PtdInsP₂, PtdInsP and PtdIns was achieved in approx. 3 min by ion pair chromatography on a short (2mm x 75 mm) RP-column. As the lipid moieties play no essential role for the biological functions of PIs, analysis of deacylated PIs was preferred. Cells were extracted with chloroform/ methanol/ aqueous HCl according

to the standard procedure, followed by deacylation of the phospholipids and LC-MS analysis. HEK 239 cells and neutrophils from whole blood were used to study the changes in PtdIns(3,4,5)P₃ levels obtained upon treatment with different plant substances and extracts.

Due to the speed of the assay, it can be integrated into a screening for modulators of PI3K activity.

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PC292

Metabolic stress is not likely to be a significant contributor to physiological TRP channel activation in blowfly photoreceptors

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In blowflies and fruitflies TRP channels can be activated by metabolic stress induced either by hypoxia or mitochondrial uncoupling (1). This has been observed either as changes in photoreceptor membrane potential, membrane current or as changes in extracellular ionic composition. Initial implicit idea that a specific signalling pathway was responsible for TRP channel activation was later rejected by findings that the activation was caused by impairment of PIP₂ metabolism due to the lack of ATP, which in turn resulted in continuous activation of the TRP channels (2). Even though the mechanism of channel activation due to metabolic stress is elucidated, the question, which remained unanswered, was whether the photoreceptor cells ever encounter the degree of metabolic stress needed to activate the TRP channels under physiologic conditions. To assess this we measured the degree of mitochondrial impairment due to the level of hypoxia needed to induce the changes of extracellular ionic composition. Measurements of the redox states of respiratory pigments (flavoproteins and cytochromes a, a₃, b and c) in the eyes of blowflies (*Calliphora vicina*) were done with time-resolved absorption spectroscopy in combination with a principal components analysis-based spectral deconvolution (3). The extracellular concentrations of K⁺, Na⁺ and Ca²⁺ ions ([K⁺]_o, [Na⁺]_o and [Ca²⁺]_o) were measured using the ion-selective microelectrodes. After the onset of anoxic conditions, established around a fly in under 2 s, the changes in [K⁺]_o, [Na⁺]_o and [Ca²⁺]_o started to appear with the latency of 8.06 ± 1.38 s, 10.38 ± 2.48 s and 13.11 ± 3.09 s, respectively (mean ± s.e.m., n=7 if not stated otherwise). On the other hand the first detectable changes in the respiratory pigments redox states, those of cytochrome c, started at 1.79 ± 0.19 s. Moreover the changes of cytochrome c reached 39.4% ± 5.4 % of the maximal reduction by the time changes in ionic composition were even detectable. Such values are well outside the normal physiological range that occurs during intense illu-

mination, which is $7.4 \pm 0.2\%$ ($n=15$) and can only be observed when environmental P_{O_2} falls below 20 hPa. From this we conclude that under physiological conditions metabolic stress is not likely to be a significant contributor to TRP channel activation.

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PC293

Generation and characterisation of PtdIns(3,4)P2 transgenic bioprobe to explore spatio-temporal regulation of PtdIns(3,4)P2 accumulation in T lymphocytes

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PI 3-kinases have been implicated in a wide range of cellular functions in T lymphocytes by experiments employing pharmacologic or genetic approaches to oppose the lipid kinase catalytic function. These experiments however, do not yield data that distinguish between lipid products of PI 3-kinase. Although the major lipid product of PI 3-kinases is PtdIns(3,4,5)P3, levels of PtdIns(3,4)P2 also rise within T lymphocytes following cellular activation [1]. The pleckstrin homology domains of some proteins can bind these lipids separately, for example the tandem PH domain protein (TAPP) 1 will bind PtdIns(3,4)P2 but not PtdIns(3,4,5)P3 [2]. This observation suggests that there may be distinct functions for different phosphoinositides. To explore the biology of PtdIns(3,4)P2 we have fused the TAPP1 PH domain to fluorescent reporters such as GFP and RFP to create a lipid specific bioprobes. We have expressed RFP-TAPP in T cell lines and human primary CD4+ T cells to image the distribution of PtdIns(3,4)P2 within cells, responding to different activation stimuli. Further we have placed RFP-TAPP under the control of the mouse Vav promoter to create transgenic mice expressing RFP-TAPP in their T cell compartment. These studies should enable non-invasive, real-time imaging of PtdIns(3,4)P2 in live cells responding to physiological stimuli.

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PC294

The relationship between noradrenergic Ca^{2+} waves and excitatory junction potentials in the rat anococcygeus

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The rat anococcygeus muscle is densely innervated by sympathetic nerves. When electrically stimulated, these nerves release noradrenaline, producing a biphasic excitatory junction potential (EJP) and contraction (Creed *et al.*, 1975). The first component of the EJP is a depolarisation, thought to be produced by the effect of increased free cytosolic $[Ca^{2+}]$ on Ca^{2+} -activated Cl^- channels (Bramich & Hirst, 1999). Noradrenergic transmission in intact tissue produces intracellular Ca^{2+} transients in individual smooth muscle cells in the form of waves and synchronised whole cell flashes (Amos *et al.*, 2005). However, the relationship between the EJP and the different Ca^{2+} transients, and the extent to which local Ca^{2+} increase affects the local membrane potential, is unknown. To explore this relationship, the anococcygeus was isolated from male Wistar rats (200 - 400 g) and smooth muscle membrane potentials were measured using sharp microelectrodes, while intracellular Ca^{2+} was monitored simultaneously using a confocal microscope and the Ca^{2+} indicator Oregon Green 488 BAPTA-1. The indicator was delivered to individual smooth muscle cells through a microelectrode as a membrane impermeable salt. Dye coupling between adjacent smooth muscle cells was not observed with this method. In 9 cells (from 5 preparations) studied with this method, EJPs could be elicited with a distinct first component, but only 6 of these showed Ca^{2+} waves (trains of 1 - 6 pulses, 1 - 10 Hz, 0.2 ms pulse width). Where waves initiated within the field of view, the initiation times of the rise in Ca^{2+} and the first component of depolarisation were well correlated (Spearman's rank correlation coefficient +0.717, $P < 0.01$). There was however no correlation between the peak amplitude of the first component of the EJP and that of the point of initiation of the Ca^{2+} increase. The threshold (frequency of stimulation, number of pulses in a train) for generating the first component and waves varied; varying the stimulation frequency or number of pulses, 6 of these 9 cells exhibited first components of depolarisation at thresholds where waves were not observed, while 2 displayed waves without the first component and in 1 cell, waves and first components appeared at the same threshold. These results suggest that the first component of the EJP in the intact organ is not the result of local Ca^{2+} waves within one smooth muscle cell. Since the input resistance of anococcygeus muscle is only 18 - 29 M Ω (Creed *et al.*, 1975), the EJP is instead likely to reflect Ca^{2+} and electrical events in a population of electrically-coupled smooth muscle cells.

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PC295

Fatty acid amide hydrolase (FAAH) inhibitor URB597 activates peroxisome proliferator-activated receptor gamma

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The fatty acid ethanolamide family (FAEs) are signalling lipids with anti-nociceptive and anti-inflammatory properties whose physiological concentration is mainly regulated in vivo by the Fatty Acid Amide Hydrolase (FAAH). URB597 is a potent and selective inhibitor of this enzyme. FAEs are thought to act primarily via the cannabinoid receptors CB1 and CB2, but recent evidence suggests that the biological effects of these compounds may involve acting as ligands for the Peroxisome Proliferator-Activated receptors (PPARs). PPARs, of which there are three isotypes (α , β and γ), are members of the nuclear receptor family of ligand-activated transcription factors. The α and γ isotypes have been reported to have anti-inflammatory and neuroprotective functions in addition to their well characterized role in intermediary metabolism. In this study we used the potent and selective FAAH inhibitor URB597 to determine whether intracellular elevation of FAE levels induced by blocking FAAH-mediated FAE metabolism would lead to activation of PPAR α and γ . A Peroxisome Proliferator Responsive Element (3XPPRE)-Luciferase construct was transfected either alone, or in combination with expression constructs for either PPAR α or γ into SH-SY5Y human neuroblastoma cells which are FAAH positive or HeLa cells which have no significant FAAH activity. In HeLa cells, luciferase activity from the PPRE-driven reporter was not altered by treatment with URB597 (10 μ M), the PPAR α agonist WY1463 (10 μ M) or the PPAR γ agonist Rosiglitazone (10 μ M). The lack of effect of either WY1463 or Rosiglitazone indicated that HeLa cells do not express either functional PPAR α or PPAR γ . In SH-SY5Y cells, luciferase activity was elevated from 0.903 $\pm$ 0.310 to 1.663 $\pm$ 0.070 (P <0.05, one-way ANOVA followed by Bonferroni's Post Hoc test) after treatment with 10 μ M URB597. 10 μ M Rosiglitazone significantly increased luciferase activity to 3.390 $\pm$ 0.305 (P <0.001) whilst 10 μ M WY1463 treatment had no effect. These data suggest that URB597 is capable of activating the PPRE-luciferase reporter possibly acting via PPAR γ in SH-SY5Y cells. We then tested the effects of URB597 on PPRE-Luciferase activation in HeLa cells transiently co-transfected with constructs expressing either Human PPAR α or PPAR γ . 10 μ M URB597 treatment failed to increase luciferase activity in cells co-transfected with PPAR α , whereas in cells co-transfected with PPAR γ , luciferase activity was increased from 5.206 $\pm$ 0.373 to 9.546 $\pm$ 0.479 (P <0.001) by 10mM URB597 and 11.700 $\pm$ 1.060 (P <0.001) by 10 μ M Rosiglitazone. These results indicate that URB597 can induce PPAR γ but not PPAR α activation in cells lacking discernable FAAH enzyme activity. Further investigations are required in order to assess whether URB597 is a ligand for PPAR γ or if it acts via an indirect mechanism.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC296

G protein-coupled receptor kinase 2 selectively regulates endogenous histamine H₁ receptor signalling in myometrial smooth muscle cellsJ.M. Willets¹, A.H. Taylor¹, H. Shaw², J.C. Konje¹ and J. Challiss²¹*Reproductive Sciences Group, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK and*²*Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, UK*

The human uterus contains large numbers of mast cells located in close proximity to the myometrial smooth muscle cells (Sivridis et al., 2001), suggesting that they may be a target tissue. Mast cells release a wide variety of inflammatory mediators such as histamine, which causes weak uterine contractions (Cruz et al., 1989). We have previously demonstrated that histamine stimulated Ca²⁺ signalling in ULTR cells, an immortalized human uterine smooth muscle cell line, occurs via the H₁ receptor (Willets et al., 2007). In this study we have used confocal imaging of the fluorescent biosensor eGFP-PH_{PLC δ} (Willets et al., 2005) to assess the regulation of H₁ histamine receptor phospholipase C (PLC)-stimulated signalling in ULTR cells.

Histamine (1 μ M to 1mM) produced rapid but transient, concentration-dependent translocations of eGFP-PH_{PLC δ} from the plasma membrane to the cytoplasm, indicative of IP₃ production. To quantify H₁ receptor desensitization ULTR cells were stimulated with an approximate EC₅₀ concentration of histamine (10 μ M, for 30 sec) before (R1) and after (R2) exposure to maximal agonist concentration (Rmax, 100 μ M) for 60 sec, with 5 min washes between challenges. This protocol produced a significant attenuation of the R2 response compared to R1 (34 $\pm$ 6%, n = 7; means $\pm$ SEM, p <0.01; one-way ANOVA/Bonferroni's multiple comparison test), indicative of receptor desensitization. R2 responses returned to the R1 level when cells were washed for 15 min after maximal agonist challenge. To determine whether G protein-coupled receptor kinases were involved in H₁ receptor desensitization, ULTR cells were transfected with siRNA (10 nM) against GRK2 (5'-GGCAGCCAGU-GACCAAAAtt-3'), GRK6 (5'-GGACACAAAAGGAU-CAAGtt-3'), or a negative control siRNA (Ambion, UK) for 48 h. Endogenous GRK2 and GRK6 expression was reduced by (80 $\pm$ 2 %, n = 4 and 83 $\pm$ 5 % n = 4, respectively), while the negative control siRNA had no effect. Using the R1/Rmax/R2 protocol, siRNA-mediated suppression of GRK2 almost completely prevented H₁ receptor desensitization, whilst GRK6 depletion was ineffective.

Further studies showed that siRNA-mediated GRK2 depletion did not affect the desensitization of endogenously expressed B₂ bradykinin and type 1 angiotensin II receptors. These data suggest that endogenous GRK2 may play an important role in the regulation of H₁ histamine receptor signalling in human uterine smooth muscle.

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PC297

Role of transcription factor Nrf2 in arsenite-induced upregulation of HO-1 expression

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Inorganic arsenic is an environmental contaminant and a known human carcinogen. Arsenite (trivalent) is thiol-reactive and more toxic than arsenate (pentavalent). Low levels of arsenite cause oxidative stress in cells and evoke cellular defense responses that lead to the up-regulation of stress-response proteins such as heme oxygenase-1 (HO-1) and the molecular chaperone Hsp70. HO-1 expression is strongly induced in response to a variety of agents in addition to arsenic. Previous studies have implicated the transcriptional enhancers Nrf2 (1), AP-1, and HSF1 in the arsenite-induced murine ho-1 gene activation, although their signaling pathways and relative contributions have not been fully elucidated. We used wild-type and Nrf2-deficient murine embryonic fibroblasts to investigate the induction of HO-1 in response to low levels of arsenite. We found that Nrf2^{-/-} cells are more sensitive than Nrf2^{+/+} cells to arsenite toxicity, and that Nrf2 plays a crucial role in the early activation of ho-1 transcription. In Nrf2^{-/-} cells, ho-1 gene activation, mainly mediated via AP-1 and HSF1, increased slowly, and during hours 4-12, the HO-1 protein levels were only half of those attained in the Nrf2^{+/+} cells. Our results suggest that genistein-sensitive tyrosine kinase and JNK activities play significant roles in the AP-1 signaling pathway, but not in the Nrf2 or HSF1 activation pathways. Inhibitors of ERK1/2, p38MAPK, and PKC had negligible effects on the arsenite-induced HO-1 expression levels. Our findings provide the first evidence that Nrf2 plays an important role in early HO-1 induction and in protecting the cells from arsenite toxicity, and that the Nrf2-independent pathway contributes to long-lasting activation of the ho-1 gene.

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PC298

Insulin and Dexamethasone depolarize hormone deprived H441 human airway epithelial cells by different molecular mechanisms

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The mechanisms underlying the hormonal regulation of human airway epithelial Na⁺ and Cl⁻ conductance (G_{Na} and G_{Cl} respectively) were explored by recording membrane currents from H441 cells using the perforated patch recording configuration (see Clunes *et al.*, 2004). Studies of cells maintained in hormone-free medium (prepared using dialysed serum), showed that the resting membrane potential (V_m) was essentially identical to E_K

(-82 mV) and was unaffected by reducing [Na⁺]_o to 10 mM (NMDG⁺ replacement; control: -83.8 ± 7.1 mV; low Na⁺: -91.4 ± 6.6 mV, n = 5, mean ± s.e.m.) or lowering [Cl⁻]_o to 29 mM (gluconate replacement; control: -79.9 ± 2.3 mV; low Cl⁻: -78.9 ± 2.4 mV; n = 5). Raising [K⁺]_o to 134.5 mM (Na⁺ substitution) caused depolarization (control: -80.1 ± 4.3 mV; high K⁺: -21.8 ± 2.3 mV; n = 8; P < 0.001; Student's paired t test). G_{Na} and G_{Cl} are therefore negligible under these conditions and the electrical properties of hormone-deprived H441 cells are dominated by G_K. Studies of cells maintained (~24 h) in medium supplemented with dexamethasone (0.2 μM) or insulin (20 nM) showed that both hormones depolarized V_m by ~20 mV (P < 0.001 for both). As anticipated (Clunes *et al.* 2004), lowering [Na⁺]_o to 10 mM hyperpolarized dexamethasone treated cells (control: -60.0 ± 2.4 mV; low Na⁺: -106.2 ± 6.4 mV) confirming that this glucocorticoid depolarizes V_m by increasing G_{Na}. Lowering [Cl⁻]_o had no effect V_m (control: -65.9 ± 3.8 mV; low Cl⁻: -58.0 ± 6.1 mV; n = 4) indicating that dexamethasone does not control G_{Cl}. However, lowering [Na⁺]_o had no effect upon insulin-treated cells (control: 63.3 ± 5.0 mV; low Na⁺: -62.4 ± 4.5 mV) whilst reducing [Cl⁻]_o caused further depolarization (control: -57.6 ± 2.2 mV; low Cl⁻: -39.9 ± 2.5 mV; n = 5; P < 0.001); the depolarizing response to insulin thus reflects an increase in G_{Cl}. Subsequent studies showed that the response to dexamethasone was mimicked by transient expression of constitutively active serum and glucocorticoid-regulated kinase 1 (SGK1) whilst the response to insulin was reproduced by an active form of the phosphatidylinositol trisphosphate-dependent kinase (PI3K). Insulin and dexamethasone thus appear to depolarize H441 cells by evoking SGK1-dependent control over G_{Na} and and PI3K-dependent control over G_{Cl} respectively. Moreover, although PI3K-dependent control of ENaC is well documented (e.g. Tong *et al.*, 2004) the present data indicate that, at least in hormone-deprived H441 cells, SGK1 can increase G_{Cl} without affecting G_{Na}. Clunes, MT, Butt, AG & Wilson, S.M. (2004). *J. Physiol.* 557, 809-819.

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PC299

Mechanism of arachidonic acid action on syntaxin/Munc18

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The ubiquitous process of membrane fusion in eukaryotes is executed by a host of essential conserved factors, including membrane associated SNARE proteins such as syntaxins. SNARE complex formation brings membranes into close apposition for fusion. The lipid metabolism of the surrounding bilayer, and highly conserved cytosolic components such as Munc18, are also important.

The interaction of Munc18 with syntaxin is essential for exocytosis. Knock-out of Munc18 results in dramatically decreased secretion (Verhage *et al.*, 2000) yet paradoxically, initial in vitro experiments suggested that the syntaxin/Munc18 interaction is

incompatible with SNARE assembly. It was recently found that Munc18 inhibition of neuronal syntaxin1 can be overcome by arachidonic acid (Rickman and Davletov, 2005), suggesting that this common second messenger disrupts syntaxin/Munc18 association.

We now show that arachidonic acid can stimulate syntaxin1 in the absence of Munc18 (Figure 1). Although arachidonic acid relieves inhibition of Munc18-bound syntaxin (Fig2A), it cannot dissociate this syntaxin1/Munc18 complex (Fig2B). Upon arachidonic acid-induced syntaxin1/SNAP25 association, Munc18 remains attached to syntaxin1, and a Munc18/syntaxin/SNAP25 complex is formed (Fig2C). We show for the first time that neuronal SNAREs can be isolated from brain membranes assembled with endogenous Munc18 (Fig2D,E) consistent with Munc18's role in downstream vesicle docking and fusion events (Toonen et al, 2006).

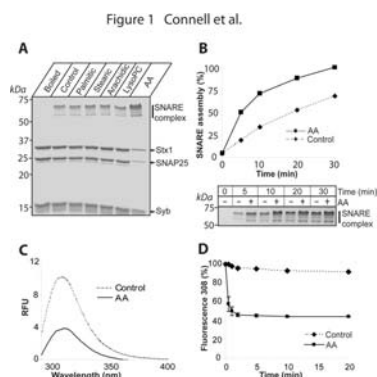


Figure 1 Connell et al.

Fig1. Action of arachidonic acid (AA) on syntaxin1 (Stx1). (A) SNARE reactions in the presence of 100 μ M saturated palmitic (C16:0), stearic (C18:0), arachidic (C20:0) acids, brain lysophosphatidylcholine (LysoPC) and unsaturated AA (20:4). Coomassie-stained gel. (B) AA-induced acceleration of SNARE assembly. (C), (D) Rapid AA-triggered change in syntaxin1 intrinsic fluorescence (excitation 280 nm).

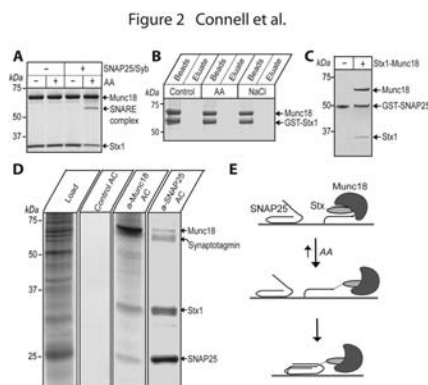


Figure 2 Connell et al.

Fig2. Interaction of native Munc18 with syntaxin1 persists following arachidonic acid-induced SNAP25 engagement. Coomassie-stained gels. (A) AA allows SNARE assembly with syntaxin1/Munc18. (B) GST-syntaxin1 association with brain Munc18 is not disrupted by 100 μ M AA or 1 M NaCl. (C) Incubation of GST-SNAP25 with syntaxin1/Munc18 binary complex, in the presence of 100 μ M AA, leads to pull-down of both syntaxin1 and Munc18. (D) Munc18 co-purifies with syntaxin1/SNAP25 from brain extract (load) during preparative affinity chromatographies (AC) using anti-Munc18 or anti-SNAP25 BrCN beads. (E) AA allows SNAP25 engagement by syntaxin without Munc18 dissociation.

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We acknowledge Sew-Yeu Peak-Chew for protein identification by mass spectrometry.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC300

Glycan legislation in the human intestine. Microflora control of host mucosal glycosylation

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The regulation of intestinal mucosal barrier glycosylation by the indigenous resident microflora has been proposed. Glycan legislation has been demonstrated in mice (1) and is a major event associated with the initial colonisation of the gut and ensures normal maturation of intestinal glycosylation profiles in addition to intestinal angiogenesis (2, 3). Little experimental evidence has been provided to demonstrate that the same processes are relevant in the human gut. Recent analysis of the glycosylation of the human mucus barrier has identified a library of glycan structures associated with distinct regions of the intestine (4), suggesting a regional glycosylation programme.

We sought to address the glycan legislation hypothesis in man using the mucosa of patients undergoing intestinal diversion surgery. This operation diverts the faecal flow away from the distal colon and provides the opportunity to assess mucosal glycosylation with and without contact to the indigenous microflora. Intestinal mucosa from patients with ulcerative colitis (n=39), Crohn's disease (n=24) and control (n=31) groups, with (n=34) and without (n=50) the diversion operation was collected from the same location in each case. The mucosa was tested for the expression of a series of known mucosal barrier glycosylated antigens. Glycan antigens were detected using a series of antibodies and plant lectins; Tn (antibody and Vicia Villosa lectin), sialyl, Tn, TF antigen (peanut lectin), sialyl Lewis_x, sialyl-Lewis_x, sulphoLewis_x, O-acetylated sialic acids (antibodies PR3A5 and 6G4), poly-N-acetyl-Lactosamine (Maackia amurensis lectin I), alpha1-2 (Ulex europeae lectin) and alpha1-6 linked (Aleuria Aurantia... agglutinin) fucose, alpha2-3 (Maackia Amurensis lectin II) and alpha2-6 (Sambucus Nigra lectin) linked sialic acids and Sda (Datura Bifloris lectin). Mucins MUC1, 2, 3A/B, 4, 5AC, 5B, 6, 7, 8, 11, 12 and 13 were detected with a series of gene specific antibodies. In addition metabolic labelling experiments with [3H]-glucosamine and sodium-[35S]-sulphate were used to monitor glycoprotein biosynthesis.

Specific changes in mucosa from diverted patients were found detected for O-acetylated sialic acids with the 6G4 antibody and for the Sda antigen detected with the Datura bifloris lectin. No other glycan structure was altered. No change in MUC gene product expression was found. The response was related to the diversion operation and not to the normal or disease (UC or CD) profile.

The data suggest that there is a selective and microflora related regulation of mucosal glycosylation corresponding to the glycan legislation hypothesis.

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We wish to thank the Medical Research Council for funding this project.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC301

Functional characterization of Actinobacillus pleuropneumoniae urea transport protein, ApUT

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Urea transporters (UTs) effect rapid flux of urea across biological membranes. In the mammalian kidney, UT activity is essential for effective urine concentration. In bacteria, UT-mediated urea uptake permits intracellular urease to degrade urea to ammonia and CO₂, a process which either buffers acid loads or provides nutrient nitrogen. In many bacterial species, uptake of urea and its metabolism play a critical role in pathogenesis. Although UT's have been identified and cloned from mammals and bacteria, their function has not been well characterized, limiting our ability to determine, when structural information becomes available, the detailed mechanism of UT urea transport. We have characterized the urea transport channel protein ApUT from Actinobacillus pleuropneumoniae. Expression of ApUT in Xenopus oocytes induced a 3 fold increase in 14C urea uptake compared to water injected controls and this response was completely inhibited by phloretin (500µM). Kinetic analysis of inside-out membranes enriched in ApUT showed ~28 fold increase in urea permeability ($3.25 \pm 0.37 \times 10^{-4}$ cm/s) compared to control vesicles ($0.114 \pm 0.023 \times 10^{-4}$ cm/s). Urea transport across the channel was phloretin inhibitable (75 % inhibition), and the site of inhibition may be located on the extracellular side. Glycerol and urea analogues such as methylamine, dimethylurea, formamide, acetamide, methylurea, propanamide and ethylamine did not permeate across ApUT. Furthermore, ApUT did not conduct protons or ammonia. Urea transport through ApUT shows saturable kinetics with a km of 357 mM. In addition to

urea ApUT also conducts water. Furthermore, phloretin inhibition studies suggest that both urea and water permeates through the same pore.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC302

A comparative study of the phytochemical and antimicrobial properties of the leaves and roots ethanolic extracts of Landolphia owariensis

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A comparative study of the ethanolic extract of the leaves and roots of Landolphia owariensis were subjected to phytochemical screening and antimicrobial activities on E. coli, Staphylococcus spp. and Proteus spp. using dehydrogenase assay method. The phytochemical screening indicated the presence of alkaloids, flavonoids, saponins and tannins in both leaf and root extracts with cyanogenic glycosides present in the root extracts. When the bacterial isolates were exposed to both extracts at different concentrations (20-200 µg/ml) in nutrient broth, their response was concentration dependent. In all the bacteria isolates dehydrogenase activity was progressively inhibited at concentration of 20µg/ml - 200µg/ml for the leaf and 150 - 2500 µg/ml for the root extracts respectively, with total inhibition observed from 700 µg/ml for Proteus spp. and 1800 µg/ml for E. coli for the leaves extract while total inhibition was observed from 2000 µg/ml for both Staphylococcus spp. and Proteus spp. and 2480 µg/ml for E. coli. The observed antimicrobial properties was believed to be due to the presence of alkaloids, flavonoids and tannins, which were identified in the leaf and root extracts. The findings may be of clinical relevance and further substantiates the traditional use of L.owariensis leaves and roots as antimicrobial agent.

Table 1: Phytochemical profil of the leaf and root extracts of L. owariensis

plant part of L. owariensis	saponins	Tannins	Alkaloids	Flavonoids	Cyanogenic glycosides
Leaf Root	++	+++	++	++	ND +

Keys: ++ = Highly present

+ = Present

ND = Not present

Table 2: Dehydrogenase activities in the control Bacterial tests for the leaf and root extracts

Test organisms	Dehydrogenase activities mg
Staphylococcus Spp	0.660
proteus spp.	0.509

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PC303

Over-expression studies of caprine growth hormone in *Escherichia coli*

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Growth hormone (GH) gene of a common local caprine breed "Beetal" was expressed in *E. coli* BL21 Codon Plus (DE3) RIPL, using T7-promoter based pET22b expression vector. Expression of GH gene with native base sequence did not produce the GH to any detectable levels as analysed by SDS-PAGE, but substitution of certain bases in the 5' region of the gene resulted in dramatically high level expression of GH. A series of constructs, pETCST 1-19, were made by substituting one, two or three bases in the initial stretch of 24 bases of the coding region. The cells transformed with the constructs pETCST 8, 9, 12 and 13, on induction with IPTG produced GH at levels of over 50% of the total cell proteins. The expression levels were generally seemed to be related to the possibility of secondary structure formation in the 5'-end region of the transcribed mRNA. Quantitative PCR studies showed that the levels of mRNA in the cells transformed with the various constructs were similar, thus substantiating that the differential expression levels occurred at translation level. Expression patterns obtained as a result of auto-induction with lactose were similar to those obtained with IPTG induction. The over-expression results in the formation of inclusion bodies, from which the growth hormone was refolded into biologically active form.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC304

Polar localization of acetate kinase in *Escherichia coli*

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The acetate kinase (AckA) and phosphotransacetylase (Pta) pathway provides a central metabolic route for acetate production

and utilization in many microorganisms. AckA interconverts acetate and acetyl-phosphate within a pathway that affects diverse cellular processes including growth, motility, chemotaxis and biofilm formation. Here we report unipolar and bipolar localization with distinct fluorescent foci of an AckA-DsRed (red fluorescent protein) fusion protein in *Escherichia coli*. The fusion protein rescued growth phenotypes caused by an ackA null mutation, demonstrating that polar localized AckA is functional. Polar localization was not altered by cephalixin treatment, suggesting that AckA foci are physically distinct from methyl-accepting chemotaxis proteins. Silencing of the ackA-pta operon using an expressed antisense RNA system did not alter flagella appearance, but inhibited motility and altered biofilm formation. Polar AckA positioning within bacteria may provide gradients of signalling molecules required for directional cellular responses.

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We thank Kjell Hultenby for help with EM, Mary Berlyn at the Coli Genetic Stock Center for sending strains.

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PC305

Polyamine metabolism - a potential target for natural and synthetic chemoprevention?

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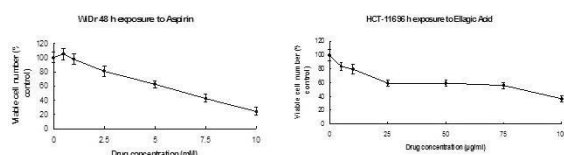
Colorectal cancer remains one of the most common causes of cancer mortality in Western society and currently has a five year survival rate of 50% (Boyle and Ferlay, 2004). Therefore alternative strategies are needed to alleviate the burden being placed in the health-care system. An attractive solution is chemoprevention. Over the last decade there have been a number of natural and synthetic compounds which have been found to have chemopreventative properties, such as non-steroidal anti-inflammatory drugs (NSAIDs) and natural compounds such as flavonoids and curcumin. Ellagitannins are compounds which are found in abundance in many foods but in particular in soft fruits and are naturally metabolised to ellagic acid (EA). The mechanism of action of these compounds is still unclear, however it is possible that they may work through the inhibition of a general cell growth pathway and we hypothesise that the polyamine biosynthesis may be the pathway involved.

Polyamines are natural polycations which are found in almost all cells and at increased concentrations in cancer cells and tissues (Kingsnorth et al 1984). The metabolism of the polyamines is tightly regulated and the key rate-limiting enzyme is ornithine decarboxylase (ODC).

The aims of this study were to (i) determine and compare the cytotoxicity of NSAIDs and EA in a range of colorectal cancer cell lines and (ii) to establish the extent of involvement of polyamine metabolism in the cytotoxic process.

Using 4 colorectal cancer cell lines we established the cytotoxicity of the NSAIDs and EA using MTT (Fig. 1), Trypan Blue exclusion and protein determination were used to monitor cell growth. Polyamine analysis was carried out by HPLC.

Aspirin and EA exposure resulted in dose-dependent cytotoxicity. IC50 values were calculated from MTT assays (Fig.1) of 5.5 mM for aspirin and 75 µg/ml for EA. Cell number, viability and protein content decreased with increasing exposure (results not shown). Analysis of polyamine concentrations showed that the total intracellular polyamine content decreased after exposure to these compounds. There was inhibition of ODC activity and that there was an increase in the catabolism of polyamines, at least in the presence of the NSAIDs. Thus, it seems that, at least, part of the mechanism of cytotoxicity of these compounds is modulation of the polyamine pathway.



Effect of chemopreventative agents on colorectal cancer cells Mean $\pm$ SEM (n=18) EA 96h exposure, aspirin 48 h exposure
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Aberdeen Royal Hopsitals NHS Trust, COST Action 922.

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PC306

Role of some newly synthesized tetrahydronaphthalinthiazol derivatives as anticancer compounds

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Title only.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC307

A natively unfolded proline-rich domain in ASPP2 regulates its protein interactions by intramolecular binding to the Ank-SH3 domains

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ASPP2 is a pro-apoptotic protein that stimulates the p53-mediated apoptotic response (1). The C-terminal Ankyrin and SH3

domains in ASPP2 mediate numerous protein-protein interactions. In addition, ASPP2 contains a proline-rich domain between residues 693-918, whose structure and function are unclear (2). Here we show using biophysical methods that the proline-rich domain of ASPP2 is natively unfolded. CD spectroscopy gave a spectrum typical for a random coil, which did not change upon increasing the temperature. Analytical ultracentrifugation studies showed that the protein is a monomer. However, in analytical gel filtration experiments the protein was eluted earlier than expected for globular protein. The results of different computational predictions of ASPP2 693-918 also revealed that the domain is mostly natively unfolded. Next, we studied the role of this domain in protein-protein interactions. Using peptide arrays bearing overlapping peptides derived from known ASPP2 binding proteins we demonstrate that only the Ank-SH3 domains, but not the proline-rich domain, mediate the interactions of ASPP2 with its partner proteins. Instead, we found using fluorescence spectroscopy and pull-down binding studies that the proline-rich domain makes an intramolecular interaction with the Ank-SH3 domain of ASPP2. We have mapped the precise sites that mediate this intramolecular domain-domain interaction using a second peptide array.

According to our model, the intramolecular interaction between the ASPP2 domains has an important role in regulating the intermolecular interactions of ASPP2 with its partner proteins. When the proline-rich domain is bound to the Ank-SH3 domains, it competitively inhibits their interactions with their partner proteins. Following a yet unknown regulation mechanism, the intramolecular domain-domain interaction is released and the Ank-SH3 domains become available to bind their target proteins. Samuels-Lev, Y., O'Connor, D. J., Bergamaschi, D., Trigianti, G., Hsieh, J. K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell 8, 781-794.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC308

The molecular mechanism of FGFRs in gastrointestinal carcinomas and its application for cancer therapeutics

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The fibroblast growth factor receptor (FGFR) is transmembrane tyrosine kinase receptor responsible for mediating cellular responses to FGFs signaling. They regulate a multitude of cellular processes, including cell growth, differentiation, migration and survival, and have been implicated in pathological processes including angiogenesis, wound healing and tumorigenesis. These receptors are widely expressed in many tissues and different cell types, and the temporal control of their expression is an important mechanism for regulating physiologically relevant signals.

Here we have demonstrated that the gene expression of FGFR1 and FGFR3 in colorectal carcinoma cells is in reciprocal relationship. We have also showed that the disruption of FGFR1 expression by introducing of FGFR1 siRNA was effective in elevating FGFR3 expression and tumor suppressive activities. However, molecular details involved in such a switch are largely unknown. Nevertheless, to our knowledge, Therefore, FGFR1 may confer a selectable advantage on clones of cells in colorectal tumorigenesis, favoring proliferation, whereas FGFR3 may have the effect of an unfavorable negative regulation of progression of the carcinomas to malignancy, promoting differentiation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC309

Structure of the 26S proteasome

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The 26S proteasome is a multi-subunit protein complex which degrades proteins targeted for breakdown by ubiquitin conjugation. It plays a fundamental role by controlling the levels of highly important proteins such as regulators of the cell cycle and apoptosis. It is formed of a proteolytically active 20S core with two 19S regulatory particles (19S-RP) at each end. Despite its crucial importance, the molecular processes for the 19S-RP ubiquitin recognition, unfolding of the target protein and its translocation into the 20S core for degradation are still not fully understood. However this knowledge is required for the full exploitation of the 26S proteasome as a therapeutic target.

There is structural information already available on the 26S proteasome. The structure of the mammalian 20S core has been determined by x-ray crystallography (1) while the structure of the complete 26S proteasome has been studied by electron microscopy and image analysis (2). However, although the structural organisation of the 20S core is well established, it is still not clear how the different protein subunits within the 19S RP are organised, how they recognise and bind the ubiquitinated substrates, unfold the target protein or how they interact with the 20S core.

We have obtained 3D structures of double (two 19S-RP capping the 20S core) and single capped (only one 19S-RP at one end of the 20S core) proteasome complexes, from human erythrocytes, by electron microscopy and single particle image analysis. The crystallographic structure of the 20S core can be docked into the resulting 3D map of the double-capped 26S proteasome by aligning the symmetry axis common to these structures. The correctness of this docking is further reinforced by inspection of the densities corresponding to each 20S core subunit. These subunits are characterised by detailed differences in their 3D structure, which in the 3D map of the double-capped 26S proteasome can be seen to match their corresponding individual shapes. The clear identification of subunits within the 26S proteasome 3D maps also allows the interactions between the 20S and the 19S complexes to be inferred. The comparison of the 3D maps

obtained for the single and double capped forms of the proteasome reveals an opening of the entry channel into the 20S core associated with binding of the 19S-RP, which appears to be achieved by a small displacement to higher radius of each α -subunit resulting in a considerably better agreement between the densities of the 3D map in this region and the crystallographic coordinates of the 20S core. Furthermore, the current 3D maps of the 26S proteasome allow us for the first time to propose likely locations and spatial distributions of a number of the subunits of the 19S-RP.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC310

Promotion of differentiation and apoptosis by militarinone A

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Neurodegenerative diseases are a major challenge for aging societies, and the development of small, non-peptidic molecules with neurotrophic activity is of vital importance as such compounds easily cross the blood-brain barrier. We recently discovered that militarinone A (MiliA), a small fungal metabolite from *Paeclomyces militaris*, was able to stimulate neuronal outgrowth in PC12 cells within 24 hours (1). Application of MiliA to other cells such as the murine neuroblastoma cell line N2a, however, resulted in immediate onset of apoptosis.

In summary, the fungal metabolite MiliA induces differentiation in PC-12 cells by persistent activation of pathways that are also involved in NGF-mediated differentiation, namely the PI3-K/PKB and the MEK/ERK pathways as well as by activation of the transcription factor CREB. The continuous activation of these pathways finally led to up-regulation of p53, release of AIF from mitochondria, and activation of the c-Jun/AP-1 transcription factor that was also described as a "killer on the transcriptional leash" (2). Consequently, PC-12 cells succumbed to cellular death 48 to 72 hours post-treatment.

Application of MiliA to other neuronal cells as N2a, however, resulted in immediate onset of apoptosis by nuclear translocation of AIF, and activation of caspases and c-Jun/AP-1. The main difference between these two cell types was found to be the basal expression of p53, being very high in N2a, but low in PC-12.

It was recently reported that basal levels of p53 positively control production and mitochondrial accumulation of AIF (3). High expression of AIF could be an advantage for survival, as it helps to detoxify oxidative radicals as long as it is localised inside the mitochondria. But, on the other side, it pre-disposes these cells to a more efficient killing upon insults that affect mitochondrial integrity. N2a cells that showed a high expression of p53 fit into this category.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC311

Epidemiology of cancers in Isfahan – Iran: A retrospective study

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More than 11 million people are diagnosed with cancer every year. It is estimated that there will be 16 million new cases every year by 2020. World Health Organization estimates that 7.6 million people died of cancer in 2005 representing 13% of deaths worldwide. Between 2005 and 2015, 84 million more people could die if urgent action is not taken. Cancer is not simply or even primarily a problem of rich countries. More than 70% of all cancer deaths occur in low and middle income countries. Assessment of the quality of cancer treatment depends upon the availability of accurate and relevant information about the process and outcomes of care for patients.

This is an observational and retrospective study the subjects included cancer inpatients at the Omid Center Data has been collected through medical records of patients baseline checklist in the period 2001-2006.

Breast cancer in female, colon cancer in male and leukemia for both men and women are the most common cancers in Isfahan. Age of breast cancer is 30-60 and colon cancer is 40 and above. Cancer is the uncontrolled growth and spread of cells that may affect almost any tissue of the body. Lung, colorectal and stomach cancer are among the five most common cancers in the world for both men and women. In this research among men, colon cancer is the most common cancer and for women, the most common cancer is breast cancer.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC312

Level of M2-pyruvate kinase in pancreatic cancer cells is influenced by acidic pH, glucose deprivation and hypoxia

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Hypoxic and nutritionally deprived areas are thought to contribute to resistance of tumour cells to apoptosis in pancreatic cancers. Tumour M2-pyruvate kinase, a tumour associated isoenzyme of pyruvate kinase, is elevated in patients with pancreatic cancer. This study aims to measure tumour M2-pyruvate kinase (TuM2-PK) in different human pancreatic cancer cell line

following exposure to acidic pH, glucose deprived and hypoxic conditions.

TuM2-PK expression was measured in cell lysate supernatant of HMEF, MCF7, Panc 1 and Colo357 human cell lines using sandwich type ELISA (ScheBo® Tumour M2-PK™) and Western blot. Panc1 and Colo 357 were grown in acidic pH (6.5), glucose free medium (DMEM) and hypoxic (1% Oxygen) condition. TuM2-PK was expressed as mUnits/million cells.

Mean TuM2-PK values between different cell lines were compared using two-way ANOVA and unpaired T test. Correlation between TuM2-PK levels and cell densities was made using Pearson's coefficient.

M2-PK expression was significantly higher in Colo357 cells compared to HMEF, MCF 7 ($p < 0.05$) and Panc 1 cells ($p < 0.001$). Correlation between TuM2-PK levels and different cell densities was negative [MCF7 ($r = -0.382$, $p = 0.06$), Panc-1 ($r = -0.096$, $p = 0.065$) and Colo357 ($r = -0.528$, $p < 0.01$). Tumour M2-PK expression was significantly enhanced in Colo357 cells ($p < 0.05$) at acidic pH or glucose deprived or hypoxic condition in the culture medium compared to normal conditions.

TuM2-PK can be measured in different cell lines using ELISA. In-vitro expression of TuM2-PK correlates with low cell density. Acidic, glucose deprived and hypoxic conditions around tumour cells enhances M2-PK expression.

Sincere thanks to ScheBo Biotech, Geissen, GERMANY for funding the cost of TuM2PK ELISA Kit and DF4 anti M2-PK antibody.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC313

In vivo characterisation of cytohesin 7/EFA6D oncoprotein, a novel member of the cytohesin family of ARF6 GEFs

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The potential of certain types of cancer cells to spread to secondary sites within the body, or metastasize, as well as other critical cellular events, has previously been shown to be regulated by the monomeric GTPase ADP-ribosylation factor (ARF) 6 [1]. Being a small GTPase, ARF6 cycles between a GDP- and a GTP-bound conformation, and this cycling is required for ARF6-dependent signalling events. ARF6 has no intrinsic GDP/GTP exchange activity or GTPase activity, and so its GDP/GTP cycle is regulated entirely by its interaction with additional regulatory proteins. One such group of these regulatory proteins is a family of phosphoinositide (PI) binding proteins termed cytohesins. Cytohesins function as guanine nucleotide exchange factors (GEFs) for ARF GTPases [2], thereby increasing the amount of ARF6 that is present in its GTP-bound form. We propose that Exchange Factor for ARF6 (EFA6)D, initially identified as a marker for hepatocellular carcinoma cells [3], is a novel member of the cytohesin family, which we have termed cytohesin 7. The expression of cytohesin 7 has been shown to be upregulated

in colon cancer and downregulated in prostate cancer [3,4]. In this study we have functionally characterised cytohesin 7 in order to understand how the activation of ARF6 by cytohesin 7 alters the phenotype of the cancer cells. Through localisation studies in PC12 and HeLa cells of GFP-tagged proteins, we have identified that cytohesin 7 is localised to the plasma membrane, and that this localisation is dependent on both its coiled coil (CC) domain and its PI-binding PH domain. Through an in vivo GST effector pull down assay, we have shown that cytohesin 7 functions as a GEF for ARF6, but not ARF1, and that this function is dependent on the presence of a functional Sec7 catalytic domain. Furthermore, exogenous expression of cytohesin 7 in HeLa cells promotes cytoskeleton reorganisation possibly through ARF6 activation. Using artificially membrane-targeted deletion constructs of cytohesin 7, we have also shown that the GEF function of cytohesin 7 towards ARF6 is dependent on its localisation to the plasma membrane. Future studies will be directed towards understanding the role of cytohesin 7 in ARF6 associated cellular functions in normal and diseased states.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC314

Apoptosis induced by benzo[a]pyrene in rat liver F258 epithelial cell line involves a modulation of the exchanger Na^+/H^+ via lipid rafts

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Polycyclic aromatic hydrocarbons (PAH), such as benzo[a]pyrene (B[a]P), are ubiquitous genotoxic environmental pollutants. Their DNA-damaging effects lead to apoptosis induction, but the early events of this signalling are still poorly understood. Our previous works have demonstrated the primordial role of NHE1 in B[a]P induced apoptosis in rat liver F258 epithelial cell line (Huc et al., *Faseb J*, 2004; *Cancer Res*, 2007). The aim of this study was to further

elucidate how NHE1 is activated by B[a]P, and more specifically to investigate the role of plasma membrane (caveolae) in this activation.

Two cholesterol-depleting agents, methyl- β -cyclodextrin and cholesterol oxidase, which are generally used for disrupting lipid rafts, were used in this study. Our results demonstrated that these two agents significantly decreased B[a]P (50nM)-induced apoptosis. Likewise, intracellular pH measurements showed that both the alkalization and the acid efflux due to NHE1 activation upon B[a]P were also inhibited. This pointed to a possible role of lipid rafts in NHE1 activation. To gain further insight into such a role, NHE1 localization was studied after lipid raft extraction from cell lysates in Triton X-100 at 4°C on a sucrose density gradient. Our findings indicated that B[a]P not only affected raft composition, notably by decreasing cholesterol concentration, but also led to NHE1 delocalization outside these microdomains, these effects being prevented by cholesterol repletion. In total, our data provide evidence that B[a]P acts on microdomain composition, notably by altering cholesterol content, and that this alteration participates to NHE1 activation, thereby potentiating apoptosis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC315

Characterization of glycoforms of the stress-related Orp150 involved in NLS-dependent nuclear protein import

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Changes of cellular glycosylation, such as the increased expression of the oncofetal Thomsen-Friedenreich antigen (galactose β 1,3N-acetylgalactosamine α -TF), are common in cancer and pre-cancerous conditions (Campbell, et al 2001). We have previously shown that lectins that bind TF can have marked effects on proliferation. The edible mushroom (*Agaricus bisporus*) lectin (ABL) has an anti-proliferative effect (Yu et al 1993) that relates to its internalisation and inhibition of NLS-dependent nuclear protein import mediated via its interaction with an N-terminally truncated form of the stress glycoprotein Orp150 (Yu et al, 2002). The aim of the present study is to characterise Orp150 glycosylation and investigate further the role of Orp150 in nuclear protein import.

Immunoblotting of protein extracts from HT29 colon cancer cells showed the presence of two Orp150 isoforms of which the lower molecular weight isoform is stress inducible by glucose starvation. The higher molecular weight isoform is constitutively expressed. Selective membrane permeabilisation with digitonin showed release of only the constitutive Orp150 form implying its cytoplasmic localisation (Fig 1). Lectin affinity purification of protein extracts from a glucose starved cell preparation with Jacalin (a TF and sialyl-TF-binding lectin from jack fruit) yielded selective extraction of the constitutive Orp150 form suggesting differential glycosylation of the two Orp150 isoforms (Fig 2). siRNA treatment of HT29 cells resulted in 62% reduction of

Orp150 expression and subsequently 89% reduction (% nuclear fluorescence, without heat stress 53.97, after heat stress 75.44, siOrp150 + heat stress 56.26, $P < 0.0001$) of the nuclear translocation of Hsp70 in response to heat stress, thus confirming a role for Orp150 in NLS-dependent nuclear protein import. Further studies will investigate the possible role of O-glycosylation in Orp150 function. This study suggests that there are two Orp150 isoforms, one is a truncated cytosolic form and the other is a stress inducible form. The cytosolic form is involved in NLS-dependent nuclear protein import.

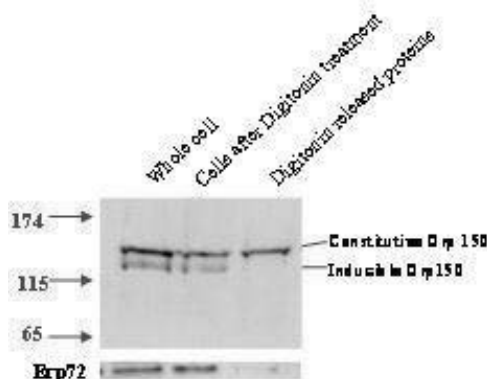


Fig 1. Orp150 immunoblot of HT29 cells shows selective release of constitutive but not the inducible Orp150 form by digitonin permeabilisation. Erp72 is an ER localised protein.

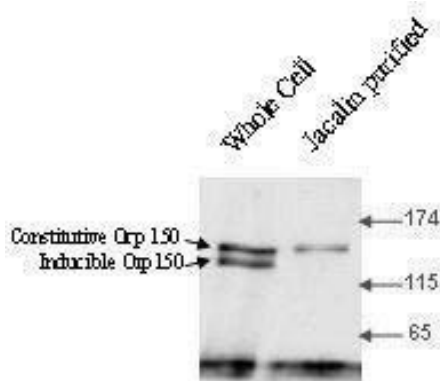


Fig 2. Jacalin affinity purification of glucose starved HT29 cell preparation followed by Orp150 immunoblot shows extraction of the constitutive Orp150 isoform.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC316

Identification of cancer stem cells in brain and prostatic tumours

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Although conventional cancer therapies such as drug and radiation treatments may eradicate the majority of a tumour mass it has been suggested that a highly proliferative cell population remains.

This led to the hypothesis that it is this population of cells, which are purely responsible for tumour growth. These cells have been termed cancer stem cells (CSCs) due to the fact that they share the properties of quiescence, self-renewal and differentiation with normal stem cells (Miller et al, 2005).

If solely this population of cells drives the growth of cancer, then this may provide an explanation as to why many cancers are resistant to conventional cancer therapies and highlights the need for therapies targeted at cancer stem cells. The search for markers of these cells led to the discovery of a cell surface protein CD133 (Yin et al. 1997).

CD133 is a five-transmembrane cell surface glycoprotein. Cells express CD133 shows self-renewal and proliferative features of stem cells and CSCs. The expression of CD133 disappears when the cells become committed to differentiation (Rizzo et al, 2005) suggesting that it is unique to stem cells.

The aim of our research is to identify and quantify the expression of CD133 in normal and cancerous brain and prostate cell lines and tissues using quantitative RT-PCR. We aim to isolate mRNA from archival formalin-fixed paraffin-embedded (FFPE) tissues and assess CD133 expression in benign and cancerous prostate samples provided by the pathology laboratory (University Hospitals Morecambe Bay NHS Trust). The research aims to add further support for isolation of good quality mRNA from FFPE tissues. There is an abundant source of FFPE material available in laboratories and if such tissues are amenable to gene expression studies then this would open up a vast archive of material for analysis.

Our results suggest that inactivation of CSCs may provide a novel therapeutic strategy in glioma and prostate tumours. Further study will focus on characterisation of the signalling pathways of CSCs in order to distinguish CSCs from normal stem cells.

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The University of Central Lancashire

University Hospitals Morecambe Bay NHS Trust

The Royal Preston Hospital

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PC317

Importance of telomerase and its core subunits in glioma

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Expression of telomerase influences the effectiveness of treatment in cancers. Telomerase is composed of two core subunits, telomerase reverse transcriptase (hTERT) and an RNA subunit (hTR) (Dong et al., 2005), the enzyme functions by utilizing hTR products as a template for the synthesis of new telomeric repeat sequences (Wong and Collins, 2003).

The molecular chaperone Hsp90 regulates the maturation, stability and folding of telomerase. It is part of a foldosome, which is required for telomerase activation. An increase in telomerase activity has been shown to correspond with an increase in the molecular chaperones required for the foldosome (Holt et al, 1999). Therefore studying the expression of hTERT and hsp90 in glioma cell lines and tissues may aid the pathological measurements of the disease and may provide a more accurate patient prognosis.

The aim of our research was to analyse the expression of hTERT and hsp90 (α and β subunits) in glioma cell lines and tissues using quantitative RT-PCR and to detect telomerase activity. We found that all cancerous cell lines and tissues expressed hTERT and both subunits of hsp90. The control cell line NHA (Normal human Astrocyte) and the normal brain tissues did not express hTERT and very low levels of hsp90 (α and β) was detected in the normal samples. Telomerase activity in cell lines was consistent with hTERT expression but in the tissues only 43% of the cancerous tissues showed active telomerase.

Several small molecule inhibitors of Hsp90 have been identified that can deplete cellular levels of multiple oncogenic client proteins simultaneously by enhancing their ubiquitination and proteasome-mediated degradation. The activity of Hsp90 inhibitors has been well validated in preclinical cancer models. We are aiming to assess some of these inhibitors on glioma cell lines and tissues. Given the redundancy and complexity of the molecular abnormalities present in most cancers, the ability of Hsp90 inhibitors to alter the activity of multiple oncogenic targets may provide a unique therapeutic benefit to patients.

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PC318

Purification and characterization of a novel monocot lectin with mitogenic and *in vitro* anti-proliferative activity from *Arisaema utile*

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A lectin with the potent mitogenic and *in vitro* anti-proliferative activity has been purified from tubers of a wild monocot plant *Arisaema utile* Schott by affinity chromatography on asialofetuin linked amino-activated silica beads. *Arisaema utile* lectin (AUL) gave a single band in SDS-PAGE at pH 8.3 corresponding to subunit Mr 13.5 kDa. The native molecular mass as determined by gel filtration chromatography was 54 kDa, suggesting a homotetrameric structure. Like other monocot lectins, AUL gave multiple bands in isoelectric focusing and in native PAGE at pH 8.3. AUL was inhibited by N-acetyl-D-lactosamine (LacNAc), a disaccharide and asialofetuin, a complex desialylated serum glycoprotein. When treated with denaturing agents, the lectin was stable in the presence of urea (3 M), thiourea (4 M) and guanidine HCl (4 M). The lectin had no requirement for divalent metal ions i.e. Ca²⁺ and Mn²⁺ for its activity. AUL was a glycoprotein with a carbohydrate content of 1.2%. Amino acid analysis revealed high content of aspartic acid, glutamic acid, glycine and threonine and a very low amount of methionine but complete absence of cysteine. Amino acid modification studies of AUL revealed tryptophan and tyrosine residues involved in lectin-sugar interaction. The lectin showed potent mitogenic response towards human lymphocytes. The mitogenic activity of AUL was even more than that of Con A, a standard well-known plant mitogen. AUL exhibited a fluorescence emission maximum (λ_{max}) at 340 nm upon excitation at 295 nm. Using Far UV CD spectra the estimated secondary structure was 37% α -helix, 25% β -sheet and 38% random contributions. *In vitro* anti-proliferative activity of AUL was tested on eleven different human cancer cell lines viz. MCF-7 (Breast), SK-N-SH (CNS), 502713 (Colon), Colo-205 (Colon), HCT-15 (Colon), HT-29 (Colon), SW-620 (Colon), Hep-2 (Liver), IMR-32 (Neuroblastoma), DU-145 (Prostate) and PC-3 (Prostate). The concentrations of AUL which produced 50% inhibition (IC₅₀) of cancer cell lines viz. SW-620, HCT-15, SK-N-SH, IMR-32, Colo-205 and HT-29 at 38, 42, 43, 49, 50 and 89 μ g/ml. respectively. AUL was found specific for LacNAc in the present study, which is reported as one of the very important cancer marker. Future investigations are focused to exploit lectins using appropriate animal models of human diseases and other agents (e.g., synthetic compounds) to reveal the synergistic effect of these agents to prevent cancer and make a better prediction to which type of protocol, the anti-proliferative effect of plant lectins is most likely to be successful.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC319

Differential effect of 2-aminoethoxydiphenyl borate on the cell proliferation and apoptosis in cancer cells

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2-Aminoethoxydiphenyl borate (2-APB) is a widely used agent in the studies of IP3 receptor and store-operated channel signaling (Bootman et al., 2002; Flemming et al., 2003). We previously demonstrated that 2-APB significantly inhibits the HEK-293 cell growth and the mechanism is not related to the IP3 receptors (Xu et al., 2005). Recently, we also found TRPC1 and TRPC5, are involved in the vascular smooth muscle cell proliferation and migration (Xu et al., 2006) and neointimal growth (Kumar et al., 2006). However the effect of 2-APB and the involvement of TRP channels on the cancer cell growth and death are not clear. In this study we used cell proliferation and apoptosis assay kits to examine the effects of 2-APB on the cancer cells including human breast adenocarcinoma cell line MCF-7, human lung carcinoma cells A549, human ovarian cancer cells SKOV-3 and human hepatocellular liver carcinoma cell line HepG2. The cells were grown in DMEM-F12 (Gibco, UK) medium containing 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin, and were maintained at 37°C under 95% air and 5% CO₂. Cells were seeded onto 96-well cell culture plates for experiments. The unpaired student's t test was used to assess the statistical difference.

The cell proliferation of SKOV-3 cells and HEK-293 cells were significantly inhibited by 2-APB in a concentration dependent manner after 24 h treatment. The minimum effective concentration occurred at 0.5 µM and the EC₅₀ was 5.9 µM. However, the effect of 2-APB on the proliferation of MCF-7 and A549 cells was not concentration-dependent. 2-APB at 10, 50 µM displayed as an antiproliferative effect on MCF-7 cells, but no effect was observed at a higher concentration (100 µM). Moreover, the lung and liver cancer cells (A549 and HepG2) were resistant to 2-APB. The antiproliferative effect were not related to the gap junctional blocking effect of 2-APB, since carbenoxolone, a gap junction blocker, did not change the effect of 2-APB. In addition, 2-APB also significantly evoked the apoptosis of the cancer cells detected by anti-histone-complexed DNA fragmentation assay. The data indicate 2-APB inhibits cancer cell growth by its antiproliferative and pro-apoptotic mechanisms. 2-APB sensitive TRP channels or store-operated channels may be the potential drug targets for anticancer therapy. The differential sensitivity to 2-APB suggests 2-APB or its analogues could be developed as specific agents for some types of cancer.

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PC320

Antibody Fc engineering for optimal immune system engagement

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Antibody Fc gamma receptors (FcγRs) are present on a wide variety of effector cell populations, including NK cells, dendritic cells (DC), neutrophils, and macrophages. We have previously engineered the Fc domains of antibodies to enhance their affinity for FcγRIIIa, leading to dramatic enhancements in ADCC. While NK cell-mediated lysis occurs predominantly through a single activating receptor FcγRIIIa, activation of antigen presenting cells such as DC and macrophages can also be influenced by the activating receptor FcγRIIa and inhibitory receptor FcγRIIb. In an effort to optimally tune antibodies for effector function, we have engineered a panel of Fc variants with a variety of unique FcγR affinities and specificities, including selective engagement of FcγRIIIa and FcγRIIa over FcγRIIb. Results indicate that whereas NK cell-mediated killing is correlated strongly with FcγRIIIa affinity, phagocytosis by macrophages is dependent on binding to both FcγRIIa and FcγRIIIa. Surprisingly, our data show that absolute FcγRIIb affinity does not negatively impact phagocytosis. Overall these variants have the potential to improve anti-cancer therapy by increasing not only innate effector functions, but also by enhancing adaptive anti-tumor responses, a novel feature of engineered therapeutic antibodies.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC321

Formation of ethylated DNA adducts in calf thymus DNA following exposure to mainstream and sidestream cigarette smoke

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Cigarette smoking is associated with the development of numerous human cancers in various tissues. Cigarette smoke is a complex mixture of chemicals and contains more than 50 known or probable human carcinogens. The majority of carcinogens present in cigarette smoke require metabolism to form the reactive species that have the potential to react with DNA, resulting in

the formation of DNA adducts. Interestingly, it has been recently suggested that there was an uncharacterized direct-acting ethylating agent present in cigarette smoke [1]. Since alkylation at the N-7 position of guanine in DNA is the predominant reaction site, N7-alkylguanine represents a good biomarker for determining exposure to alkylating agents. In our previous study, N7-ethylguanine (N7-EtG) has been detected in human urine and was found to be highly associated with cigarette intake of smokers [2]. But the source of this direct-acting ethylating agent in tobacco smoke is still unclear. In the present study, we aimed to investigate the characteristics of the direct-acting ethylating agent present in cigarette smoke by measuring the formation of N7-EtG following exposure of DNA to cigarette smoke *in vitro*. Cigarettes were smoked as specified under the Federal Trade Commission (FTC) standard conditions. Both the particulate and vapor phases of mainstream and sidestream smoke were collected. After sampling, the aqueous extracts of cigarette smoke were added to calf thymus DNA and the mixtures were incubated. The levels of N7-EtG formed were then quantitated by liquid chromatography tandem-mass spectrometry (LC-MS/MS) with on-line solid phase extraction [3]. Our results showed that N7-EtG was formed *in vitro* and was highly correlated with the number of cigarettes treated. Moreover, this uncharacterized direct-acting ethylating agent was found to be mainly present in the particulate phase of mainstream smoke, indicating it poses a greater health threat to smoker themselves than others.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC322

Determination of 8-oxo-7,8-dihydro-2'-deoxyguanosine in human serum by isotope-dilution LC-MS/MS with automated solid-phase extraction

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Level of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) in biological fluids has been widely used as a biomarker to assess oxidative lesions to DNA (1). Commercially available ELISA tests could provide a fast and simple method to measure 8-oxodGuo in human serum although the overestimations attributed to a lack of specificity of antibodies has been frequently observed (2).

Interestingly, there are little reports of mass spectrometry techniques attempting to measure 8-oxodGuo in serum. Herein we develop an isotope-dilution LC-MS/MS method coupled with an on-line solid-phase extraction (on-line SPE) to measure serum 8-oxodGuo. Serum sample of 200µl was firstly added 15N5-8-oxodGuo as an internal standard, followed by the addition of acetonitrile (ACN) to remove the proteins from serum. After centrifugation, the supernatant was collected, dried and re-dissolved in 5%MeOH/0.1% FA for on-line SPE LC-MS/MS analysis.

This method allows for fast and high-throughput analysis of serum 8-oxodGuo because it does not use a column-based purification process; it is also relatively inexpensive by using common laboratory reagents. The results showed that the overall mean recovery of 8-oxodGuo in serum was ~97 % by our newly developed on-line SPE LC-MS/MS with protein precipitation method. This on-line SPE LC-MS/MS method is now ready for comparison tests with the commercial ELISA tests using healthy volunteer's serum samples.

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PC323

ADP Hunter™ Plus: an optimized assay format with minimized compound interference for kinase primary screening using ADP accumulation

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Commonly used methods for kinase screening either rely on antibody detection of a phospho-epitope, or monitoring ATP depletion as the result of kinase activity *in vivo*. We have developed an improved, generic kinase assay format that monitors the accumulation of ADP as the result of kinase activity. This approach was initially developed as a very rapid, kinetic application for assay development and compound profiling and characterization. However, the assay formulation has recently been optimized to provide features more suitable for primary screening applications. The benefits of this approach include gain-of-signal output, high DTT tolerance, high ATP tolerance and the ability to use whole protein substrates. The detection of ADP is based on an enzyme-coupled reaction converting ADP to a fluorescent signal. This has traditionally been challenging with issues such as compound interference and overall performance. We will present data demonstrating assay improvements and performance with respect to compound screening.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC324

Citrate metabolism in prostate cancer: NADPH generated via cytosolic aconitase/isocitrate dehydrogenase pathway in lipogenesis

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Prostate cancer is the commonest male cancer in the west, with mortality second to that of lung cancer. The role played by lipogenesis in cancer biology is receiving increasing interest in the research arena (Kuhajda, 2006) whilst in prostate cancer fatty acid synthase is considered to be a metabolic oncogen (Baron et al., 2004). Understanding lipogenesis and citrate-related cytoplasmic metabolism in prostate cancer cells formed the basis of the present research (Mycielska et al., 2006). In particular, the project studied the role played by citrate metabolism, via the c-aconitase/cICD pathway, in lipogenesis.

We adopted PNT2-C2 and PC-3M cell lines to represent normal and highly metastatic prostatic epithelia, respectively. Spectrophotometric assays were used to study NADPH production, free fatty levels and cICD activity in these cell lines, under various conditions. Real-time PCR was used to study mRNA expression of the major NADPH producing enzymes.

NADPH levels were significantly higher in cancer cells compared to normal prostate cells (0.025 ± 0.0066 and 0.015 ± 0.0047 mmols/100 μ g protein, respectively). In the presence of oxalomalate, inhibitor of the c- (cytosolic) aconitase/cICD pathway, the NADPH levels in cancer were reduced to those seen in normal prostate cells. A similar reduction in NADPH levels was seen when cancer cells were treated with an iron chelator (DFO). C-aconitase, known also as iron regulatory protein (IRP), acquires enzymatic activity in the presence of high intracellular iron, known to be associated with cancer. Therefore, DFO was used to inhibit c-aconitase activity. The same inhibitors (2.5 mM oxalomalate and 0.5 mM DFO) reduced significantly the synthesis of free fatty acids in prostate cancer cells (37.7 ± 1.1 % and 28.5 ± 8.5 % respectively) but had no effect on normal cells. cICD mRNA expression was 38 ± 0.7 % higher in PNT2-C2 cells compared to PC-3M cells, however activity of cICD measured as rate of NADPH production was strongly dependent on the inhibition/activity of FAS. In the presence of FAS inhibitors the level of NADPH resulting from cICD activity was significantly higher, by 20 ± 0.7 %, in PC-3M cells treated with inhibitor.

The obtained results would suggest that: (1) c-aconitase/cICD pathway becomes active in cancer cells (2) c-aconitase/cICD provides NADPH to FAS for de novo fatty acid synthesis (3) c-aconitase activity is dependent on the intracellular levels of Fe.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC325

Genotoxic effects of PCB 52 and PCB 77 on cultured human peripheral lymphocytes

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Polychlorinated biphenyls (PCBs) are industrial chemicals that have been released into the environment, resulting in widespread and persistent contamination. PCBs exist as 209 different congeners depending on the chlorine substitution on the biphenyl rings, and the physical properties and toxic effects of different PCB congeners may be structure-dependent. It has been suggested that environmental contaminants may cause DNA damage directly and/or by generation of reactive species such as electrophilic metabolites or free radicals (Xue and Warshawsky, 2005). Potential of PCBs for genotoxicity has been controversial (Belpaeme et al, 1996). The present study was designed to determine genotoxic effects of ortho-substituted, noncoplanar congener, 2,2',5,5'-tetrachlorobiphenyl (PCB 52), and a non-ortho-substituted coplanar congener, 3,3',4,4'-tetrachlorobiphenyl (PCB 77) on cultured human peripheral lymphocytes. DNA damage was assessed by use of the comet assay (alkaline single-cell gel electrophoresis). The six healthy male non-smoking donors (mean age 29.3 ± 2.3 years) were used in the study. Subjects had not been exposed to radiation or drugs six months prior to the study. Whole peripheral blood samples were collected by venipuncture. The isolation of lymphocytes was performed by centrifugation in a density gradient of lymphorep (15 min, 280xg) and the cells washed with RPMI-1640 medium supplemented with 10% fetal calf serum. Cell viability was determined with the use of 0.4% trypan blue. After cell cultures were prepared, test groups were treated with different concentrations of PCB 52 (0.2 and 1 μ M) and PCB 77 (1 and 10 μ M), and vehicle control group was treated with DMSO for 1 hour at 37 °C in a humidified carbon dioxide incubator. DMSO concentration in the culture medium was not more than 0.1% (v/v). Positive control group was treated with 50 μ M H₂O₂ for 5 min on ice. Fifty cells per slide and two slides per sample were scored to evaluate DNA damage for each concentration of the tested PCB congeners. Results were statistically analyzed by Student's t-test. The cells were visually classified into four categories on the basis of extend of migration such as undamaged (UD), low damaged (LD), moderate damaged (MD) and high damaged (HD). Total comet scores (TCS) were calculated as: $1 \times \text{HD} + 2 \times \text{LD} + 3 \times \text{MD} + 4 \times \text{HD}$. The results obtained were compared against to negative control group. The highest concentration of PCB 52 and 77 significantly increased DNA breakage in human lymphocytes ($p < 0.05$). However, lower concentrations of either PCB congeners did not produce any significant DNA damage in the comet assay. Our results indicate that both noncoplanar PCB 52 and coplanar PCB 77 increased DNA damage, but the planarity of the PCB congeners did not affect the direction of action, although the ortho-substituted congeners were found to be more potent.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC326

Characterisation of recombinant human fatty aldehyde dehydrogenase: Implications for Sjögren-Larsson syndrome

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Fatty aldehyde dehydrogenase (FALDH) is an NAD⁺-dependent oxidoreductase involved in the metabolism of long-chain fatty alcohols. Enzyme activity has been implicated in the pathology of diabetes and cancer. Mutations in the human gene inactivate the enzyme and cause accumulation of fatty alcohols in Sjögren-Larsson syndrome, a neurological disorder resulting in physical and mental handicaps. The major form of FALDH from human microsomes was expressed in *E. coli* and purified by anion-exchange and metal-chelate chromatographies. Using an *in vitro* activity spectrophotometric assay based on NAD⁺ reduction an optimum pH of ~9.5 and temperature of ~35 degrees C were determined. Medium- and long-chain fatty aldehydes were converted to the corresponding acids and kinetic parameters determined. The enzyme showed high activity with heptanal (227 ± 7 nmol/min/mg), tetradecanal (183 ± 19 nmol/min/mg), hexadecanal (195 ± 25 nmol/min/mg) and octadecanal (180 ± 25 nmol/min/mg) with lower activities for the other tested substrates. The enzyme was also able to convert some fatty alcohol substrates to their corresponding aldehydes and acids, with decanol at 44 ± 3 nmol/min/mg and hexadecanol at 64 ± 3 nmol/min/mg. A structural model of FALDH has been constructed, and catalytically important residues have been proposed to be involved in alcohol and aldehyde oxidation: Gln-120, Glu-207, Cys-241, Phe-333, Tyr-410 and His-411. These results place FALDH in a central role in the fatty alcohol/acid interconversion cycle, and provide a direct link between enzyme inactivation and disease pathology caused by accumulation of alcohols.

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PC327

Chronic neuroendocrine stimulation induces Ca²⁺ homeostasis adaptation in long-lasting cultured adult heart cells

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Cultured adult cardiac myocytes represent an important approach to study the failing heart on the cellular level. We have developed a long-term culture of adult rat ventricular myocytes based on serum-free medium and substrates coated by extracellular matrix proteins allowing the sustained adenovirus-mediated expression of the Ca²⁺ sensor "inverse pericam". We used this single-cell model for the current work to investigate the effects of chronic neuroendocrine Gq-coupled stimulation on cellular and subcellular Ca²⁺ signalling. We assessed the loading state of internal Ca²⁺ stores and the activity of transport systems in Fura-2 loaded myocytes following caffeine applications (10mM), while spatially resolved Ca²⁺ imaging (realtime confocal microscopy) was undertaken to characterise the spatio-temporal features of Ca²⁺ sparks and waves. The control cells displayed a 4-fold augmentation of the wave frequency from day 6 onwards. Also on day 6, the spark distribution changed from a cytoplasmic spread into a sub-plasma membrane ring. Interestingly we could measure a 2-fold increase of the size of the spark spread from day 1 onwards. Furthermore, on days *in-vitro* 3 and 6, we observed decreased amplitudes of caffeine-induced Ca²⁺ transients. The latter ones displayed two distinct decay time constants: a slow one due to Na/Ca exchangers and a rapid one influenced to a large extent by sarcoplasmic reticulum (SR)-Ca²⁺ ATPases (SERCA), the precise role of which in the failing heart is still under controversy. After one week of culture, we could measure a 2-fold increase of the rapid decay time constant. Chronic stimulation with phenylephrine (10-100µM) or endothelin-1 (100nM) led to a rapid (2 days) dedifferentiation of the myocytes into so called myoballs, which formed spreading complexes from day 6 onwards, the spontaneous activity of which was predominantly waves. In the one week old Gq-coupled stimulated cells, the decreased amplitudes of caffeine responses were abolished. On the contrary, the rapid decay time constant was increased by 50% compared to control cells at the same stage. We conclude that chronic neuroendocrine stimulation leads to a remodelling of the cellular Ca²⁺ handling, especially in terms of SR loading and activity of the SERCA pumps, which could be a key target for the hormones tested. Moreover, neuroendocrine stimulation of adult myocytes might thus be an important part of cardiac remodelling *in-vivo* that can be mimicked and studied in an *in-vitro* model of adult myocytes.

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PC328

Probing the mechanical properties of cardiac titin by molecular combing and atomic force microscopy

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Left ventricular diastolic dysfunction in patients with congestive heart failure is associated with significant morbidity and mortality and is characterised by impaired relaxation, increased myocardial stiffness and ventricular dilatation. In cardiac muscle, the cytoskeletal protein titin is responsible for the development of passive tension in the myocyte and is present as two alternatively spliced isoforms. The isoform ratio is thought to determine the passive tension in the myocyte (1). We wanted to characterise the mechanical properties of titin isoform populations by applying a tensile force to isolated titin molecules.

Titin was extracted and purified from ferret left ventricular tissue (2). Tensile force was applied to isolated titin molecules by employing a molecular combing technique (3) whereby titin molecules partially adsorbed to a poly-L-lysine coated mica surface are exposed to forces of ~800pN by a receding meniscus. Visualisation of uncombed titin molecules by atomic force microscopy revealed a highly coiled, spring-like conformation. In contrast, combed molecules were straightened and aligned. Uncombed titin monomers ranged in length from 0.6 to 1.4µm which we suggest is due to the presence of splice variants. The mean contour lengths were increased following molecular combing ($2.24 \pm 0.16 \mu\text{m}$ uncombed vs $2.60 \pm 0.21 \mu\text{m}$ combed, $p < 0.05$ $n=42$) and axial height profiles of entire titin molecules revealed localized areas of thinning. Mean axial heights were reduced following combing ($0.62 \pm 0.21 \text{ nm}$ uncombed vs $0.58 \pm 0.22 \text{ nm}$ $p < 0.001$ $n=4000$). The molecular combing technique is capable of aligning and elongating titin molecules and would be appropriate for the determination of mechanical properties of titin in disease.

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harboured by microparticles, we have recently shown that Sonic Hedgehog is present in MPs generated from activated/apoptotic human T CEM lymphocytes (1).

Microparticles were isolated following serial centrifugations. Eahy 926 endothelial cells were grown for 24h in absence or presence of 10 µg/mL microparticles pre-incubated or not either with PI3-kinase inhibitor (LY294002, 20 µM), MEK 1/2 inhibitor (U0126, 10 µM), cyclopamine (30 µM), a specific antagonist of the Sonic Hedgehog receptor (Patched), or siRNA of Patched. Cell lysates were analyzed by Western blot. Also, cells were used for direct measure of nitric oxide, whereas oxidative stress was determined by flow cytometry. In another set of experiments, after 24h of intravenous injection of microparticles into mice, endothelium-dependent relaxation was determined in aortic rings. Also, ischemia/reperfusion was induced in mice by ligating the left anterior descending coronary artery proximal to its origin and endothelial function of distal coronary artery was assessed. Finally, the effect of microparticles on angiogenesis was determined by Matrigel assays and vessel sprouting in mice aortic rings.

Here, we show that Sonic Hedgehog carried by microparticles induces nitric oxide release from endothelial cells at the basal level and after a bradykinin-stimulation (20 µM) (2.47 and 2.6 fold, respectively) and triggers changes in both the expression and the phosphorylation of enzymes related to the nitric oxide pathway, and also decreases production of reactive oxygen species (38.6 ± 1.4 % of positive cells in treated vs. 51.4 ± 0.2 % in control). When PI3-kinase and ERK signalling were specifically inhibited, the effects of microparticles were reversed. In vivo injection of microparticles in mice was also able to improve endothelial function (EC_{50} ; 5.3×10^{-8} M of acetylcholine in control vs. 6.5×10^{-8} M in treated mice) by increasing nitric oxide release (1.4, 1.92 and 2.6-fold in blood, heart and lung, respectively) and it reversed endothelial dysfunction after ischaemia/reperfusion. Silencing the effects of Sonic Hedgehog with either cyclopamine or siRNA of Patched, strongly reduced production of nitric oxide elicited by microparticles (34.6%) in endothelial cells. Finally, microparticles promotes angiogenesis, these effects being abolished by Sonic Hedgehog pathway inhibition.

Taken together, we propose that the biological message carried by microparticles harbouring Sonic Hedgehog may represent a new therapeutic approach against endothelial dysfunction during acute severe endothelial injury.

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PC330

Circulating microparticles from septic shock patients exert protective role on vascular function

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Microparticles (MPs) are membrane vesicles released during cell activation or apoptosis. Elevated levels of circulating MPs have

PC329

Sonic hedgehog carried by microparticles corrects endothelial injury and promotes angiogenesis through nitric oxide release

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Microparticles are small fragments generated from the plasma membrane after cell stimulation. Among the candidate proteins

been detected in pathological states associated with vascular dysfunction. The present study was designed to characterise MPs from both septic shock and healthy subjects and to investigate their effects on vascular functions.

MPs were obtained from whole blood of patients ($n = 32$) or healthy ($n = 16$) subjects by serial centrifugations. Then, their counts and cellular origins are determined by flow cytometry in platelet-free plasma. Markers of endothelial (CD146), platelet (CD41 and P-selectin: CD62P), and leukocyte (L-selectin: CD62L) activation were measured. Nitric oxide (NO) and superoxide anion (O_2^-) were measured by electronic paramagnetic resonance technique (ERP). MPs either from patients or healthy subject were injected i.v to mice and then, vascular reactivity was assessed in aorta by myography. Circulating levels of MPs, endothelial- and platelet-derived MPs, as well as P-selectin+ and L-selectin+ MPs were increased in septic patients. No difference in NO or O_2^- production was detected in mouse aortas treated with MPs from either septic or healthy subjects. MPs either from patients or healthy subjects were injected i.v to mice and then, vascular reactivity was assessed in aorta. Interestingly, the sensitivity of contraction in response to serotonin was increased in aorta taken from mouse treated with septic MPs compared to those with healthy MPs. This effect was not modified in presence either of NO-synthase inhibitor, LNA, or cyclooxygenase (COX)-2 inhibitor, NS398. In other hand, the non-selective COX inhibitor, indomethacin, either reduced or abolished contraction to serotonin in aorta from mice treated with healthy and septic MPs, respectively. Contraction to $CaCl_2$ on arteries exposed to KCl-depolarization and relaxation to the Rho-kinase inhibitor on pre-contracted vessels were identical in aorta taken either from healthy or septic MPs-treated mice.

In conclusion, we demonstrate that septic patients displayed increased circulating of MPs especially those originated from non activated and activated platelet, endothelial and activated leukocytes. Interestingly, septic MPs in vivo enhanced reactivity to serotonin without affecting nitrosative or oxidative stresses within the aorta. Thus, septic MPs may be rather protective against vascular hyporeactivity accounting for hypotension in septic shock patients.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC331

Blockade of agonist- and flow-induced EDHF activity by ascorbate in the bovine ciliary artery under conditions of constant pressure

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We have shown previously that ascorbate has two distinct actions in the bovine perfused ciliary artery: a blockade of agonist (bradykinin or acetylcholine)-induced endothelium-derived hyperpolarising factor (EDHF) activity and an elevation of tone, resulting from blockade of flow-induced EDHF (Nelli *et al.*, 2004, Stirrat *et al.*, 2006). Both effects of ascorbate require the presence of flow and are abolished in the presence of zero or low flow. These studies were conducted using an open perfusion system where it was not possible to hold pressure constant when

varying the rate of flow. The aim of this study was to make use of a pressure myograph to determine if the flow-dependent actions of ascorbate could still be elicited when pressure was held constant.

Segments of bovine extraocular ciliary artery were cannulated at both ends, mounted in a pressure myograph and perfused at flow rates of 2.5, 5 or 10 ml min⁻¹ using a peristaltic pump. Pressure was held constant at 100 mm Hg by adjusting the height of the outflow tube and external diameter was monitored throughout. In some experiments EDHF activity was isolated by blocking nitric oxide synthase and cyclooxygenase using L-NAME (100 μ M) and indomethacin (10 μ M), respectively. Bradykinin (1 nmol) was injected into the perfusion fluid to evoke endothelium-dependent vasodilatation. In all experiments, $n > 6$.

When pressurised to 100 mm Hg the external diameter of ciliary artery segments was $1,609 \pm 100$ μ m. Infusion of ascorbate (50 μ M) induced constriction that was dependent upon the rate of flow: 1 ± 38 , 113 ± 26 and 242 ± 52 μ m at flow rates of 2.5, 5 and 10 ml min⁻¹, respectively. Treatment with L-NAME and indomethacin induced a constriction which did not vary with flow rate: 106 ± 24 , 84 ± 30 and 131 ± 23 μ m at flow rates of 2.5, 5 and 10 ml min⁻¹, respectively. Under these conditions, the ascorbate-induced constrictions were generally enhanced: 146 ± 55 , 135 ± 33 and 457 ± 25 μ m at flow rates of 2.5, 5 and 10 ml min⁻¹, respectively. Bradykinin-induced, EDHF-dependent dilatation did not vary with flow rate: 59.8 ± 4.7 , 56.9 ± 4.1 and 51.8 ± 3.8 % at flow rates of 2.5, 5 and 10 ml min⁻¹, respectively. Ascorbate, reduced these dilatations in a flow-dependent manner to 41.4 ± 10.9 , 39.2 ± 5.1 and 0 ± 0 % at flow rates of 2.5, 5 and 10 ml min⁻¹, respectively.

These findings demonstrate that when the bovine ciliary artery is held at constant pressure, the ability of ascorbate to inhibit agonist- and flow-induced EDHF remains flow-dependent.

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PC332

Role of TNF-alpha in type II diabetes-induced endothelial dysfunction

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We previously reported that tumor necrosis factor-alpha (TNF) contributes to endothelial dysfunction in the prediabetic Zucker obese rat. To determine if TNF plays a similar role in endothelial dysfunction in fully developed diabetes, we tested the hypothesis that impairment in coronary endothelial function in type II

diabetes (Zucker diabetic fatty rat, ZDF) is due to the overexpression of TNF via TNF receptors (TNFRs). In ZDF rats, dilation (isolated arterioles) to endothelium-dependent vasodilator, acetylcholine (ACh), was blunted compared to lean rats, but dilation to the endothelium-independent vasodilator sodium nitroprusside (SNP) was comparable. Neutralizing antibodies to TNF (72 hr treatment; 24 mg/kg, i.p.) restored NO-mediated coronary arteriolar dilation in ZDF rats, but did not alter endothelium-dependent vasodilation in lean rats. Expression of TNFR1 (mRNA) was significantly increased in coronary arterioles in ZDF vs lean, but TNFR 2 mRNA expression was similar in the two groups. Western blotting shows the protein expression of TNF was higher in both heart and coronary arterioles in ZDF rats than that in lean rats. NAD(P)H oxidase activity and H₂O₂ production increased significantly in ZDF rats, but anti-TNF decreased both NAD(P)H oxidase activity and H₂O₂ production. These results demonstrate the endothelial dysfunction occurring in type II diabetes is the result of effects of the inflammatory cytokine TNF, mainly through the overexpression of TNF-R1, which subsequently activates NAD(P)H oxidase and H₂O₂.

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PC333

The effects of subjects personality on heart rate variability during stress

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The purpose of this study was to investigate the effects of subjects personality on heart rate variability (HRV) when stress was induced by examination. 105 healthy female students with a mean age of 21.51 $\pm$ 0.09 years volunteered for the experiment. To study the role of probabilistic forecasting of results in the development of the real life stress affect on the heart rate control, each student was asked to predict her mark. Groups were formed based on

the difference between the expectations and the actual mark (group 1: expectation = actual mark; group 2: expectation > actual mark; group 3: expectation < actual mark) [2]. HRV was recorded after 10-minute supine rest for 5 minutes while subjects were resting in a supine position [1]. The students were examined in the following sequence: recording of HRV at rest and shortly before real life stress. The values were compared by Mann-Whitney test (U) and sign test (Z).

Mean values of M, LF, HF and LF/HF for the different groups are presented in Table 1. In the present study significant differences during control day were observed in M between group 2 and group 3 (U=265.50; p=0.03) in LF between groups 1 and 3 (U=194.00; p=0.01) and between groups 2 and 3 (U=243.00; p=0.02). The M was significantly higher in group 1 than in group 3 (U=186.00; p=0.007) during stress day. Significant differences during stress day were observed in LF between groups 1 and 3 (U=206.00; p=0.02), in HF between groups 1 and 3 (U=169.00; p=0.003) and between groups 2 and 3 (U=254.50; p=0.02), in LF/HF between groups 1 and 3 (U=203.00; p=0.02). The differences in LF/HF were significantly larger between groups 1 and 3 (U=207.00; p=0.02) and between groups 2 and 3 (U=276.00; p=0.04).

The main finding of this study on healthy young subjects is that a mild real life stressor, is capable to produce complex changes in major cardiovascular regulatory mechanisms. This result also suggests that susceptibility to stress would presumably be different from person to person and be affected by probabilistic forecasting of results.

Table 1. Descriptive Statistics of RR Interval Variabilities in Healthy Subjects During Stress and Control Days

Parameter		group 1	group 2	group 3
LF	Control day	1581.21 $\pm$ 244.06	1424.45 $\pm$ 202.62	697.35 $\pm$ 111.43
LF	Stress day	1320.33 $\pm$ 164.13	1299.08 $\pm$ 152.35	761.50 $\pm$ 98.60
HF	Control day	966.17 $\pm$ 151.37	851.63 $\pm$ 159.88	550.71 $\pm$ 133.42
HF	Stress day	583.13 $\pm$ 88.97	637.70 $\pm$ 103.79*	257.65 $\pm$ 56.15*
LF/HF	Control day	2.55 $\pm$ 0.31	2.28 $\pm$ 0.24	2.60 $\pm$ 0.59
LF/HF	Stress day	3.35 $\pm$ 0.32*	2.77 $\pm$ 0.24*	4.60 $\pm$ 0.67*
M	Control day	0.833 $\pm$ 0.019	0.813 $\pm$ 0.020	0.745 $\pm$ 0.029
M	Stress day	0.722 $\pm$ 0.016*	0.736 $\pm$ 0.014*	0.666 $\pm$ 0.018*

Data presented as mean $\pm$ SE.

* – significant difference between control day and stress day (p<0.05). Task Force (1996). *Circulation* 96 (5), 1043-1065.

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PC334

Spontaneous Ca²⁺ transients in cardiac-like cells of the rat pulmonary vein

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The pulmonary vein is widely regarded as an important source of ectopic activity triggering atrial arrhythmias (Haissaguerre *et al.*, 1998). In many mammalian species, the vein is surrounded by a sleeve of cardiac-like cells that extends from the atria to the lungs (Best *et al.*, 1961). These cardiac-like cells show intrinsic electrical activity (Chen *et al.*, 2000); however, the mechanisms

underlying the initiation of this activity remain unclear. It has been suggested that intracellular Ca^{2+} may influence the electrical activity in cardiac-like cells, thus we have examined Ca^{2+} signalling events in the pulmonary vein.

Male Sprague Dawley rats (180–300g) were sacrificed by cervical dislocation. The pulmonary vein (0.7–1 cm long and 0.3–1 mm outside diameter) was dissected and loaded with $10\text{ }\mu\text{M}$ fluo-4 AM. The tissue was then placed in bath solution comprising (in mM); NaCl, 150; KCl, 5.4; HEPES, 10; Glucose, 10; MgCl_2 , 1.2; CaCl_2 , 1.8, pH 7.4 with NaOH, and imaged using a Zeiss Axioskop microscope equipped with a Hamamatsu CCD camera. Images were acquired using WinFluor (University of Strathclyde) and fluorescence was expressed as $\Delta\text{F}/\text{F}_{\text{min}}$, with n being the number of tissues studied. Student's paired *t*-test was used for statistical comparison and $P < 0.05$ was considered to be significant.

In all control tissues, spontaneous Ca^{2+} transients were observed within individual cardiac-like cells. The activity was generally asynchronous in nature yielding a mean frequency of 1.4 ± 0.02 Hz and amplitude $\Delta\text{F}/\text{F}_{\text{min}} = 0.11 \pm 0.01$ ($n = 47$). Upon removal of extracellular Ca^{2+} spontaneous activity continued, although there was a small but significant reduction in both the frequency (from 1.4 ± 0.1 to 1.1 ± 0.1 Hz, $n = 7$, $P < 0.05$) and amplitude (from $\Delta\text{F}/\text{F}_{\text{min}} = 0.11 \pm 0.02$ to 0.09 ± 0.02 , $P < 0.05$). However, when the SR was depleted of Ca^{2+} by continued exposure to caffeine (20 mM), the spontaneous Ca^{2+} transients were almost completely abolished, with the frequency being reduced from 1.4 ± 0.1 to 0.03 ± 0.03 Hz ($n = 7$, $P < 0.01$). In the presence of ryanodine (20 μM) the frequency was reduced from 1.4 ± 0.1 to 0.1 ± 0.04 Hz ($n = 7$, $P < 0.01$), and the amplitude from $\Delta\text{F}/\text{F}_{\text{min}} = 0.15 \pm 0.03$ to 0.02 ± 0.01 ($P < 0.01$). The IP_3 receptor blocker 2-aminoethoxydiphenyl borate (20 μM) also reduced the frequency from 1.6 ± 0.2 to 0.4 ± 0.2 Hz ($n = 5$, $P < 0.05$) and amplitude from $\Delta\text{F}/\text{F}_{\text{min}} = 0.05 \pm 0.01$ to 0.01 ± 0.004 ($P < 0.05$). Inhibiting the SR Ca^{2+} ATPase with cyclopiazonic acid (20 μM) produced a decrease in frequency from 1.4 ± 0.2 to 0.2 ± 0.1 Hz ($n = 7$, $P < 0.01$) and amplitude from 0.10 ± 0.03 to 0.03 ± 0.02 $\Delta\text{F}/\text{F}_{\text{min}}$ ($P < 0.05$).

This study has shown that spontaneous Ca^{2+} transients occur in individual cardiac-like cells of the pulmonary vein under resting conditions. Furthermore, our results indicate that SR Ca^{2+} release via both the ryanodine and IP_3 receptors is important for producing this spontaneous activity.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC335

Renal actions of urotensin II in the anaesthetised spontaneously hypertensive rat (SHR)

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Urotensin II (UII) has been implicated in cardiovascular and renal disease [1] but its physiological role has yet to be fully explored. We have shown that UII has a marked effect on renal haemodynamics, resulting in a decrease in glomerular filtration rate (GFR) in Sprague Dawley rats [2]. At lower doses, which had

only minimal effects on GFR, UII also increased fractional excretion of Na^+ (FE_{Na}) [3]. Renal UII receptor (UT) mRNA expression is increased in SHR compared with Wistar-Kyoto (WKY) rats [2], suggesting that SHR may be more sensitive to UII. Hence, the aim of this study was to determine the renal response to UII in the SHR.

Inactin anaesthetised (100 mg/kg, i.p.) male SHR and control normotensive WKY rats ($n = 7$ per group) were infused with 0.154M NaCl containing ^3H inulin (0.3 $\mu\text{Ci}/\text{h}$) and *para*-aminohippuric acid (3mg/h) via a jugular vein catheter for 4 hours at 50 $\mu\text{l}/\text{min}$. Animals were then divided into three groups which received either vehicle, rUII at 0.6 or 6 $\text{pmol}\cdot\text{min}^{-1}\cdot 100\text{g body weight}^{-1}$ for one hour. The infusate was then switched back to saline for a further hour. Urine samples were taken every 15 mins via a bladder catheter and blood samples were taken once per hour via a carotid artery catheter for the measurement of electrolytes. Animals were killed humanely at the end of the experiment.

Mean arterial blood pressure was higher in SHR (214 ± 2) compared with WKY rats (117 ± 2 mmHg, $P < 0.001$) and was unaffected by rUII. Basal effective renal blood flow (ERBF), GFR, urine flow (UV) and sodium excretion (U_{NaV}) rates were lower in SHR compared with WKY rats during vehicle infusion. Rat UII infused at 6 $\text{pmol}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$ induced comparable reductions in the ERBF of SHR and WKY (Table 1). However, the reduction in GFR was significantly greater ($P < 0.05$) in SHR; reductions in UV and U_{NaV} also tended to be greater. Interestingly, FE_{Na} increased in WKY rats but fell in SHR. The effects of rUII at 0.6 $\text{pmol}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$ were similar but of smaller magnitude (data not shown).

These data suggest that SHR are more sensitive to the renal haemodynamic effects of UII. The reduction in FE_{Na} observed in SHR in response to rUII contrasts with the increase in FE_{Na} seen in WKY, suggesting that UII may contribute to renal sodium retention in the SHR.

	WKY ($n = 7$)	SHR ($n = 7$)
Δ ERBF ($\text{ml}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$)	-1.4 ± 0.3	-1.5 ± 0.3
Δ GFR ($\text{ml}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$)	-0.3 ± 0.1	$-0.6 \pm 0.1^*$
Δ UV ($\mu\text{l}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$)	-5.3 ± 0.9	-9.2 ± 1.7
Δ U_{NaV} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$)	-0.6 ± 0.2	-1.3 ± 0.3
Δ FE_{Na} (%)	0.6 ± 0.2	$-0.5 \pm 0.3^*$

Table 1. Maximal difference (Δ) between rats receiving vehicle and rUII infusion at 6 $\text{pmol}\cdot\text{min}^{-1}\cdot 100\text{g bwt}^{-1}$, * $P < 0.05$ WKY vs SHR.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC336

Peroxyntirite mediates LPC-induced enhancement of late cardiac sodium currents

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In cardiac myocytes, the opening of voltage-activated sodium (Na_v) channels induces a fast-activating inward Na^+ current which mediates the rapid action potential upstroke. The slowly

inactivating (or late) component of the Na^+ current (I_{LNa}) remains active during prolonged depolarisation and contributes to the action potential plateau. Lysophosphatidylcholine (LPC) accumulates in the ischaemic myocardium and is arrhythmogenic, at least in part via effects on I_{LNa} . Here, we examined the mechanisms underlying the effects of LPC on I_{LNa} , using recombinant $\text{Na}_v1.5 \alpha$ subunits expressed in HEK293 cells. Na^+ currents were studied by whole-cell patch-clamp. Currents were evoked by 40ms step depolarisations to -40mV (holding potential -140mV) every 5s. Data were taken from current values obtained 10ms after initiating the depolarisation. Accordant with previous reports, 5 μM LPC enhanced I_{LNa} . Thus, normalised (I/I_0) I_{LNa} was 2.53 ± 0.58 ($n=7$) after a 6min exposure to LPC, compared to 0.99 ± 0.13 ($n=8$) in cells perfused without LPC ($P<0.05$). Enhancement of I_{LNa} was abolished by the antioxidant ascorbic acid (200 μM) and by the NADPH oxidase inhibitor DPI (5 μM). Normalised I_{LNa} following LPC exposure in the presence of these agents was 1.21 ± 0.08 ($n=7$) and 1.16 ± 0.16 ($n=5$) respectively ($P<0.05$ compared to LPC alone in each case). Although these data suggest a role for NADPH oxidase derived superoxide in mediating the response to LPC, the superoxide donor pyrogallol (100 μM) did not affect I_{LNa} . Normalised I_{LNa} following exposure to pyrogallol was 1.15 ± 0.05 ($n=8$; $P>0.05$ compared to control). Enhancement of I_{LNa} was also abrogated when cells were pretreated with the NOS inhibitors L-NAME (500 μM) and 7-nitroindazole (300 μM). Normalised I_{LNa} following LPC exposure in the presence of these agents was 0.88 ± 0.06 ($n=6$) and 1.37 ± 0.14 ($n=6$) respectively ($P<0.05$ in each case compared to LPC alone). Despite this, the NO donor SNAP (200 μM) evoked no change in I_{LNa} . Normalised I_{LNa} following exposure to SNAP was 1.11 ± 0.10 ($n=6$; $P>0.05$ compared to control). However, SNAP and pyrogallol combined enhanced I_{LNa} , to 2.16 ± 0.35 ($n=8$; $P<0.05$ compared to control), suggesting peroxynitrite formation as a mediator of the response to LPC. Supporting this, the peroxynitrite scavenger FeTPPS (50 μM) abolished the response to LPC (normalised I_{LNa} in response to LPC in the presence of FeTPPS was 1.15 ± 0.11 , $n=4$; $P<0.05$ compared to LPC alone). We conclude that peroxynitrite formation provides a novel mechanism by which LPC regulates Na^+ channel function.

Experiments were supported by a Project Grant (PG/05/042) to IMF from the British Heart Foundation (BHF). Equipment was provided by the BHF and the Royal Society. HEK293 cells stably expressing hNaV1.5 were kindly provided by Dr. J. Makielski (University of Wisconsin).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC337

GFRP over expression in endothelial cells attenuates LPS and cytokine-induced nitric oxide production

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GTP-cyclohydrolase 1 (GTP-CH1) catalyses the first and rate limiting step for the de novo production of tetrahydrobiopterin

(BH4), an essential cofactor for nitric oxide synthase (NOS). Interestingly, the GTP-CH1-BH4 pathway is emerging as a potential regulator in a number of pathologies associated with nitric oxide (NO) over production. GTP-CH1 activity can be inhibited by BH4 through its protein-protein interactions with GTP-CH1 regulatory protein (GFRP) and transcriptional and post translational modification of both GTP-CH1 and GFRP have been reported in response to proinflammatory stimuli but the functional significance of GFRP/GTP-CH1 interactions on NO pathways has not yet been demonstrated. We aimed to investigate whether over expression of GFRP could affect NO production in living cells.

We have over-expressed N-terminally -Myc tagged recombinant human GFRP in the murine endothelial cell line, sEnd 1. BH4 and nitrite levels were measured both basally and following stimulation with proinflammatory cytokines and lipopolysaccharide. GFRP over expression had no significant effect on basal BH4 nor NO levels but significantly attenuated the rise in BH4 and NO observed following cytokine stimulation of cells.

This study demonstrates that GFRP can play a direct regulatory role in iNOS mediated NO synthesis and suggests that the allosteric regulation of GTP-CH1 activity by GFRP may be an important mechanism regulating BH4 and NO levels in vivo.

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PC339

α_1 -acid glycoprotein enhances sodium channel activation in stressed cardiac myocytes

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α_1 -acid glycoprotein (AAG) is an acute phase protein whose plasma concentration increases in response to trauma, inflammation and bacterial infection (Fournier et al. 2000). We have previously reported that AAG reverses the sodium channel blockade induced by amitriptyline, a tricyclic antidepressant, and by quinidine, a class I antiarrhythmic agent (Ma & Henry 2001), and by cocaine (Ma et al. 2006), but AAG alone has no

effect on sodium channels when the cells were bathed in physiological solution. Here we report a further discovery that AAG enhances sodium channel activation in cardiac myocytes placed in modified physiological solution with extracellular Na^+ reduced to 18mM from 138mM, and with the calcium and potassium currents blocked by Cd^{2+} and Cs^+ . Under these conditions the cardiac myocytes were unable to function normally, such as generating action potentials, and the homeostasis of Na^+ and other ions, and the normal physiological functions of Na^+ transporters were severely disturbed (Pieske et al. 2003; Bers et al. 2003). We therefore use the term “stressed cardiac myocytes” to describe these cells, and the term “cytoprotective” to describe the enhancement of sodium channel activation by AAG.

Voltage gated sodium current (I_{Na}) was recorded using whole cell patch clamp techniques to evaluate the effect of AAG. Guinea pig cardiac ventricular myocytes were isolated by enzymic digestion and continuously perfused at room temperature with normal or modified physiological solution. With extracellular Na^+ reduced to 18mM, AAG 25.6 μM not only reversed the I_{Na} blockade induced by 0.8 μM amitriptyline, but also increased the current beyond the control level (Fig1): with control as 1, blockade was 0.46 ± 0.21 , and the reversal effect of AAG was 1.39 ± 0.25 ($N=4$). When recordings were carried out in physiological solutions with 138mM extracellular Na^+ , cells generated action potential (AP) under current clamp, and AAG reversed the effects of amitriptyline on AP to control level, but not beyond. We conclude, therefore, that AAG may protect cardiac myocytes from physiological or pathological stress, and that this may have clinical significance.

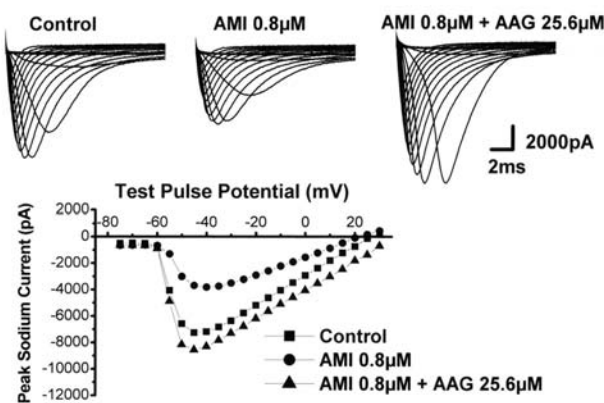


Fig1. The effect of amitriptyline and amitriptyline (AMI) plus AAG on sodium channels recorded in modified physiological solution. I_{Na} was elicited by a 20 ms depolarisation to test potentials between -75mV and 30mV from a potential of -90mV. I_{Na} under control, AMI and AMI plus AAG are shown in the top panel. The peak value of the currents is plotted against the test pulse potentials below. The keys indicate drug concentrations applied.

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PC340

Expression of *START (STARD1-D15)* family members during differentiation of human THP monocytes to macrophages

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Steroidogenic acute regulatory-related lipid transfer (*START*) domains have been found in 15 mammalian proteins, *STARD1-D15*; the *START* domain is a lipid binding domain, implicated in lipid metabolism and trafficking and cell signalling. Cholesterol-trafficking *START* proteins (*STARD1, D3, D4, D5*) may play a role in macrophage cholesterol homeostasis and, by extension, atherosclerosis, and, by virtue of their lipid binding regions, represent potential drug targets. In this study, we describe qualitative changes in expression of *STARD1-D15* during differentiation of human THP-1 monocytes to macrophages, which is accompanied by marked increases in cellular lipid content.

Human THP-1 monocytes were differentiated into macrophages, by incubation with phorbol 12-myristate 13-acetate (PMA; 250nM) for up to seven days in RPMI medium containing 10% FBS. Marked changes in cellular phenotype were attended by significant and maximal increases in macrophage phospholipid (5.1-fold, $72.8 \pm 16.4 \mu\text{g}/4.5 \times 10^6 \text{ cells}$ versus $14.3 \pm 6.24 \mu\text{g}/4.5 \times 10^6 \text{ cells}$; $p < 0.05$, $n=4$), total cholesterol (3.3-fold, $40.1 \pm 7.7 \mu\text{g}/4.5 \times 10^6 \text{ cells}$ versus $12.3 \pm 3.69 \mu\text{g}/4.5 \times 10^6 \text{ cells}$; $p < 0.05$; $n=4$) and triacylglycerol (5.8-fold, $89.7 \pm 25.7 \mu\text{g}/4.5 \times 10^6 \text{ cells}$ versus $15.4 \pm 4.96 \mu\text{g}/4.5 \times 10^6 \text{ cells}$; $p < 0.05$, $n=4$) at day 4, compared with monocytic control cells. Using the qualitative polymerase chain reaction (PCR) method and specific primers we found 12 of the 15 known *START* member genes to be expressed in THP-1 macrophages; expression of *STARD6, STARD14* and *STARD15* were undetectable using this methodology. Sequence analysis confirmed the expression of *STARD1*, hitherto thought restricted to steroidogenic tissues, in THP-1 macrophages; the presence of *STARD1* precursor protein (37kDa) was also detected by Western blotting. Expression of *STARD1* and *STARD3* appear up-regulated during macrophage differentiation (0-7 days), compared with the housekeeping gene, GAPDH; no apparent changes in the other sterol-trafficking proteins, *STARD4* or *STARD5* were evident during this process. Examination of the phospholipid trafficking subfamily of *START* proteins suggested that *STARD2*, but not *STARD7, D10* or *D11*, was up-regulated during macrophage differentiation, relative to GAPDH. Members of the RhoGAP *START* family members (*STARD8, STARD12* and *STARD13*) did not appear to be regulated during macrophage differentiation, but, intriguingly, *STARD9* was appeared to be induced during this period. Our future studies will investigate the therapeutic potential of key members of this novel family of lipid trafficking proteins by investigating their ability to resolve macrophage lipid accumulation and, potentially, atheroma formation.

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PC341

In vitro study of cardiac troponins as biochemical markers of anthracycline-induced cardiotoxicity

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Risk of cardiotoxicity is the most serious drawback of the clinical usefulness of anthracycline antineoplastic antibiotics. Early, accurate and preferably non-invasive detection of cardiac injury is therefore of crucial importance. Previously we have shown that cardiac troponin T (cTnT) is a useful biochemical marker of anthracycline-induced cardiotoxicity in vivo, using a model of chronic daunorubicin-induced heart failure in rabbits (1).

In the present study, we examined the release kinetics of cTnT and cTnI using an in vitro model of isolated rat neonatal ventricular cardiomyocytes (NVCM, 72-hour treatment with 0.1 – 3 µM of daunorubicin). Daunorubicin-exposed cardiomyocytes typically increased beating frequency, which was in groups with 1 and 3 µmol/l followed by irregular activity, discontinuation of cellular monolayer and eventually contractile arrest. While in culture media of control cells LDH activity was spontaneously increasing over time, cTnT and cTnI concentrations remained unchanged throughout the experiment. LDH activity and both troponin concentrations were dose- and time-dependently increasing in response to daunorubicin exposure and were negatively exponentially related to cardiomyocyte viability. With 3 µM daunorubicin, the relative increase of AUC of cTnT and cTnI was 2.4 and 5.3-fold higher than the increase of LDH activity, respectively (Table 1).

Hence, the troponins can become not only the bridge between the clinical and experimental studies of drug-induced cardiotoxicity but also the linkage between the preclinical experiments in vitro and in vivo.

Table 1 – Cardiomyocyte viability after the 72h daunorubicin exposure (means ± S.E.M.) and cumulative release of LDH, cTnT and cTnI over the 72h incubation period (AUCs – given as medians)

	Cardiomyocyte viability (Living cells*1000/well)	LDH (IU/l/min) AUC 0 - 72h	cTnT (ng/ml) AUC 0 - 72h	cTnI (ng/ml) AUC 0 - 72h
Control	707 ± 52 (100.0 %)	297.0 (100.0 %)	65.5 (100.0 %)	12.4 (100.0 %)
Daunorubicin 0.1 µM	638 ± 91 (-10.5 %)	295.0 (-0.7%)	68.3 (+4.4%)	15.2 (+23.1%)
Daunorubicin 0.3 µM	537 ± 60* (-24.4 %)	362.0 (+21.9 %)	88.9 (+35.8 %)	32.9 (+165.8 %)
Daunorubicin 1 µM	189 ± 26* (-73.6 %)	912.2* (+207.2 %)	407.8* (+522.7 %)	195.6* (+1482.9 %)
Daunorubicin 3 µM	72 ± 35* (-89.8 %)	962.5* (+224.1 %)	506.6* (+673.6 %)	213.2* (+1624.6%)

Upper lines – absolute values; lower lines – relative changes compared to control. NVCM were seeded 4 days before the start of incubation in an amount of 800,000 cells/well.

* statistical significance against control (ANOVA; P<0.05).

Simunek T. et al. (2003) Cancer Chemother Pharmacol. 52, 451-455.

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PC342

Human atrial cell morphology is linked to heterogeneous distribution of Ca²⁺ sparks

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Differences in EC-coupling between mammalian atrial and ventricular cells and intracellular calcium events associated with mechanical shortening have been reported (1,2). This involves the elementary calcium release event, the calcium spark, that initiates whole cell calcium waves which, in atrial cells, has been shown to be mainly localised to sub-sarcolemmal regions at the cell periphery. It has also been shown however that narrower atrial cells, with a less well organised t-tubular system, display a “U”-shaped calcium wave form which originates at the cell periphery, whilst wider atrial cells, with a more organised t-tubular system display a “W” shaped calcium wave with varying points of origin of the calcium wave (3).

As human atrial cells have a different morphology to rat atrial cells, the spatially restricted calcium signal observed in rat atrial cells (1,2) may be of limited clinical significance. To test whether calcium waves in human atrial cells have a spatially restricted initiation site we analysed sparks from atrial cells isolated from human tissue obtained during bypass operations. After enzymatic digestion of atrial appendage, cells were loaded with fluo 4-AM and the spatio-temporal properties of sparks recorded with a confocal microscope in linescan (50-60 Hz) and fast x-y (20-40 Hz) acquisition mode. Origin and IDL software was used for analysis.

Two types of cell were observed, healthy resting and calcium-overloaded cells. Calcium sparks were observed at the periphery and the centre in both cell types. Spark summation in both regions produced localised sub-cellular waves with larger summation in overloaded cells.

In resting cells sparks were more variable in amplitude and less frequent in central regions. Noradrenaline (NA) produced calcium waves with a linear front. In contrast, spark frequency and amplitude distribution in overloaded cells was more uniform in both central and peripheral regions with “V” shaped spontaneous waves. This suggests that coupling between calcium release units was increased in overloaded cells.

These results imply that in resting cells there is a difference in release channel activity at the periphery and centre of the cell giving a different spark frequency. This difference is eliminated in overloaded cells and resting cells stimulated with NA.

The morphology of human atrial cells and the uniform shape of waves in stimulated resting cells suggests that they have a more organised t-tubule system than atrial cells from other mammalian

species. Observations made in other mammalian atrial myocytes may thus be of limited relevance to human atrial function. Mackenzie et al (2004), *Journal of Cell Science*, 117:6327-6337. Bootman et al (2006), *Journal of Cell Science*, 119:3915-3925. Kirk et al (2003), *Journal of Physiology*, 547:441-451.

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PC343

Transverse tubule density in cardiac myocytes is regulated during pathologic, but not physiologic remodeling

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The cardiomyocyte (CMC) adapts to stress by cellular hypertrophy. However, the type of stress (physiological or pathological) has important consequences for remodeling. Previous studies indicated that pathologic remodeling is accompanied by a reduced transverse tubule (TT) density. Here, we examined the effects on TT-density after exercise training (ExTr) in two models of cardiac dysfunction (heart failure and metabolic syndrome), and in failing hearts that were unloaded with angiotensin II type 1 antagonism (Losartan). Healthy, post-myocardial infarction (MI) heart failure and metabolic syndrome adult rats were exercised for 8 weeks on a daily treadmill running program that increased exercise capacity by ~50%. Isolated CMCs were stained with Di-8-ANEPPS to image the TT-system with confocal microscopy. In healthy rats, ExTr associated with increased CMC length (sedentary $101 \pm 3 \mu\text{m}$ vs. ExTr $112 \pm 5 \mu\text{m}$) and unchanged relative TT-density (sedentary 0.2 ± 0.04 vs. ExTr 0.21 ± 0.04). In rats with heart failure 12 weeks post-MI, cell length increased (sham $101 \pm 3 \mu\text{m}$ vs. MI $130 \pm 7 \mu\text{m}$), and TT-density decreased (sham 0.2 ± 0.04 vs. MI 0.1 ± 0.01). ExTr from weeks 4-12 after MI reduced pathologic hypertrophy (MI sedentary $130 \pm 7 \mu\text{m}$ vs. MI ExTr $117 \pm 4 \mu\text{m}$). However, TT-density remained low (MI sedentary 0.1 ± 0.01 vs. MI ExTr 0.13 ± 0.03). In separate experiments, Losartan lowered blood pressure in heart failure rats, but did not cause any changes in TT structure. Nor did it add any additive effects when combined with ExTr. In contrast to heart failure, CMCs from metabolic syndrome rats (LCR; low capacity running) showed depressed contractile function, but had smaller cell sizes (LCR sedentary $118 \pm 2 \mu\text{m}$ vs. controls (HCR (high capacity running) sedentary $124 \pm 2 \mu\text{m}$). Metabolic syndrome was associated with reduced TT-density (LCR sedentary 0.18 ± 0.01 vs. HCR sedentary 0.21 ± 0.02). ExTr induced a greater hypertrophy in HCR than LCR (LCR $125 \pm 3 \mu\text{m}$; 6% vs. HCR

$141 \pm 4 \mu\text{m}$; 14%), but did not change TT-density in any group. Thus, CMC TT-density is sustained in physiologic, but not in pathologic hypertrophy. Moreover, cardiac dysfunction in metabolic syndrome is also associated with reduced TT-density. ExTr reduces pathological hypertrophy in heart failure after MI, induces physiological growth in metabolic syndrome hearts, but has little effect on TT-density.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC344

Effects of the Kv7 channel activator retigabine on native K⁺ currents in murine hepatic portal vein

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KCNQ-encoded K⁺ channels (Kv7.x) define neuronal excitability and cardiac rhythmicity, however, there remains little information on Kv7 channels in the vasculature. In 2003, Ohya *et al* (2003) reported expression of KCNQ1 in murine portal vein (mPV) myocytes. Our subsequent study showed that the Kv7 selective blocker XE991 inhibited a sustained K⁺ current in mPV myocytes, depolarised the membrane potential and increased contractile activity (Yeung & Greenwood, 2005). The aim of the present study was to characterise further the impact of Kv7 channels in the mPV K⁺ current using retigabine, which activates Kv7.2-7.5 but not Kv7.1.

Female BALB/c mice (6-8 weeks) were sacrificed by cervical dislocation and the portal vein excised. Cells were obtained by enzymatic dispersion for electrophysiological recordings and immunocytochemistry. Currents were recorded using the whole-cell voltage-clamp technique with an external solution of (mM): NaCl 126, KCl 5, MgCl₂ 1, CaCl₂ 0.1, glucose 11, HEPES 10, adjusted to pH 7.2 with NaOH. The internal solution contained (mM): KCl 130, MgCl₂ 1, ATP 2, HEPES 10, EGTA 5, adjusted to pH 7.2 with KOH. Currents were evoked by stepping to various test potential (V_T) from a holding potential (V_H) of -60 mV. Qualitative PCR of mPV tissue showed KCNQ1 and KCNQ4 gene expression. This was confirmed by antibody staining for Kv7.1 and Kv7.4 confined predominantly to the plasmalemmal region. In electrophysiological studies retigabine had bimodal effects on the mPV K⁺ current. At test potentials between -60 mV and -20 mV the peak K⁺ current was enhanced (20 μM increased current at V_T -20 mV by 46 ± 12 pA $n=8$). However at more depolarised V_T retigabine inhibited the outward current (eg 20 μM reduced the current at V_T +40 mV by 230 ± 36 pA, $n=8$). Inclusion of 4-AP (5 mM) was sufficient to ablate the inhibitory effect of retigabine yet still retain the current enhancement. Currents in the presence of 20 μM retigabine were inhibited by 10 μM XE991 ($37 \pm 5\%$ at +40 mV, $n=6$). $V^{1/2}$ of XE991-sensitive currents calculated from conductance-voltage plots was -7 ± 2 mV ($k = 15 \pm 1$ mV, $n=6$). In contrast the $V^{1/2}$ of XE991-sensitive currents with no pretreatment with retigabine was 1.7

± 3.5 mV ($n=4$). In isometric tension recordings 20 μ M retigabine reduced the amplitude ($74 \pm 4\%$, $n=4$) and increased the frequency ($71 \pm 9\%$, $n=4$) of inherent contractile responses. These data show that retigabine has pronounced effects on contractile activity and has bi-modal effects on membrane currents, which suggest that ion channels encoded by KCNQ4 have a significant impact in this vessel.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC345

Combined protective effect of nitric oxide donor and antioxidants in the rat heart

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Ischemic and pharmacological preconditioning (IP) can successfully limit ischemia/reperfusion (I/R) injury. Since they consist of interventions carried out before an experimental infarction, their clinical importance is limited. Unlike IP, ischemic postconditioning (PostC) consists of a series of brief coronary occlusions followed by reperfusion starting 10-15 s after the beginning of reperfusion (1). Thus PostC can be performed at a selected time after an ischemia.

Both a release of reactive oxygen species (ROS) and a reduced production of nitric oxide (NO) during reperfusion contribute to I/R injury (2). This research aims at investigating whether it is possible to induce protection with administration of NO-donors or antioxidant compounds during the first minutes of reperfusion and whether protection is enhanced by the simultaneous administration of the two types of compounds or of hybrid molecules having both effects.

Experiments were performed on 40 Langendorff-perfused rat hearts from animal decapitated after general anaesthesia with ketamine. The hearts underwent 30 minutes of ischemia and 2 hours of reperfusion (I/R). Control Group 1 ($n=10$) underwent I/R only. In Group 2 ($n=6$) antioxidant (AOX) compound 2,2,5,7,8-pentamethylcroman-6-olo (1 μ M) was added to the buffer solution for 20 min at the beginning of reperfusion. In Group 3 ($n=7$) AOX was replaced by NO-donor 4-[(dimethylamino)methyl]furoxan-3-carbossamide (1 μ M) and in Group 4 ($n=8$) AOX and NO-donor have been given together at the same concentrations. Finally in Group 5 ($n=9$) the hybrid compound 4-((N-((6-idrossi-2,5,7,8-tetrametilcroman-2-il)metil)-N-metilamino)metil)furossan-3-carbossamide (1 μ M) was infused for 20 min.

Data are mean $\pm$ SEM. While in the Group 1, the infarct size was $53\pm 3\%$, it was not significantly modified in the hearts of Group

2 ($48\pm 8\%$) and 3 (37 ± 7). In Group 4 infarct size was significantly ($p<0.01$, unpaired "t" test) reduced to $26\pm 6\%$. Finally in Group 5 the hybrid (1 μ M) significantly ($p<0.01$) reduced the infarct size to $22\pm 5\%$. After ischemia, diastolic contracture was greater than in the baseline conditions in the Group 1 and after AOX (Group 3), while it was unchanged in all other groups. The results suggest that if AOX and NO-donor properties are concentrated in a hybrid molecule, the protection obtained with 1 μ M of this compound is the same as that obtained with 1 μ M of AOX plus 1 μ M of NO-donor given together.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC346

Macrophage expression of Steroidogenic Acute Regulatory protein (StAR; StarD1) enhances apoAI-dependent cholesterol efflux

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Mitochondrial sterol 27-hydroxylase (CYP27A) plays a major role in macrophage cholesterol homeostasis by metabolizing cholesterol to polar oxysterols, which can ligate liver X receptor α (LXR α) and upregulate genes involved in cholesterol efflux including ATP binding cassette transporter A1 (ABCA1). Here, we exploit the cholesterol trafficking properties of Steroidogenic Acute Regulatory protein (StAR; StarD1), to increase the rate-limiting step of cholesterol transport to the inner mitochondrial membrane where CYP27A resides, and describe the impact of this on macrophage cholesterol efflux mechanisms.

Stably transfected RAW 264.7 macrophages, containing either pCMV.5 or pCMV.5_StarD1, were generated by hygromycin selection. Murine StarD1 amplicon (355bp) was detected by PCR only in cells transfected with pCMV.5_StarD1; sequencing of this fragment confirmed its identity. Mature StarD1 protein (30kDa) was detected only in lysates from macrophages transfected with pCMV.5_StarD1, and not in empty vector controls, or wild type RAW 264.7 macrophages. Expression of StAR significantly enhanced (1.5 fold; $p<0.001$) macrophage cholesterol efflux to apoAI (20 μ g ml⁻¹), but not HDL, in the presence of dibutyryl cAMP [(Bu)₂cAMP (0.3mM)], compared to empty vector, or wild type controls. Macrophages stably transfected with mutated murine StAR (Arg181Leu), which abrogates the mitochondrial cholesterol trafficking properties of this protein, did not show any enhancement of cholesterol efflux. Inhibition of CYP27A1, using GW2739297x (1 μ M), blocked the impact of StAR on apoAI-dependent cholesterol efflux. Further, treatment with the LXR antagonist (geranyl geranyl pyrophosphate, ammonium salt) negated the effect of StAR. At the end of the efflux period (24h), cellular levels of ABCA1 mRNA and protein were lower in cells expressing StAR, compared with cells expressing the

empty vector, consistent with lower cholesterol content and higher cholesterol efflux from these cells. No changes in expression of ABCG1, ABCG4 or ABCA7 relative to GAPDH were detected by PCR in either cell type.

Thus, introduction of StAR into macrophages exerts a markedly beneficial influence upon cholesterol efflux mechanisms, and may provide an avenue for the development of anti-atherogenic therapeutics.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC347

Intermittent bradykinin infusion affords myocardial protective effects akin to postconditioning: Role of B2 receptors and mitochondrial K⁺ channels

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Bradykinin (BK) triggers preconditioning via a pathway which include B2 receptors, nitric oxide (NO), protein kinase G (PKG), mitochondrial KATP channels (mKATP) and reactive oxygen species (ROS). Postconditioning (PostC) allows post-ischemic accumulation of autacoids, which trigger protection. We tested if PostC-triggering includes BK and its downstream pathway. Two-hundred rats were anesthetized with ketamin and decapitated, then isolated hearts underwent 30 min ischemia and 120 min reperfusion. Infarct size was evaluated using nitro-blue-tetrazolium staining. Data are mean±SEM. One-Way ANOVA and post-test Tukey's HSD were used for statistical analysis. In Control hearts infarct size was 61±5% of risk area. PostC (5 cycles of 10 s reperfusion/ischemia) reduced infarct size to 22±4% (p<0.01). The infarct-sparing effect of PostC was abolished by the following antagonists given for 3 min only, while performing PostC maneuvers: a) the B2 BK receptor-antagonists HOE140 (100 nM) or WIN64338 (100 µM); b) the NO synthase-inhibitor L-nitro-arginine-methylester (100µM); c) the PKG-blocker 8-bromoguanosine-3',5'-cyclic-monophosphorothioate (1 µM); d) mKATP blocker 5-hydroxydecanoate (100 µM). Since 3 min of BK-infusion (100nM) did not reproduce PostC-protection, protocols with Intermittent-BK infusion were used to mimic PostC: a) 5 cycles of 10 s oxygenated-no-BK/oxygenated+BK buffer; b) 5 cycles of 10 s oxygenated-no-BK/hypoxic+BK buffer. Both protocols with Intermittent-BK attenuated infarct size (36±5% and 38±4%, respectively; p<0.05 vs Control and NS vs PostC for both; NS vs each other). Intermittent-BK protection was abolished by the same antagonists used to prevent PostC-protection. Intermittence of re-oxygenation only (5 cycles of 10 s oxygenated/hypoxic buffer) did not reproduce PostC. Yet, cardioprotection was triggered by intermittent mKATP activation with diazoxide (30µM), but not by intermittent ROS generation with purine/xanthine oxidase. However, ROS scavengers [N-acetyl-L-cysteine (4 mM) or 2-mercaptopropionylglycine

(300 µM)], given for 3 min only, abolished either PostC-, Intermittent BK- or diazoxide-induced protection.

Intermittent targeting of specific cellular sites (i.e. BK B2 receptors and mKATP channels) during early reperfusion triggers PostC-protection via a ROS signaling. Since neither intermittent oxygenation nor exogenous ROS generators can trigger protection, it is likely that metabolites are washed-out and that exogenous ROS do not reach the right compartment. Therefore, intermittent autacid accumulation and ROS compartmentalization may play a pivotal role in PostC-triggering.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC348

Hypoxia exposure and chronic noradrenaline stimulation in neonatal rat cardiomyocytes: β_3 adrenergic receptor function and effect on cell viability

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β_1 adrenergic receptors (β_1 ARs) increase cardiac function through G_s-protein coupling, whereas β_3 ARs coupled to G_i-protein decrease contractility (1). We have previously shown that chronic noradrenaline (NA) stimulation induced a decrease in β_1 ARs and an increase in β_3 ARs at the functional, genomic and protein levels, which can explain the reduction in cardiac function observed in heart diseases (2). Indeed, circulating catecholamines are increased in heart diseases leading to an alteration of the cardiac function, a decrease in β_1 AR density and an elevation in β_3 AR level (1). The aim of this work was to study the function and expression of β_1 and β_3 ARs in neonatal cardiomyocytes exposed to normoxia (NX), NX and 100µM NA (NX/NA), hypoxia (HX, 0.5% O₂) or HX/NA for 24h. In addition, cell viability was performed using LDH assay following 24h NA or 2µM Cl31 treatment in NX and HX. Functional studies were performed using dobutamine (Dob, β_1 agonist) on cAMP accumulation and Cl 316243 (Cl31, β_3 agonist) on forskolin-induced cAMP response. β_1 and β_3 AR mRNA levels were determined by real time PCR.

NA pretreatment in NX and HX decreased Dob-induced cAMP accumulation (maximal response: 36±8 vs 167±16%, P<0.001 and 51±10 vs 91±21%, P<0.05, n=6, respectively), without changing the sensitivity. HX downregulated β_1 AR response (vs NX, P<0.001) and β_1 AR mRNA level (57±3% vs 100%, P<0.01, n=4). The mRNA level was similarly decreased (≈ 60%) following NA treatment in NX (P<0.01) and HX (P<0.05). No functional β_3 AR response was observed in NX and HX. NA pretreatment increased Cl31-mediated inhibition of forskolin response in NX and HX, with a higher maximal inhibition in HX/NA (Imax: 43±3% vs 23±4%, P<0.05, n=6). Inversely, the sensitivity decreased (pIC₅₀: HX/NA, 8.16±0.36, vs NX/NA, 9.15±0.26, P<0.05). β_3 AR mRNA level was upregulated following chronic NA stimulation in NX (157±52% vs 100%, P<0.05) and HX (193±77% vs 53±16%, P<0.01). Furthermore, HX reduced β_3 AR mRNA expression (53±16% vs 100% NX, P<0.05, n=4). Chronic

β_3 AR stimulation had no effect on LDH level in NX (97 ± 6 vs 100% , $n=5$) and did not induce cell survival against HX-induced cell death (230 ± 23 vs $254 \pm 9\%$), whereas chronic NA treatment induced an increase in LDH activity in NX ($170 \pm 18\%$, $P < 0.05$) and HX ($332 \pm 23\%$, $P < 0.05$).

In conclusion, the further upregulation of the β_3 AR function following chronic NA stimulation in HX in comparison to NX/NA indicates that the β_3 AR subtype plays an important role in heart diseases. However, chronic β_3 stimulation did not induce cell survival against cell death by necrosis in our model of ischemic heart disease, although this subtype did not promote cell death. Gauthier et al (2006), *Pharmacol Ther.* 111: 652-673

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PC349

Electrophysiologically detailed models for transmural heterogeneity and propagation in the rabbit ventricles

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Electrical differences between three primary cell types – epicardial (epi), midmyocardial (M) and endocardial (endo) – account for transmural heterogeneity of action potentials (AP) in the ventricular wall, and are believed to be important in normal functioning as well as arrhythmogenesis in the heart. We develop a new family of electrophysiologically detailed computer models to explore the effects of transmural heterogeneity and intercellular electrotonic interactions in the rabbit ventricular wall. The LabHeart model of AP in a single rabbit ventricular cell was modified to incorporate new experimental data (Rozanski *et al.*, 1997; Tsuji *et al.*, 2000; Xu *et al.*, 2001) on the transmural heterogeneity of the rabbit ventricle. Densities and kinetics of several ionic currents (I_{Na} , I_{CaL} , I_{to} , I_{K1} , I_{Ks} , I_{Kr} , $I_{Cl(Ca)}$) were updated. Single cell models were validated to reproduce experimentally measured electrical restitution properties of epi, M and endo cells (Idriss & Wolf, 2004). Finally, the 3 single-cell models were incorporated into a 1D tissue model with an intercellular gap junctional conductance chosen to give the transmural AP propagation velocity of 0.25 m/s as reported in rabbit. The models reproduce single cell AP durations (APD), transmural APD dispersion patterns and pseudo-ECGs with feasible T-waves and QT intervals similar to those observed in experiments (Yan *et al.*, 2001; Idriss & Wolf, 2004). In the intact tissue model, different profiles of APD dispersion (including those masking the M cell region) can be simulated by changing relative sizes of the epi, M and endo regions, which shows the importance of intercellular electrotonic interactions in modulating the transmural heterogeneity of the ventricular wall. Different proportions of the three cell types can quantitatively account for differences in APD

reported between male and female rabbits. Our electrophysiologically detailed models provide a powerful computational tool for non-invasive studies of electrical phenomena within the heterogeneous ventricular wall, such as functionally induced re-entry and the role of gender differences in drug dosing during its pharmacological treatment aimed at changing the transmural APD dispersion.

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PC350

Sorcini mediates effects on L-type Ca^{2+} channel inactivation independently of CaMKII in rabbit ventricular myocytes

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Sorcini is a 22 kDa EF hand Ca^{2+} -binding protein expressed in cardiac tissue that modulates excitation-contraction (EC) coupling in cardiomyocytes by interacting with RyR2, NCX and I_{CaL} . I_{CaL} is a critical Ca^{2+} influx pathway which dictates the Ca^{2+} load of the cell. One way of controlling Ca^{2+} influx is by regulating current inactivation. Slowing of current inactivation and increases in peak current underlie I_{CaL} facilitation which occurs during increases in stimulation rate. This effect contributes to the positive contraction-frequency relationship and is thought to be the result of CaMKII-mediated phosphorylation of I_{CaL} . Sorcini has also been shown to modulate I_{CaL} inactivation kinetics and so this study was performed to investigate the effects of CaMKII and sorcini on I_{CaL} .

Rabbit ventricular myocytes were whole-cell patch clamped using Cs- and TEA-rich solutions with 50 mM EGTA (170 nM free Ca^{2+}) in the patch pipette to spatially restrict diffusion of Ca^{2+} to ~40nm and prevent overlap of K^+ and Na^+ currents with I_{CaL} , which was activated by a depolarising voltage step (-80 mV to 0 mV).

Myocytes displayed I_{CaL} facilitation following an increase in stimulation frequency from 0.1 Hz to 1 Hz: the peak current ratio of beat 5 and beat 1 (B5/B1) was 1.2 ± 0.02 ($n=8$) and inactivation kinetics were slowed from 6 ± 0.5 ms (beat 1) ($n=8$) to 13.3 ± 2 ms (beat 5) ($n=8$, $P < 0.05$). 5 μ M KN-93 (a CaMKII inhibitor) abolished current facilitation: B5/B1 was 0.88 ± 0.02 ($n=8$, $P < 0.05$ vs Control), whilst inactivation kinetics were not significantly prolonged by KN-93 (Beat 1, 13.1 ± 3.7 ms, $n=8$; Beat 5, 19.3 ± 2.8 ms, $n=8$, $P > 0.05$). This data confirms the involvement of CaMKII in mediating facilitation of I_{CaL} .

Sorcini (3 μ M) dialysed into the cell via the patch pipette, prolonged I_{CaL} inactivation and did not prevent current facilitation

(Control beat 1, 6 ± 0.5 ms, $n=8$; Sorcin beat 1, 12.7 ± 2.3 ms, $n=6$, $P<0.05$; Control beat 5, 13.3 ± 2 ms, $n=8$; Sorcin beat 5, 21.5 ± 2 ms, $n=6$, $P<0.05$).

We investigated the involvement of CaMKII in the actions of sorcin by using KN-93. B5/B1 was significantly lower in the presence of KN-93 (Sorcin, 1.18 ± 0.05 , $n=6$; Sorcin + KN-93, 0.92 ± 0.02 , $n=7$, $P<0.05$), suggesting that facilitation was inhibited. However, current inactivation was still prolonged at beat 5 compared to beat 1 (Sorcin + KN-93 beat 1, 8.8 ± 0.4 ms; Sorcin + KN-93 beat 5, 18.8 ± 2.2 ms, $n=7$, $P<0.05$), suggesting that despite inhibition by KN-93, sorcin can still influence current inactivation kinetics.

This data suggests that sorcin does not modulate ICaL kinetics via the CaMKII pathway and that I_{CaL} facilitation operates within a cellular microdomain.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC351

Progression of heart failure correlates with a progressive increase in protein expression and activity of CaMKII δ

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CaMKII δ plays a pivotal role in the regulation of cardiac excitation-contraction coupling. Work by ourselves and others suggests that CaMKII δ is elevated in hypertrophy and heart failure (HF). However, it is unclear whether this elevation correlates with progression of heart failure. This study compares CaMKII δ protein levels and enzyme activity at two separate time points (8 weeks and 32 weeks) following coronary artery ligation (under anaesthesia) of rabbit hearts. Basal left ventricular homogenates (LVHs) were used for global CaMKII δ protein determination and activity assays. Sarcoplasmic reticulum (SR) preparations were also studied from the 8 week animals to measure localised CaMKII δ expression and activity. CaMKII δ protein expression was measured by quantitative immunoblotting using a CaMKII δ -specific polyclonal antibody (Abgent) and signals were normalised to GAPDH. Low n numbers ($n=6$) were used to specifically identify whether significant changes could be detected early at both time points. In the 32 week model CaMKII δ protein is significantly elevated in HF vs sham (0.11 ± 0.01 vs 0.08 ± 0.012 , $p=0.011$) whereas in the 8 week model using similarly low n numbers there is no detectable change in CaMKII δ (HF, 0.118 ± 0.053 vs sham, 0.117 ± 0.036). This contrasts with SR-associated CaMKII δ expression which is significantly elevated in the 8 week model (~ 2.5 -fold HF c.f. sham). CaMKII δ activity in the same LVHs was then measured using an assay kit (Upstate). In the 32 week model using similarly low n numbers as before, CaMKII δ activity was significantly up-regulated in HF vs sham (0.52 ± 0.14 vs 0.4 ± 0.11 pmol phosphate incorporated/min/ μ g total protein, $p=0.009$). At 8 weeks, although CaMKII δ activity associated with the SR was significantly increased (HF, 0.84 ± 0.18 vs sham, 0.35 ± 0.05), initial activity measurements from LVHs show no

significant change (HF, 0.63 ± 0.15 vs sham, 0.53 ± 0.18 , $n=3$). These results suggest that differences may exist between local (SR) and global measurements of CaMKII δ from the same experimental group. They also suggest the extent of increase in global CaMKII δ expression and activity in LVHs is associated with the duration of heart failure.

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PC352

Immunomodulatory effects of testosterone on fractalkine expression in human aortic endothelial and smooth muscle cells

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Cardiovascular disease is the leading cause of death in men in the UK, with atherosclerosis as the major underlying cause. Atherosclerosis is a chronic inflammatory disease and it has been documented that men with angina and heart failure often have lower levels of circulating testosterone (Eckardstein & Wu 2003). Evidence from clinical trials has shown that testosterone replacement therapy can improve symptoms of cardiovascular disease, thus indicating a potential role of testosterone as anti-inflammatory therapy in atherosclerosis (Pugh et al 2004). Fractalkine (CX3CL1) is a novel member of the chemokine superfamily and exists as two distinct forms, a membrane-bound adhesion molecule and a cleaved peptide chemotactic for cells expressing its receptor (CX3CR1). Evidence suggests that fractalkine plays a role in atherogenesis, and analysis of atherosclerotic lesions by immunocytochemistry has demonstrated its presence (Greaves et al 2001). Fractalkine is expressed on the surface of vascular cells and has been found to be up-regulated by pro-inflammatory cytokines in vitro, suggesting a role in the onset of atherosclerosis (Ollivier et al 2003, Chapman et al 2000). In order to assess whether testosterone has a modulatory effect on fractalkine expression following pro-inflammatory cytokine treatment, cell culture models have been used. Primary human aortic endothelial (HAEC) and smooth muscle cells (HASMC) cultured at 1×10^5 cells/ml between passages 3-8 were used to investigate in vitro production and expression of fractalkine following treatment with TNF- α and IFN- γ (0-100ng/ml) for up to 48 hours. Preliminary data obtained through immunocytochemistry, confocal microscopy, RT-PCR and ELISA supports the up-regulation of fractalkine under inflammatory conditions. Anti-inflammatory effects of testosterone in this in vitro system would provide further support for the use of testosterone therapy in patients with cardiovascular disease.

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PC353

Role of Nrf2 in transforming growth factor- β 1 mediated macrophage migration inhibitory factor and heme oxygenase-1 induction in aortic smooth muscle cells

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Anti-inflammatory properties of transforming growth factor- β 1 (TGF- β 1) may account for its protective role against atherosclerotic plaque rupture. The transcription factor Nrf2 mediates induction of cytoprotective genes such as heme oxygenase-1 (HO-1) via activation of antioxidant response elements (ARE). HO-1 catabolises the pro-oxidant heme to generate the vasodilator carbon monoxide and antioxidants biliverdin and bilirubin. Macrophage migration inhibitory factor (MIF) has multiple functions in inflammatory processes, exhibits catalytic thiol-protein oxidoreductase activity and plays a role in redox homeostasis. The present study investigates whether TGF- β 1 induces HO-1 and MIF expression in cultured wild-type (WT) and Nrf2 deficient (KO) mouse aortic smooth muscle cells (MASMC). In addition, nuclear translocation of Nrf2 and p53, a transcription factor which mediates apoptosis, were investigated in human aortic smooth muscle cells (HASMC) treated with TGF- β 1. Western blot analyses revealed that HO-1 and MIF expression were significantly enhanced by TGF- β 1 (0-10 ng/ml, 0-12 h, $p < 0.01$, $n = 5$) in WT but not Nrf2 KO MASMC. Induction of HO-1 in HASMC by TGF- β 1 (5 ng/ml, 12 h) was significantly ($p < 0.05$, $n = 3$) attenuated by scavenging of superoxide by superoxide dismutase (200 U/ml) or following inhibition of NADPH oxidase by diphenylionium (2 μ M). TGF- β 1 (0-10 ng/ml, 2h) elicited significant ($p < 0.05$, $n = 3$) nuclear translocation of Nrf2 and attenuated nuclear accumulation of p53 in response to hydrogen peroxide generated by glucose oxidase (10 mU/ml, 2h) in HASMC. Our novel findings suggest that TGF- β 1 may afford protection in aortic SMC by enhancing Nrf2 mediated induction of cytoprotective antioxidant genes such as HO-1 and MIF and attenuating p53 activation in response to oxidative stress. Therapeutic strategies to modulate TGF- β 1 in the vessel wall may be of benefit in atherogenesis and restenosis.

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PC354

Acute activation of nitric oxide synthase by estrogen receptor agonists in human vascular endothelial cells: Role of extracellular signal-regulated kinase, protein kinase A and Akt

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Estrogen protects premenopausal women against heart disease; however, hormone replacement therapy following menopause has been linked to breast cancer and has limited benefits on the cardiovascular system. Dietary phytoestrogens, such as genistein and the metabolite equol, may serve as selective estrogen receptor (ER) modulators that prevent vascular endothelial dysfunction through enhanced generation of the vasodilator nitric oxide. The present study investigates whether equol or specific ER agonists acutely modulate intracellular signalling pathways involved in the activation of endothelial NO synthase (eNOS). Confluent cultures of human umbilical vein endothelial cells (HUVEC) were treated (100 nM, 0-10 min) with either equol, propylpyrazoletriol (PPT), an ER α agonist, or diarylpropionitrile (DPN), an ER β agonist, in Krebs buffer containing L-arginine (100 μ M) and lysates collected for Western blot analyses. Statistical significance of quantified data derived from $n = 3-6$ independent cell cultures were evaluated using Student's unpaired t tests. Immunoblotting revealed that eNOS-Ser1177 was acutely phosphorylated by equol, DPN and PPT at 2-10 min with concurrent phosphorylation of Akt and extracellular signal-regulated kinase (ERK1/2) and cAMP-responsive element binding protein (CREB). Inhibition of phosphoinositol-3-kinase/Akt by LY294002 (10 μ M, 30 min) or cAMP-dependent protein kinase by H89 (1 μ M, 30 min) resulted in a significant attenuation of eNOS phosphorylation by equol (2 min, 100 nM, $p < 0.05$, $n = 3$). Our present study provides the first direct evidence that equol can acutely phosphorylate eNOS in HUVEC via PKA, Akt and ERK1/2 activation. These acute intracellular signalling events mediate the enhanced NO generation and vascular relaxation elicited by isoflavones and specific ER agonists.

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PC355

Ruthenium red inhibits ryanodine receptor-mediated Ca^{2+} flux and Ca^{2+} spark activity with different time courses

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Ruthenium Red (RuR) blocks conduction of Ca^{2+} through single cardiac ryanodine receptor (RyR2). Studies on isolated channels indicate that RuR acts by decreasing open probability without changing single channel conductance (Lukyanenko et al., 2000; Xu et al., 1999). This study examines the time course of RuR action on Ca^{2+} efflux from the sarcoplasmic reticulum (SR) of rabbit ventricular myocytes and compares the timecourse of the block with that the effects on Ca^{2+} sparks activity. Rabbit cardiac myocytes were enzymatically isolated from New Zealand White male rabbits killed with an intravenous injection of sodium pentobarbitone (100 mg kg⁻¹). Ca^{2+} fluxes were studied on populations cells using a cuvette-based system and Ca^{2+} sparks were monitored in single cells using confocal microscopy. For the cuvette studies, aliquots of 2×10^6 cells were permeabilised by brief exposure to 0.01 mg β -escin and the cells were placed in a cuvette containing a mock intracellular solution (1.2ml) of the following composition (mM): 0.05 EGTA, 25 Hepes, 0.9 Mg²⁺, 100 KCl, 0.01 Na⁺, pH 7.0 (20–22°C). Free [Ca^{2+}] was monitored with the addition of 10 μM Fura2 (free acid). After addition of 30 mmol of Ca^{2+} , 20mM caffeine was added to induce a large Ca^{2+} leak. Under these conditions the [Ca^{2+}] within the cuvette increased to $540 \pm 23 \text{ nM}$ (n=4). Addition of 4 μM RuR caused a rapid reduction in Fura-2 fluorescence reflecting RuR block of RyR2. The Fura-2 signal reached a steady state after ~10mins (mean value $238 \pm 35 \text{ nM}$, n=4). The block was evident immediately with a decrease to $82 \pm 4\%$ of control occurring in the 1st minute and reaching $37 \pm 6\%$ of control by 4mins.

In parallel confocal experiments, β -escin-treated cardiomyocytes were perfused with mock intracellular solutions containing ~600nM Ca^{2+} and 300 mM EGTA. On exposure to 4 μM RuR, spark frequency was not significantly different from control after 2 mins, while slightly lower ($84 \pm 13\%$) after 3 mins, and reduced to $54 \pm 13\%$ of control value after 5 mins. Remarkably, spark width, duration and amplitude remained unchanged from control values. This suggests that RyR2-mediated Ca^{2+} flux is more sensitive to RuR than Ca^{2+} sparks. The reason for this discrepancy is unknown, one possibility is that there may be a significant population of RyR's that are not associated with the diadic cleft and do not participate in Ca^{2+} sparks. These RyR2 may be more sensitive to block by RuR.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC356

Cardiac geometry and architecture affect dynamics of re-entrant scroll waves in human virtual tissues

A.P. Benson and A.V. Holden

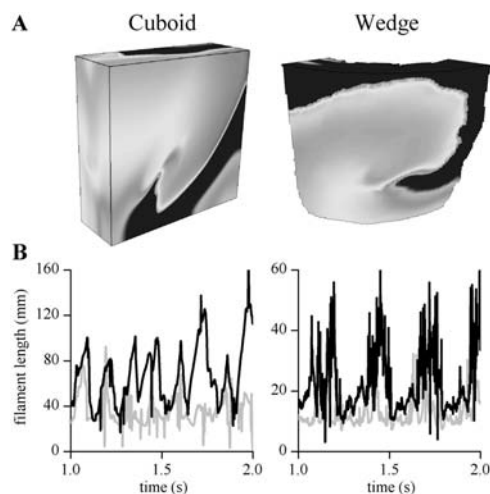
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Ventricular tachycardia (VT) and fibrillation (VF) are dangerous cardiac arrhythmias. It is thought that a single re-entrant wave of excitation (a scroll wave) that rotates around a phase singularity (a filament) underlies VT, and that breakdown of this wave into multiple wavelets results in the transition from VT to VF (Jalife 2000).

We examined effects of cardiac geometry and architecture (fibre and sheet structure - see Gilbert et al. 2007) on scroll wave dynamics by quantifying the scroll wave filament in two heterogeneous models of the human left ventricular free wall – a simple cuboid model with a rule-based architecture, and a wedge model with geometry and architecture reconstructed from human diffusion tensor MRI data. For each, excitation was described using a biophysically-detailed human model (ten Tusscher et al. 2004), and propagation of excitation was either isotropic (no architecture), anisotropic (fibre structure only) or orthotropic (fibre and sheet structure). Figure 1A shows snapshots of orthotropic re-entrant activity in the cuboid and wedge geometries after 2 s of activity.

Filament trajectories on the epicardial surfaces of the geometries were used to examine meander of the scroll wave filament. For both models, changing from isotropy through to orthotropy rescales the meander in the sheet normal direction. The lengths of the scroll wave filaments during 1 second of simulation are shown in Fig. 1B. Oscillations of filament length are evident even in isotropic simulations, a consequence of heterogeneous excitation kinetics in the models. For both models, filament length increases as anisotropy and then orthotropy are introduced. Filament curvature increases with anisotropy then orthotropy in the wedge (from 0.8 to 3.8 /mm), although this pattern is not seen in the cuboid. Conversely, filament twist increases with anisotropy then orthotropy in the cuboid (1.0 to 5.8 °/mm) but not in the wedge. Maximum twist along a single filament increases in both models as anisotropy and then orthotropy are introduced – from 51 to 445° in the cuboid, and from 64 to 161° in the wedge. Increases in filament length, curvature and twist can result in breakdown of the wave into the multiple wavelets that underlie VF (Clayton et al. 2006).

We conclude that different cardiac geometries and architectures result in different re-entrant scroll wave dynamics. Hence, simulations of VT and VF should take into account the complex geometry and fibre and sheet structure of the ventricles.



A, Snapshots of orthotropic re-entrant excitation in the cuboid and wedge models after 2 s of activity. Dark is resting tissue, light is excited. The scroll waves are rotating clockwise. B, Filament length during 1 s of activity in the two geometries for anisotropic (grey) and orthotropic (black) architectures.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC357

Transmural patterns of action potential duration are altered by activation sequence

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Background: Transmural gradients of action potential duration (APD) are known to exist in ventricular cardiomyocytes. This study investigated whether the sequence of activation across the ventricular wall would modulate transmural APD.

Methods: New Zealand White male rabbits ($n = 5$) were killed with an intravenous injection of sodium pentobarbitone (100 mg kg^{-1}). Hearts were removed, and perfused left ventricular wedge preparations were loaded with a voltage-sensitive dye (RH237). An optical mapping system was used to record transmural optical action potentials (APs) during endocardial and then epicardial stimulation at a cycle length of 350ms at 30°C . APs were sampled from points at equal distances between the two bipolar stimulating electrodes, which were arranged perpendicular to the wall. APD_{90} was calculated as the time from the steepest upstroke to 90% repolarisation. Data are presented as mean $\pm$ SEM. Comparisons between mean values for each experiment were made using a repeated measures ANOVA with a Tukey post-test.

Results: In each experiment the transmural gradient of APD_{90} was reversed following a change from endocardial to epicardial stimulation. During endocardial stimulation, mean APD_{90} was $188 \pm 0.7 \text{ ms}$ at the subendocardium, $179.8 \pm 1.6 \text{ ms}$ at the midmyocardium and $171 \pm 0.9 \text{ ms}$ at the subepicardium ($p < 0.01$ for subendocardium vs. subepicardium). During epicardial stimulation, mean APD_{90} was $176.8 \pm 2.4 \text{ ms}$ at the subendocardium, $181.4 \pm 2.4 \text{ ms}$ at the midmyocardium and $191.6 \pm 1.6 \text{ ms}$ at the subepicardium ($p < 0.01$ for subendocardium vs. subepicardium). Compared to endocardial stimulation, epicardial stimulation produced a significant lengthening of subepicardial APD_{90} ($p < 0.01$). In a subset of experiments ($n = 3$) endocardial stimulation was repeated and the transmural pattern of APD_{90} reverted to that seen originally.

Conclusions: These data demonstrate a reversal of the pattern of transmural APD_{90} following a change from endocardial to epicardial stimulation, and suggest that electrotonic influences are important in determining transmural heterogeneity of APD.

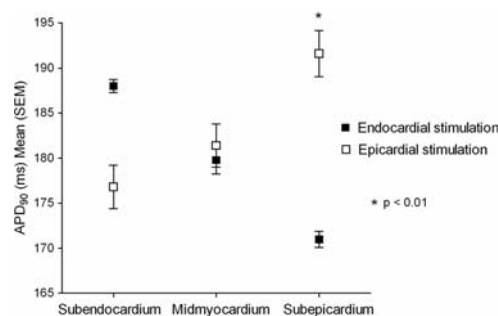


Figure 1. Mean transmural APD_{90} from five experiments during endocardial stimulation (closed squares) and epicardial stimulation (open squares).

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PC358

Discrimination of EP_1 and EP_3 prostanoid receptor systems in smooth muscle using ONO-DI-004 and ONO-AE-324

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Discrimination of EP_1 and EP_3 prostanoid receptors has mainly relied on the use of selective agonists in combination with EP_1 antagonists, although EP_3 antagonists are now emerging. We were concerned about the lack of functional data for two newer agonists, ONO-DI-004 (EP_1 -selective) and ONO-AE-248 (EP_3 -selective) (Suzawa *et al.*, 2000). Consequently, we have examined these agents on guinea-pig trachea (EP_1 preparation) and guinea-pig aorta and rat mesenteric artery (EP_3 preparations). Ring preparations obtained from male guinea-pigs (600 - 800 g) and male rats (250 - 300 g) were suspended in Krebs-Henseleit solution (95% O_2 / 5% CO_2) at 37°C in 10 ml tissue baths. Tension was recorded with a force transducer and relayed to AD

Instruments Chart software. Cumulative agonist sequences were obtained in the presence of the COX inhibitor indomethacin (1 μ M) and the TP antagonist BMS-180291 (300 nM), and under priming with 20 mM K⁺ on guinea-pig aorta.

The agonist potencies of 17-phenyl PGE₂ (EP₁ > EP₃), sulprostone (EP₃ > EP₁), ONO-DI-004 and ONO-AE-248 are shown in Table 1. EC₅₀ values for 17-phenyl PGE₂ on trachea were 0.74 - 1.3 - 2.0 nM (mean / range) and for sulprostone on aorta and mesenteric artery were 0.079 - 0.29 - 0.87 nM and 7 - 11 - 16 nM respectively. ONO-AE-248 had a shallower log concentration-response curve than sulprostone on guinea-pig aorta. The EP₁ antagonists SC-51322 (0.03 - 1 μ M) and AH-6809 (0.1 - 3 μ M) abolished established responses to 10 nM 17-phenyl PGE₂ / 1.4 μ M ONO-DI-004 on guinea-pig trachea; pA₂ values computed by the Gaddum method were 8.09 $\pm$ 0.05 / 7.99 $\pm$ 0.10 and 7.61 $\pm$ 0.04 / 7.48 $\pm$ 0.02 ($\pm$ SEM, n = 4) respectively. Comparable studies are in progress with an EP₃ antagonist.

It is clear that ONO-DI-004 and ONO-AE-248 show high selectivity for EP₁ and EP₃ receptors respectively. However, this occurs at the expense of considerable loss of potency, to the extent that it would be difficult to use these agonists in a Schild antagonism protocol. They could be used in a Cheng-Prusoff protocol (Leff & Dougall, 1993), with the advantage that their onset and offset of action are faster than those of 17-phenyl PGE₂ and sulprostone, which probably reflects their lower potencies / affinities. Table I. Relative potencies of EP receptor agonists

Preparation	Equi-effective molar ratio			
	17-Phenyl PGE ₂	Sulprostone	ONO-DI-004	ONO-AE-248
Guinea-pig trachea	1.0	5.6 $\pm$ 1.6	295 $\pm$ 15	>780 - >2000
Guinea-pig aorta	6.0 $\pm$ 0.8	1.0	>14,500 - >52,700	780 $\pm$ 240
Rat mesenteric artery *	3.5	1.0	>30	15

Values are means $\pm$ SEM; n = 4 - 9. *Between-preparations comparisons; potencies derived from mean log concentration-response curves. Suzawa T *et al.* (2000). *Endocrinology* **141**:1554-1559.

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We thank Bristol-Myers Squibb for the gift of BMS-180291. ONO-DI-004 and ONO-AE-248 were prepared for Allergan.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC359

The divergence in canine myocardial fibre orientation across transitions between positive and negative orientated cardiac sheets

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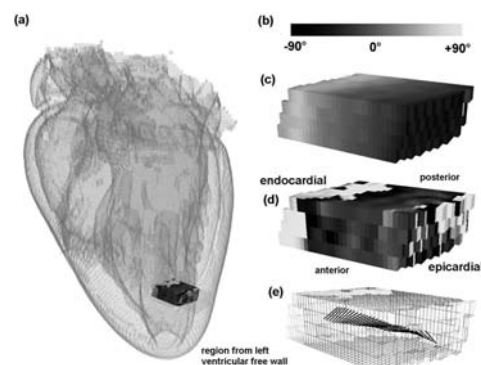
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Myocardial fibre and sheet architecture both influence the dynamics of propagation of the action potential (1,2), and, in addition, both are important determinants in the mechanism of myocardial thickening during contraction (3, 4). Diffusion Tensor - Magnetic Resonance Imaging (DT-MRI) has been validated as a method to extract fibre and sheet architecture (4). DT-MRI studies have shown that cardiac sheet structure exists as two populations, angled at -55° and +35° to the cardiac short-

axis plane, and these sheet populations are variable in distribution between canine individuals (4). The abutments of the two sheet populations are elongated circumferentially, around the left-ventricle, and are regions of large spatial gradients in sheet angle.

We examined sheet abutments in blocks of left-ventricle from DT-MRI datasets. Five cuboid regions (~30 x 20 x 5 voxels, of dimensions 0.3125 x 0.3125 x 0.9 mm) were selected from three canine hearts. Regions of rapid sheet angle change with distance were selected from throughout the left ventricle. The fibre helix, fibre transverse and sheet angles were extracted and visualised for these regions, and the fibre-orientation visualised in three dimensions. Visualisations of fibre-orientation across positive to negative sheet transitions did not reveal increased fibre-angle divergence across these zones (Figure 1). Quantitative analysis of the fibre angle divergence across sheet angle transitions was carried out on selected rows of voxels from the myocardial blocks. Voxel-to-voxel fibre divergence was found to be greater across sheet transitions (average voxel-to-voxel fibre divergence 3.5°, transition zone voxel-to-voxel fibre divergence 5.5°), but this difference was not statistically significant (at p = 0.05).

We conclude that the regular helical conformation of cardiac myofibre orientation is maintained across positive and negative sheet abutments. This has implications for the nature of fibre merging between positive and negative sheet populations (4) which must occur in a highly organised and regular fashion. This is important given the variable location of the positive and negative sheet population between individuals.



(a) Cardiac location of selected block. (b) Smooth change in the fibre helix angle during (c) discontinuous change in the sheet angle (positive to negative sheet transition) and (d) combined smooth changes in fibre orientation (black rods) with discontinuous changes in sheet angle (shaded grid). (e) Monochrome angular scale.

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DT-MRI datasets from Patrick Helm and Raimond Winslow at The Center for Cardiovascular Bioinformatics & Modeling and Elliot McVeigh at the National Institute of Health. This work is supported by the European Union through the Network of Excellence BioSim, Contract No. LSHB-CT-2004-005137, and a studentship from the MRC (SHG).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC360

Differential effect of high glucose in human mesenteric and subcutaneous arteries

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The effect of glucose on endothelial function is a critical factor in the pathology of vascular disease in diabetes. Previous studies have yielded conflicting results with increases in glucose causing attenuated vasodilatation (Bohen & Lash 1993), enhanced vasodilatation (Cipolla et al 1997) or no change in vascular tone (Brands & Fitzgerald 1998). Indeed we have recently reported an enhanced vasodilatation in the presence of high glucose (Cooper et al 2006). One possible explanation for the contrasting results is that hyperglycaemia may have different effects in differing vascular beds. Here we examine the effects of high glucose on endothelial-dependent & -independent relaxation in two sets of human arteries.

Mesenteric arteries (MA; 546±27µm, n>8) & subcutaneous arteries (SA; 643±67µm, n=7), obtained from patients undergoing elective abdominal (n=5) or breast reduction surgery (n=4) at the Glasgow Royal Infirmary, were dissected, mounted on a wire myograph, normalised & left to equilibrate (60mmHg, 37°C, 20mins). Endothelial-dependent relaxations induced by acetylcholine (ACh; 1nM-10mM) & bradykinin (Bk; 0.1nM-1mM), & endothelial-independent relaxations induced by the NO-donor glycerol trinitrate (GTN; 0.1nM-1mM) were assessed in pre-contracted vessels (EC₈₀ U46619) before and after incubation for 2h in either control (5mM glucose) or high glucose Krebs (20mM glucose). Data is expressed as mean±sem & analysed using two way ANOVA or paired t-tests.

Incubating vessels in 5 or 20mM glucose for 2 hours had no effect on basal vascular tone or vessel constriction to U46619 (EC₈₀=25±2 & 27±4kPa in 5mM glucose compared with 25±2 & 22±2kPa in 20mM glucose in MA & SA respectively, p>0.05). After incubation for 2h in 5mM glucose ACh, Bk & GTN all elicited dose dependant relaxations with maximums of 54±9, 75±8 & 71±6% respectively in MA & 75±9, 50±12 & 63±7% respectively in SA. When MA were incubated in high glucose (20mM) for 2h, relaxation to both ACh (maximum=72±9%, p=0.01) & Bk were enhanced (EC₅₀=7.8±0.3nM vs 85.1±0.4nM in 5mM glucose, p=0.01). In contrast, in SA, incubation in high glucose for 2h significantly attenuated relaxation in response to ACh (maximum=58±15%, p<0.01) & Bk (maximum=37±13%, p=0.05). GTN induced relaxations were unaffected by high glucose in both MA (maximum=75±6%) & SA (maximum=56±13%).

These results demonstrate that high glucose has no effect on the basal release of endothelial relaxants or the ability of NO to induce smooth muscle relaxation. However the effects of high glucose on endothelial-dependent relaxations are clearly dependant on the vascular bed studied. These differences may be an

adaptive response due to variations in the glucose requirements of the different vascular beds.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC361

The effect of chromium on the oxygen uptake capacity of blood

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Many metallic implants used in orthopaedics are made of stainless steel or cobalt-chromium alloys which contain 18-30% chromium. Hexavalent chromium has been shown to be the predominant form of chromium released following *in vivo* and *in vitro* corrosion of these metal implants (Merritt & Brown, 1995). Blood chromium levels may be elevated between 50 and 250 times in patients with metal hip implants (Lhotka et al., 2003). At physiological pH, hexavalent chromium exists predominantly as the chromate anion and as such can enter cells via non-specific anion channels. The anionic hexavalent chromium diffuses readily through the red blood cell (RBC) membrane and is bound by the haemoglobin probably after its rapid reduction to the cationic trivalent state within the RBC (Gray & Sterling, 1950). The aims of this study were;

i. To investigate the effect of the presence of chromium in RBCs on oxygen uptake [Oxyhaemoglobin saturation (O₂-SAT) at specific pO₂ values]

ii. To evaluate the suitability of the two commonly used anticoagulants, heparin and EDTA, for oxygen uptake studies. Blood stabilized by either EDTA or sodium heparin was spiked with clinically relevant concentrations of hexavalent chromium ranging from 0-40µg/L. The spiked blood was equilibrated with two different gas mixtures (2.5% O₂; 5% CO₂; balance N₂ and 5% O₂; 5% CO₂; balance N₂) for 20 minutes. The pH, pO₂, pCO₂ and O₂-SAT were measured using an aliquot of the blood after equilibration.

A single factor analysis of variance (ANOVA) showed no significant difference (p>0.05) between experimental O₂-SAT values in the presence and absence of hexavalent chromium in blood stabilized by either anticoagulant. No significant difference was observed between the experimental O₂-SAT values in heparinised blood and O₂-SAT values predicted from standard oxygen dissociation curve nomograms at the same pO₂ and acid-base status. In contrast, the experimental values using EDTA treated

blood were significantly higher than the theoretically expected values ($p < 0.05$).

This shows that:

- i. The presence of chromium in RBCs does not significantly affect the oxygen uptake capacity of haemoglobin.
- ii. Heparin is a more suitable anticoagulant for blood pH and blood gas analysis than EDTA. This is probably due to the ability of the latter to chelate metal ions like Ca^{2+} and interfere with blood chemistry by decreasing the CO_2 combining power of blood.

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PC362

Beneficial effects of Rho-kinase inhibition on the development of pulmonary hypertension in mice that overexpress the 5HT transporter

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Pulmonary arterial hypertension (PAH) can be characterised by vasoconstriction and remodelling of the pulmonary artery. Patients with familial PAH can demonstrate increased expression/activity of the 5HT transporter (5HTT) (1). Rho-kinase (ROCK) may play a role in vasoconstriction (2) and remodelling of the pulmonary arteries (3). In addition to this, there may be cross-talk between 5HT and the ROCK signalling pathways (4). Here we investigate the role of ROCK in chronic hypoxia-induced PAH in wild-type (WT) mice and mice that over-express the 5HTT (5HTT) (5).

Female mice (C57BL/6 x CBA, WT and 5HTT+, 5-6 months) were maintained in hypoxic (10% O_2) or normoxic conditions for 14 days. Mice were dosed daily with vehicle or the ROCK inhibitor Y27632 (30mg/kg) by oral gavage. Subsequently, under anaesthesia, systolic right ventricular pressure (sRVP) was obtained by direct, transdiaphragmatic, right ventricular cannulation. Right ventricular hypertrophy (RVH) was also assessed by determining the ratio of right ventricular free wall to left ventricular plus septum (RV/LV+S). RNA extracted from whole lung tissue was also analysed by real-time fluorogenic reverse transcription-PCR in order to determine the relative levels of ROCK 1 transcript abundance.

Under normoxic conditions 5HTT+ mice had elevated sRVP compared to WT (WT: 18.7 ± 1.2 mmHg ($n=8$), TG: 27.6 ± 3.4 mmHg, $p < 0.05$). Exposure to chronic hypoxia was found to significantly increase sRVP in both WT and 5HTT+ mice (WT hypoxia: 30.5 ± 1.8 mmHg, $n=5$, $p < 0.01$ versus normoxic. 5HTT+ hypoxic: 41.7 ± 3.1 mmHg, $n=5$, $p < 0.01$ versus normoxic). Treatment with Y27632 attenuated this response in WT (24.5 ± 1.6 mmHg, $n=8$, $p < 0.05$ versus hypoxia) and 5HTT+ mice (23.8 ± 3.1 , $n=8$, $p < 0.01$ versus hypoxia). Chronic hypoxia

also increased RVH in both WT and 5HTT+ mice (WT: 0.27 ± 0.01 , $n=12$ to 0.39 ± 0.01 , $n=15$, $p < 0.001$. 5HTT+: 0.29 ± 0.03 , $n=14$ to 0.39 ± 0.01 , $n=15$, $p < 0.001$). Dosing with Y27632 had no effect on RVH in WT mice (0.35 ± 0.02 , $n=9$) but significantly reduced RVH in 5HTT+ mice (0.33 ± 0.01 , $n=9$, $p < 0.001$ versus hypoxic). Moreover, levels of ROCK 1 transcript were significantly elevated by chronic hypoxia in 5HTT+ mice (5.9 ± 1.5 , $n=5$ to 61 ± 16.6 , $n=5$, $p < 0.01$) but not in WT mice (7.9 ± 1.4 , $n=5$ to 27.6 ± 5.7 , $n=5$).

In summary, the beneficial effects of ROCK inhibition on sRVP are more pronounced in 5HTT+ mice. Furthermore, the ability of Y27632 to reduce right ventricular hypertrophy in 5HTT+ mice but not WT mice suggests cross-talk between 5HT and ROCK signalling systems. This is consistent with the marked elevation in the expression of ROCK 1 observed in the lungs of 5HTT+ mice in response to hypoxia.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC363

TNF α and IL-1 β impair pulmonary artery endothelial dependent relaxation through different mechanisms

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Tumour necrosis factor- α (TNF α) and interleukin 1 β (IL-1 β) are implicated in inflammation-associated disturbances in vascular reactivity (1). The present study aims to determine the mechanism of impairment of pulmonary artery endothelial-dependent relaxation by TNF α and IL-1 β .

Male Wistar rats (290 g) were humanely killed by cervical dislocation. Pulmonary artery branches were dissected, cut into 2 mm rings and suspended in Krebs' buffer for measurement of changes in tension. After preconstriction with phenylephrine, acetylcholine (ACh) was added before and after 2h incubation with or without cytokines. In some experiments the selective iNOS inhibitor 1400W (1 μM), the superoxide dismutase mimetic tempol (1mM), the NADPH oxidase inhibitor apocynin (300 μM), the xanthine oxidase inhibitor allopurinol (1mM) or the eNOS cofactor tetrahydrobiopeptin BH4 (3 μM) were added 30 min before the second addition of ACh. The changes in maximal relaxation (E_{max}) and $p\text{EC}_{50}$ due to incubation were compared between treatments using ANOVA followed by Newman-Keuls' post-hoc tests.

Following treatment with TNF α (1 ng.ml⁻¹, 2h), E_{max} values for ACh-induced relaxation of the pulmonary artery were reduced by 28.8 ± 6.1 % compared with a reduction of 6.9 ± 5.7 % in

matched controls ($p < 0.05$), and pEC50 values were increased by 1.03 ± 0.17 compared with 0.35 ± 0.07 log units in controls ($p < 0.001$). These TNF α -induced changes were prevented by preincubation with either 1400W, tempol, BH4 or apocynin, resulting in Emax changes of 5.1 ± 3.7 ; 7.2 ± 5.0 and 1.1 ± 6.5 % in the case of tempol, BH4 or apocynin respectively (all $p < 0.05$ compared with TNF α alone) and pEC50 shifts of 0.45 ± 0.06 ($p < 0.01$), 0.50 ± 0.11 ($p < 0.05$) and 0.51 ± 0.04 log units ($p < 0.01$) respectively compared with TNF α alone. Preincubation with allopurinol had no significant effect on TNF α induced changes. Treatment of pulmonary artery rings with IL-1 β ($1 \text{ ng} \cdot \text{ml}^{-1}$) also led to a reduction in Emax (31.5 ± 4.2 compared with 5.6 ± 1.8 % in controls, $p < 0.001$) and an increase in the pEC50 shift (1.08 ± 0.19 compared with 0.19 ± 0.05 log units in controls, $p < 0.001$). These IL-1 β -induced changes were prevented by preincubation with tempol or apocynin resulting in Emax changes of 10.5 ± 5.7 ($p < 0.05$) and 2.8 ± 4.9 % ($p < 0.01$) respectively and a pEC50 shift of 0.29 ± 0.21 log units ($p < 0.01$) in the case of apocynin compared with IL-1 β alone. Preincubation with 1400W, BH4 or allopurinol had no significant effect on IL-1 β induced changes.

In conclusion, TNF α produces its effect through stimulation of NADPH oxidase and iNOS leading to eNOS uncoupling. IL-1 β stimulates superoxide release via NADPH oxidase; however iNOS and eNOS uncoupling do not have a major role in the IL-1 β induced impairment.

Vila E & Salaices M (2005). *Am J Physiol* 288, H1016-H1021.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC364

The pharmacological characterization of P2X receptors in rat pulmonary artery

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P2X receptors are ligand-gated cation channels that are activated by adenosine 5-triphosphate (ATP) and expressed throughout the body (North, 2002). The P2X agonist α, β -methyleneATP (α, β -meATP) evokes vasoconstriction of the rat isolated large (LPA) and small (SPA) pulmonary arteries, but possibly via different P2X receptor subtypes (Chootip *et al.*, 2002). The aim of this study was to further characterise and compare the pharmacological properties of the P2X receptors present in rat LPA and SPA.

5 mm rings of rat LPA and SPA were mounted under isometric conditions in 1 ml baths at 37°C and at resting tensions of 1 g and 0.5 g respectively. In some tissues the endothelium was removed by gentle rubbing of the intima. Tension was recorded by Grass FT 03 transducers connected to a Powerlab/4e system (AD Instruments). Contractions were elicited by addition of α, β -meATP to the bath. Data are expressed as mean $\pm$ sem and were compared by Student's t-test, one-way ANOVA and Tukey's comparison or two-way ANOVA as appropriate. Concentration-inhibition response curves for antagonists were fitted to the data by logistic (Hill equation), non-linear regression analysis.

α, β -meATP (10 μM) evoked reproducible contractions of LPA and SPA rings and the peak amplitudes were unaffected by removal of the endothelium. However, the combined mean responses in the LPA (250 ± 27 mg, $n=13$) were significantly greater than those of the SPA (144 ± 11 mg, $n=13$) ($P < 0.0001$). The contractions were not maintained in the continued presence of α, β -meATP and initially decayed at a similar rate in both tissues. However, on repeated administration of α, β -meATP, the decay of the contraction in the SPA became significantly faster than the LPA (decay from peak at 5 min: SPA- 53.7 ± 8.1 %, LPA- 19.8 ± 5.5 %, $P < 0.05$). In both preparations α, β -meATP (100 μM) induced significantly greater contractions than 10 μM α, β -meATP ($n=6$, $P < 0.05$) and continuous exposure to α, β -meATP (100 μM) for 20 min abolished contractions to subsequent addition of α, β -meATP (10 μM). Finally, the ability of P2X receptor antagonists to inhibit the contractions evoked by α, β -meATP (10 μM) was determined. When added cumulatively, each antagonist inhibited the contractions in a concentration-dependent manner and abolished them at the highest concentrations used, with a rank order of potency of NF449 > PPADS=suramin (LPA- $n=3-5$, SPA- $n=3-7$). There was no significant difference in the potency of any antagonist in the LPA compared to the SPA.

Thus, these data show that α, β -meATP evokes reproducible, transient contractile responses, with a similar pharmacological profile in the rat SPA and LPA. Furthermore, this profile is consistent with the expression of the P2X₁ receptor subtype along the length of the rat pulmonary arteries.

Chootip, K, Ness, K.E, Wang, Y., Gurney, A.M. & Kennedy, C. (2002). *Br. J. Pharmacol.*, **137**, 637-646.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC365

Pharmacological reactivity of pulmonary arteries in streptozotocin-induced diabetes

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Poor control of blood glucose in diabetes causes systemic hypertension, which contributes to the development of organ damage. The underlying cause is peripheral vasoconstriction¹ mediated by endothelial dysfunction² and enhanced smooth muscle contractility³. Studies on humans⁴ and rat lungs⁵ suggest that diabetes also affects the pulmonary circulation. We have investigated the reactivity of isolated, intrapulmonary arteries (IPA) to vasoactive agents in a rat model of diabetes.

Diabetes was induced in male Wistar rats by i.p. injection of streptozotocin (STZ; 60 mg/kg), with age-matched controls receiving equivalent vehicle. Animals were sacrificed at 3-4 months, after measuring body weight and blood glucose, and conduit and resistance (<250 μm i.d.) IPA isolated for wire myography. Experiments were performed at 37°C in solution containing (mM): NaCl 120, KCl 5, KH₂PO₄ 0.5, Na₂HPO₄ 0.5, CaCl₂ 1.8, MgCl₂

1, HEPES 10; pH 7.3. Procedures accorded with current UK legislation. Data are expressed as mean $\pm$ s.e.m. of *n* animals and compared using Student's *t*-test.

At sacrifice, STZ-treated rats weighed 270 ± 7 g with blood glucose 250 ± 26 mg/dl (*n*=17), values significantly ($P < 0.001$) different from controls (360 ± 15 g, *n*=13; 68 ± 4 mg/dl glucose). All vessels displayed concentration-dependent constriction to raised extracellular K^+ . In small vessels, the response was maximum at 50 mM K^+ and decreased at higher concentrations, whereas in conduit vessels contraction increased in amplitude up to 110 mM. Concentration-effect curves for K^+ were, however, similar in control and STZ vessels. Phenylephrine (PE) and serotonin (5HT) constricted conduit vessels with PEC_{50} values of 8.1 ± 0.2 and 6.1 ± 0.3 , respectively, and maximum responses $72 \pm 18\%$ (*n*=5) and $81 \pm 13\%$ (*n*=4) of the response to 50 mM K^+ . In small vessels, the 5HT concentration-response relationship was similar, but PE had its maximum effect at 100 nM, becoming ineffective at higher concentrations. $PGF_{2\alpha}$ constricted small IPA with $PEC_{50} = 5.6 \pm 0.1$ and maximum response $78 \pm 13\%$ (*n*=4) of the K^+ contraction, but in conduit vessels the response failed to reach maximum at 10 μ M, where it was nevertheless 100 $\pm$ 22% of the K^+ response. STZ treatment did not significantly alter the concentration-effect curves of any agonist. In contrast, STZ treatment inhibited endothelium-dependent relaxation to carbachol. This was most apparent in conduit vessels, where the maximum relaxation was reduced from $106 \pm 12\%$ (*n*=4) in controls to $44 \pm 12\%$ (*n*=3) in STZ-treated vessels ($P < 0.05$). The results imply that, like systemic vessels, diabetes causes dysfunction of the pulmonary artery endothelium, but the reactivity to a range of vasoconstrictors is preserved.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC366

The contribution of Ca^{2+} -sensitisation to P2Y receptor-mediated vasoconstriction of rat pulmonary artery

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P2Y receptors are a family of G protein-coupled receptors that are activated by endogenous nucleotides, such as ATP, UTP and UDP. In the vascular system these agonists induce vasodilation via endothelial P2Y receptors and vasoconstriction via P2Y receptors located on arterial smooth muscle cells (Chootip *et al.*, 2002). The intracellular signalling mechanisms by which vasoconstriction is induced is poorly characterised, so the aim of this study was to determine the role of Ca^{2+} -sensitisation of the contractile proteins in nucleotide-evoked vasoconstriction of rat small intrapulmonary artery (SPA).

5 mm rings of rat SPA, from which the endothelium was removed by gentle rubbing of the intima, were mounted under isometric conditions in 1 ml baths at 37°C and a resting tension of 0.5 g. Tension was recorded by Grass FT 03 transducers connected to a Powerlab/4e system (AD Instruments). Contractions were elicited by addition of agonists to the bath.

UTP and UDP (both 300 μ M) evoked slowly developing contractions, which reached a peak within 5 minutes and decayed slowly in the continued presence of agonist. The contractions were of similar amplitude when UTP and UDP were administered at 30 minute intervals. Both protein kinase C (PKC) and rho kinase have been implicated in Ca^{2+} -sensitisation of smooth muscle, therefore we investigated the effects of selective inhibitors of these enzymes. The PKC inhibitor GF 109203X (3 μ M) abolished contractions evoked by the PKC activator phorbol 12-myristate 13-acetate (PMA) (10 μ M) when applied beforehand and reversed the contractions if applied once they had developed. In contrast, it had only a small effect on the UTP and UDP-evoked responses. The rho kinase inhibitor Y27632 (10 μ M) had no effect on PMA-induced responses, consistent with a selective action against rho kinase, but caused a moderate inhibition of nucleotide-evoked contractions.

These results indicate that Ca^{2+} -sensitisation is involved in P2Y receptor-mediated contraction of rat pulmonary artery. Furthermore, rho kinase plays a greater role than PKC. Other signalling mechanisms, most likely Ca^{2+} influx and release, also appear to be involved and make a greater contribution.

Chootip, K., Ness, K., Wang, J., Gurney, A.M. & Kennedy, C. (2002). *Br J Pharmacol* 137, 637-646.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC367

Genetic factors influence dexfenfluramine-induced vasoconstriction in mouse pulmonary arteries

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Dexfenfluramine (Dfen) is an appetite suppressant associated with an increased risk of pulmonary arterial hypertension (PAH), a disease characterised by vasoconstriction and remodelling of pulmonary resistance arteries (PRAs). Dfen is a 5-HT reuptake inhibitor and substrate for the 5-HT transporter (5-HTT). Increased expression of the 5-HTT is implicated in the pathogenesis of PAH (Eddahibi *et al.* 2000, MacLean *et al.* 2004). Heterozygous loss of function mutations in the bone morphogenetic protein receptor II (BMPR-II) were found to occur at a high frequency in pulmonary hypertensive patients (Thomson *et al.* 2000). Recent evidence has indicated an interaction between 5-HT and reduced BMPR-II signalling showing that 5-HT can uncover a pulmonary hypertensive phenotype in mice deficient in BMPR-II (BMPR-II +/- mice) (Long *et al.* 2006).

We assessed the ability of dfen to contract PRAs in the mouse and whether this is affected by overexpression of 5-HTT and/or

deficiency in BMPR-II. We employed three transgenic mouse models, one overexpressing the human form of 5-HTT (5-HTT +), another BMPR-II +/- and one both 5-HTT + and BMPR-II +/- (all mice were age 5-6 months and 25-40g weight). Vasoconstriction was assessed by wire myography. Under halothane (1%; inhalation) anaesthesia, we also analysed the pulmonary haemodynamics of the 5-HTT + and 5-HTT + /BMPR-II +/- mouse using transdiaphragmatic cardiac puncture to measure systolic right ventricular pressure (sRVP). Statistical comparisons were made by one-way ANOVA followed by Tukey's multiple comparison test.

Dfen induced contraction of PRAs only at high concentrations ($> 1 \mu\text{M}$). It was significantly more potent in 5-HTT + (pEC₅₀, 5.33 ± 0.12 , n=6) and 5-HTT + /BMPR-II +/- (pEC₅₀, 5.55 ± 0.12 , n=9) mice than in wild type (pEC₅₀, 3.97 ± 0.12 , n=6) and BMPR-II +/- (pEC₅₀, 3.72 ± 0.10 , n=4) mice (p<0.001). The Emax values were not significantly different.

Wild type mice had a sRVP of 12.5 ± 1.1 mmHg, n=12 and this was significantly lower (p<0.001) than in 5-HTT + mice (31.1 ± 4.1 mmHg, n=10). 5-HTT + /BMPR-II +/- mice had a pressure of 19.0 ± 0.10 mmHg, n=11. Interestingly this was significantly lower than 5-HTT + mice (p<0.01).

Dfen-induced vasoconstriction was increased in mice that overexpress the 5-HTT both in mice with intact and deficient BMPR-II signalling. Dfen may increase extracellular 5-HT by preventing 5-HT uptake or may exert intracellular responses similar or perhaps greater than 5-HT by acting as a 5-HTT substrate.

Overexpression of the 5-HTT caused an increase in sRVP in our mouse model. This effect was reduced by a deficiency in BMPR-II. The reason for this protective effect is unclear. BMPR-II interacts with several intracellular signalling pathways including 5-HT and elucidating these will allow a better understanding of the observed phenotype.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC368

Pharmacological evaluation of the contractile phenotype of cultured mouse pulmonary arteries

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Short term culture of blood vessel explants has gained increasing interest, as it provides opportunities for new approaches to studying biological function in an integrated in vitro cell system¹. As cell phenotype can change in culture, it is essential to understand how culture affects vessel function before employing it for studies with gene knockouts. We therefore undertook

characterization of the pharmacological properties of mouse pulmonary artery explants during the first few days of culture. Intrapulmonary arteries (IPA) were harvested from Balb/c mice, divided into short segments and incubated in DMEM supplemented with 1% penicillin-streptomycin, 4.5 g/l glucose, L-glutamine, and 25mM HEPES, in 96 well plates placed in a humidified incubator at 37°C under 5% CO₂ in air. Responses to compounds known to modulate mechanical activity of IPA, and to ion channel blockers, were investigated in fresh tissue and explants after 48, 72 or 96 hours in culture, using wire myography. Data are expressed as mean $\pm$ s.e.m. of n experiments and compared using Student's t-test.

The contractile response to high potassium challenge (50mM KCl) was preserved up to 96 hours in culture. Responses to other agents are expressed as a percentage of the KCl response. Contraction to 10⁻⁵M phenylephrine (PE) was also conserved: $144 \pm 5\%$ (n=8) in fresh IPA; $117 \pm 35\%$ (n=25) at 96h. Ca²⁺-free solution did not elicit a response in fresh IPA, but promoted an increasing relaxation after 48, 72 and 96h: respectively $-5 \pm 1\%$ (n=7), $-70 \pm 37\%$ (n=12) and $-337 \pm 98\%$ (n=25). IPA at 48h were more sensitive to 10mM KCl ($76 \pm 6\%$, n=7) than fresh vessels ($8 \pm 2\%$, n=8, $P < 4.10^{-3}$). The TASK channel inhibitor, bupivacaine (10⁻⁴M), caused increasing contraction from fresh IPA ($4 \pm 5\%$, n=8) to 48h culture ($65 \pm 5\%$, n=7), which dropped at 96h even to relaxation in some vessels ($-27 \pm 39\%$, n=25). The Kv channel inhibitor 4-aminopyridine (4-AP; 10⁻³M) elicited little contractile response in fresh vessels ($7 \pm 1\%$, n=8), but pronounced contraction after 72 and 96h (respectively $104 \pm 22\%$, n=12, and $84 \pm 44\%$, n=25). Finally, although the L-type calcium channel blocker, nifedipine (10⁻⁶M), had no effect on fresh IPA, it induced relaxation that increased with the duration of culture: $-11 \pm 1\%$ (n=4) at 48h; $-33 \pm 12\%$ (n=12) at 72h; $-409 \pm 111\%$ (n=25) at 96h. Our data show that mouse IPA remain responsive to KCl and PE up to 96 hours in culture. However, responses to Ca²⁺-free solution, nifedipine and 4-AP are modified in a manner that may be explained by progressive cell depolarisation during the culture. The data are consistent with recent work¹ where increased reactivity to high potassium challenge was observed in rat pulmonary artery cultured for 4 days.

Guibert C et al. (2005). Br J Pharmacol 146, 692-701.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC369

Signalling mechanisms underlying P2Y receptor-mediated vasoconstriction in pulmonary arteries

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When endogenous nucleotides are released within the vascular lumen they can cause vasodilation via P2Y receptors on endothelial cells, but if the endothelium is damaged then they may evoke vasoconstriction via P2Y receptors on smooth muscle cells (Burnstock & Knight, 2004). The P2Y agonists ATP, UTP and UDP evoke vasoconstriction in rat pulmonary artery (Chootip

et al., 2002), but little is known about the signalling mechanisms through which they act. The aim of this study was to establish the roles of Ca^{2+} signalling pathways in these P2Y receptor-evoked contractions.

Small intrapulmonary arteries were dissected post mortem from rats (200–300g). The endothelium was removed and the arteries mounted on intraluminal wires under isometric conditions. UTP and UDP (both 300 μM) evoked slowly developing, tonic contractions, which reached a peak within 5 minutes. 300 μM ATP also evoked a slowly developing contraction, but this response was not maintained and decayed back to close to basal levels over the same time-course.

Upon removal of extracellular Ca^{2+} , the contractile response of each nucleotide was significantly decreased ($P < 0.001$) by 50–80%. The responses of UTP and UDP in the Ca^{2+} -free salt solution were significantly larger than the response of ATP in the same conditions ($P < 0.01$). In the presence of the Cav1.2 channel blocker, nifedipine (1 μM), the responses to ATP, UTP and UDP were significantly ($P < 0.001$) decreased by around 50%. The role of Cav1.2 channels on maintaining nucleotide-evoked contractions was also investigated. Nifedipine was added when the contraction to UTP or UDP had decreased from the peak and reached a plateau. Nifedipine caused a significant relaxation of the maintained contraction of both UTP and UDP such that they were 20–30% of the control response over the same time course, but in the absence of nifedipine ($P < 0.001$). Pre-administration of niflumic acid (10 μM), a blocker of Ca^{2+} -dependent Cl^- channels, significantly depressed the responses to ATP, UTP and UDP to approximately 40% of control ($P < 0.001$). Addition of niflumic acid (10 μM), 20 min after UTP and UDP had been administered and their contractions had reached a plateau, caused a significant inhibition in both cases ($P < 0.001$). There was a more marked effect on UTP compared to UDP, but this difference was not significant.

These results indicate that influx of extracellular Ca^{2+} via Cav1.2 channels, release of Ca^{2+} from intracellular stores and Ca^{2+} -dependent Cl^- channels play a significant role in the induction of the P2Y-mediated pulmonary vasoconstriction. Furthermore, these data provides evidence that Cav1.2 channels and Ca^{2+} -dependent Cl^- channels are important in the maintenance of pulmonary artery contraction in response to UTP and UDP.

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Chootip, K., Ness, K., Wang, J., Gurney, A.M. & Kennedy, C. (2002). *Br. J. Pharmacol* **137**, 637–646.

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response of rat pulmonary arteries to the TXA2 mimetic, U46619. Concentration response curves were performed in the presence or absence of the VOCC blocker, nifedipine, the IP3 receptor antagonist 2-APB and Rho-kinase inhibition. To investigate the influence of the endothelium on the contractile mechanisms experiments were performed in endothelium-intact and denuded arteries.

Male Wistar rats (200–250 g) were killed by cervical dislocation. Ring segments 0.2–0.4cm in diameter were mounted on a small vessel wire myograph with a resting tension of 9.81mN in PSS gassed with 95/5% O_2/CO_2 at 37°C. Changes in isometric tension were recorded using Powerlab data collection and Chart 5 software. Tissues were allowed to equilibrate for 1 hour then contracted with 60mM KCl then washed. Drugs were incubated for 45mins, and cumulative concentration response curves (CRC) to U46619 constructed. The endothelium was denuded by gentle abrasion of the intimal surface. Results are expressed as a percentage of the KCl-induced contraction and are the means $\pm$ S.E.M. Statistical analysis was carried out using Student's t-test and $p < 0.05$ is considered significant.

The U46619 (1nM–3mM) CRC (pEC50 and Rmax, 7.83 ± 0.04 , $130 \pm 3\%$, $n=5$) was unaffected by nifedipine (1 μM) or 2-APB (30 μM) in endothelium-intact tissue. Removal of the endothelium shifted the CRC to the left and this curve was shifted to the right and the maximum response reduced by nifedipine or 2-APB and markedly reduced by the combination of nifedipine and 2-APB, U46619 pEC50 and Rmax, -ENDO, 8.11 ± 0.06 , $128 \pm 4\%$, $n=5$; nifedipine, 7.23 ± 0.06 , $103 \pm 3\%$, $n=5$; 2-APB 7.98 ± 0.09 , $104 \pm 4\%$, $n=5$; nifedipine + 2-APB, 6.83 ± 0.12 , $57 \pm 4\%$, $n=5$. The Rho-kinase inhibitor Y-27632 (30 μM) caused a rightward shift and a reduced maximum in both endothelium-intact and endothelium-denuded rings. pEC50 and Rmax, +ENDO, 7.83 ± 0.04 , $130 \pm 3\%$, $n=5$; Y-27632, 6.58 ± 0.09 , $67 \pm 4\%$, $n=5$; -ENDO, Y-27632, 7.14 ± 0.05 , $89 \pm 3\%$, $n=5$. The combination of the three in endothelium-denuded rings abolishes the response. This study shows that Rho-kinase is involved in the contractile response to U46619 in endothelium-intact and denuded arteries. In endothelium-denuded but not intact rings the response involves Ca^{2+} influx through a nifedipine-sensitive VOCC and a 2-APB-sensitive mechanism possibly IP3-mediated Ca^{2+} release. We have reported previously in intact rings the response to U46619 involves Rho-kinase and a chloride sensitive mechanism.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC370

U46619-induced contractile responses in rat pulmonary arteries: influence of the endothelium

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Thromboxane A2 (TXA2) is a pulmonary vasoconstrictor that has been implicated in the development of pulmonary hypertension. The present study investigated the involvement of voltage-operated calcium channels (VOCC), the inositol 1,4,5-trisphosphate (IP3) receptor and Rho kinase in the contractile

PC371

The effect of over-expression of the 5-HT transporter on vascular reactivity to 5-HT: role of Ca^{2+} influx

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Mice over-expressing the serotonin transporter (5-HTT+) are pre-disposed to hypoxia-induced pulmonary hypertension.¹ Pulmonary arteries removed from 5-HTT+ mice, however, exhibit reduced sensitivity to 5-HT-induced contraction when compared to wild-type (WT). We investigated the possible mechanisms

behind this by examining the influences of membrane potential and Ca^{2+} entry in intrapulmonary arteries (IPAs) from WT and 5-HTT+ mice.

Mice (C57BL/6, 5-6 month, 25-40g) were euthanised (sodium pentobarbitone 2g/kg) and IPAs dissected out and mounted on wire myographs (Krebs; 37°C). Cumulative concentration-response curves were determined for 5-HT (10^{-9}M - 10^{-4}M) in the presence and absence of nifedipine (inhibits voltage-operated Ca^{2+} channels), NiCl_2 (inhibits store-operated Ca^{2+} entry, SOCE), 2-APB (inhibits IP3-induced Ca^{2+} release and SOCE) and at different extracellular K^+ concentrations (5, 20 and 50mM). Responses to 10 μM 5-HT were also examined in Ca^{2+} -free solution. Contractile responses are expressed as % response to 50mM KCl. Statistical analysis used Student's t-test, $p < 0.05$ considered significant. 5-HT was more potent in WT compared to 5-HTT+ (pEC50 = 6.9 ± 0.08 , $n=12$ and 5.3 ± 0.1 , $n=6$, respectively, $P < 0.001$). Removing extracellular Ca^{2+} abolished the contraction induced by sub-maximal 5-HT (10 μM) in both WT ($2.4 \pm 3.7\%$ cf. control $60 \pm 9\%$ $n=6$, $P < 0.001$) and 5-HTT+ ($8 \pm 2\%$ cf. control $70 \pm 7\%$ $n=5$, $P < 0.001$). Nifedipine (5 μM) partially inhibited 5-HT-induced contraction in WT and 5-HTT+ mice, significantly reducing the maximum response (Emax): WT: $98 \pm 7\%$ cf. $64 \pm 5\%$, $P < 0.01$; 5-HTT+: $86 \pm 5\%$ cf. $59 \pm 2\%$, $P < 0.001$. In nifedipine pEC50 values were 5.8 ± 0.2 , $n=7$ (WT) and 5.29 ± 0.06 , $n=6$ (5-HTT+), $P < 0.05$. NiCl_2 (50 μM) reduced pEC50 values in both preparations: WT pEC50 = 6.75 ± 0.06 $n=6$, cf. 7.0 ± 0.1 $n=6$ $P < 0.001$; 5-HTT+ pEC50 = 5.94 ± 0.04 $n=8$, cf. 5.65 ± 0.05 $n=8$, $P < 0.001$. 2-APB (75 μM) reduced the Emax of 5-HT-induced contraction in WT & 5-HTT+ vessels: WT Emax = $88.7 \pm 0.2\%$ $n=6$, cf. $46.6 \pm 0.1\%$ $n=7$, $P < 0.001$; 5-HTT+ Emax = $112.5 \pm 0.2\%$, $n=5$ cf. $61.2 \pm 0.4\%$, $n=6$, $P < 0.001$. 5-HT induced contractions remained significantly different in WT and 5-HTT+ at different K^+ concentrations. In 5mM K^+ pEC50 = 6.97 ± 0.08 $n=7$ (WT) cf. 5.74 ± 0.08 $n=6$ (5-HTT+), $P < 0.001$. Potency increased in 20mM K^+ to pEC50 = 7.6 ± 0.1 , $n=6$ (WT) cf. 5.98 ± 0.07 $n=6$ (5-HTT+), $P < 0.001$, but fell in 50mM K^+ to pEC50 = 7.2 ± 0.2 $n=4$ (WT) cf. 5.8 ± 0.2 $n=6$ (5-HTT+), $P < 0.001$. The results suggest that 5-HT-induced contraction requires Ca^{2+} influx and involves both voltage-dependent Ca^{2+} channels and SOCE. The difference in 5-HT potency between WT and 5-HTT+ is not related to membrane potential as it was not restored by changing extracellular K^+ . No difference in Ca^{2+} transport was found between WT and 5-HTT+ IPAs to explain the difference in sensitivity to 5-HT.

MacLean MR et al (2004). *Circulation* 109, 2150-2155.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC372

Characterisation of an extracellular Ca^{2+} -dependent, non-selective cation current in rat small pulmonary and mesenteric arterial myocytes

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An increase in intracellular calcium is the major trigger for contraction in vascular smooth muscle. However, the exact mechanisms that control Ca^{2+} homeostasis, in particular Ca^{2+} entry

mechanisms, still remain to be fully elucidated. Many studies have manipulated extracellular calcium in order to investigate these mechanisms (Robertson *et al.*, 2000; Ng & Gurney, 2001; Snetkov *et al.*, 2003). Here we present data showing the activation of a voltage independent, non-selective cation current (termed I_C) in pulmonary artery (PA) and mesenteric artery (MA) smooth muscle cells (SMCs) upon removal of extracellular Ca^{2+} (Ca^{2+}_o) and in the presence of 1 mM EGTA.

Male Wistar rats (225-300 g) were humanely killed, and small intra-pulmonary ($< 500 \mu\text{m}$) and mesenteric ($< 300 \mu\text{m}$) arteries were dissected. Cells were isolated as previously described (Smirnov *et al.*, 2002). Experiments were performed at room temperature, with a Cs^+ and low Cl^- -based pipette solution, in the perforated patch mode of the voltage clamp-technique (100 $\mu\text{g/ml}$ amphotericin B). The external solution contained (mM): 130 NaCl, 5 CsCl, 1.2 MgCl_2 , 1.5 CaCl_2 , 10 HEPES, 10 glucose (pH=7.2). 4-aminopyridine and TEA (both at 5 mM) were added to block Cs^+ flux through K^+ channels.

In PA SMCs, I_C was observed with greater frequency than in MA (152 of 160 cells in PA; 35 out of 54 cells in MA) and was larger in amplitude (with the mean current density at -100 mV equal to 97.8 ± 7.8 pA/pF in PA and 50.1 ± 9.9 pA/pF in MA, $p < 0.05$). In both tissues, I_C was significantly blocked by lanthanum (100 μM), gadolinium and cadmium (100 μM); nickel (100 μM) also significantly reduced I_C in PA SMCs (by $32.3 \pm 13.9\%$ at -100 mV, $n=7$; $p < 0.05$). Biophysical characterisation of I_C in PA SMCs suggests that I_C exhibited the following selectivity sequence $\text{Cs}^+ \leq \text{Rb}^+ = \text{K}^+ = \text{Na}^+ \leq \text{Li}^+ < \text{NMDG}$ ($n \leq 8$), suggesting weak binding within the channel pore. Ca^{2+}_o sensitivity, assessed in PA SMCs, showed that I_C was blocked by 1 μM Ca^{2+}_o by $74.5 \pm 8.8\%$ at -100mV ($n=5$). In PA SMCs, I_C activation was not significantly affected by cell pre-treatment with 1 μM thapsigargin, whereas in MA SMCs, its activation was significantly enhanced. Qualitatively similar results were observed with 10 μM cyclopiazonic acid. These results suggest that activation of I_C is functionally coupled to depletion of intracellular Ca^{2+} stores in MAs, but not in PAs. Although, due to its high Ca^{2+}_o sensitivity, the functional role of I_C is not currently clear, activation of I_C by intracellular or extracellular, yet to be identified, factors, cannot be excluded. Robertson TP *et al.* (2000). *J.Physiol.* 525:669-680.

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PC373

The effects of nitric oxide on pulmonary hypertension and vessel size distribution

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PC374

Osteoprotegerin promotes proliferation and migration of human microvascular endothelial cells

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Objective: Osteoprotegerin (OPG), a member of the TNF (tumor necrosis factor) receptor superfamily, is a key regulator of osteoclastogenesis. Recently, we reported that endothelial cells produce high level of OPG in response to *Escherichia coli* lipopolysaccharide¹. We also indicated that OPG treatment protects human microvascular endothelial cells (HMVECs) from detachment and apoptotic cell death induced by cysteine proteases that are produced by *Porphyromonas gingivalis*². These results suggest that OPG can be involved in the immunological functions of endothelial cells. However, the potential role of OPG in endothelial cell biology is unknown. In this study, we investigated the effects of OPG on HMVECs.

Methods: HMVECs were seeded in 96-well plates and incubated for 36 h in human serum-free medium (H-SFM) with increasing doses of OPG (20,100 and 500 ng/ml). Cell proliferation was assessed by a tetrazolium (WST-8) reduction assay. Cell migration was assessed using FALCON Cell Culture FluoroBlok Inserts, which are inserts coated with vitronectin or fibronectin. Two integrin receptors for vitronectin, namely, integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed on endothelial cells; in certain experiments, therefore, cells in suspension were pre-treated with anti- $\alpha_v\beta_3$ or anti- $\alpha_v\beta_5$ antibodies.

Results: The OPG treatment induced the proliferation of HMVECs in a dose-dependent manner. The cell migration assay showed that OPG induced cell migration on vitronectin in a dose-dependent manner, whereas no significant increase in migration occurred on fibronectin. In addition, a blocking antibody to $\alpha_v\beta_3$, but not to $\alpha_v\beta_5$, significantly inhibits OPG-induced cell migration. These results indicate that OPG promotes $\alpha_v\beta_3$ -dependent endothelial cell migration.

Conclusions: Our results reveal that the OPG is an important factor for the proliferation and migration of endothelial cells. Because integrin $\alpha_v\beta_3$ is highly expressed in angiogenic endothelial cells but not in quiescent endothelial cells, our results also suggest that OPG can regulate the migration of endothelial cells by influencing $\alpha_v\beta_3$ expression. This suggests that OPG plays a role in neovessel formation under physiological and pathological conditions.

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PC375

Constitutive presence of immature myocytes in resistance arteries

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Cells with irregular bodies and numerous thin long processes were described in resistance arteries (Pucovsky et al., 2003). Our recent results (Pucovsky et al., 2005) have shown that they are immature or phenotypically modulated myocytes. The aim of this work was to establish whether these cells are constitutively present in the vessel wall or are mature myocytes that have modulated their phenotype only after being isolated from the tissue. The experiments were carried out on guinea-pig mesenteric artery segments and on single cells isolated from them by enzymatic digestion. The cells were labelled by incubation with primary antibodies against a target molecule and the labelling was visualised using fluorescent secondary antibodies. The images were obtained with a laser scanning confocal microscope. Vessel segments used for electron microscopy were first placed in physiological solution containing 100 μ M nicardipine for 3 hours and then were processed, cut in sections and viewed with transmission electron microscope. Statistical significance was tested with Student's t-test. The fluorescent signal intensity of myosin light chain kinase in immature myocytes was $32.1 \pm 6.6\%$ of that in mature myocytes ($n=9$ cell pairs, $p=10^{-5}$), which is in accordance with their inability to contract. Cell isolation in the continuous presence of 20 μ M latrunculin B, actin polymerisation inhibitor, did not cause the disappearance of cells with processes from cell suspension, suggesting that some cells had processes even before isolation. The fluorescence signal of basal lamina protein collagen IV in immature myocytes was $113.3 \pm 12.1\%$ of that in mature myocytes ($n=9$ cell pairs, $p=0.305$), but the laminin fluorescence was significantly higher in immature myocytes, $131.9 \pm 9.6\%$ of that in mature myocytes ($n=8$ cell pairs, $p=0.013$), ruling out phenotypic modulation due to basal lamina shedding. The cells with thin processes were found in the tunica media of small resistance arteries using transmission electron microscopy. The results suggest that myocytes of immature phenotype are constitutively present in resistance arteries of guinea-pig.

Pucovsky V et al. (2003), *J Physiol* 552, 119-135.

Pucovsky V et al. (2005), *J Physiol* 568P, PC34.

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PC376

Effect of organ culture on noradrenaline-evoked contraction, calcium signaling and TRPC expression in rat mesenteric artery

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Organ culture of arteries is generally thought to affect the contractile phenotype, but the mechanism behind this effect is not understood. In view of the reported plasticity of TRPC expression (Bergdahl et al. 2005), we have explored the changes in calcium signal, in contractility and in TRPC expression evoked by organ culture in rat resistance mesenteric artery. TRPC1 and TRPC6 were the most abundant isoforms expressed in mesenteric artery. In arteries cultured for 2 days, TRPC1 mRNA expression decreased by $47 \pm 8\%$ ($n=4$, $P<0.01$), while TRPC6 mRNA expression increased by $94 \pm 26\%$ ($n=4$, $P<0.05$) in comparison with non-cultured arteries. Immunofluorescence analysis of protein expression by confocal microscopy confirmed the upregulation of TRPC6 proteins after 2 days of organ culture, compared with freshly isolated mesenteric arteries. Measurement of contractile responses to cumulative concentrations of noradrenaline showed that cultured arteries were significantly more sensitive to noradrenaline than freshly isolated control arteries (pD₂ values of noradrenaline were 5.83 ± 0.05 and 6.13 ± 0.05 , $n=18$, in freshly isolated artery and after 48h of culture, respectively, $P<0.01$). Neither maximum contraction nor contraction evoked by KCl depolarization were modified. Simultaneous measurement of intracellular calcium concentration and tension in fura-2 loaded mesenteric arteries indicated that the increased sensitivity of noradrenaline-evoked contraction in cultured arteries was associated with a parallel increase in calcium signal. The increased contractile response of cultured arteries to noradrenaline was abolished in the presence of the tyrosine kinase inhibitor genistein (noradrenaline pD₂ values in the presence of genistein were 5.70 ± 0.20 and 5.70 ± 0.10 , $n=6$, in freshly isolated and cultured arteries, respectively), or of PP2, a Src kinase inhibitor (noradrenaline pD₂ values in the presence of PP2 were 5.70 ± 0.10 and 5.80 ± 0.11 , $n=6$, in freshly isolated and cultured arteries, respectively). Inhibition of protein kinase C with G66976, or GF109203X did not affect the contraction induced by noradrenaline either in cultured or in freshly isolated mesenteric arteries. These results indicate that organ culture of rat mesenteric resistance artery modifies TRPC expression and results in Src kinase-dependent increase in contractile response to noradrenaline.

Bergdahl, A. et al., (2005). *Am J Physiol Cell Physiol* 288, C872-80.

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PC377

The proteolytic activity of an elastase is required for shear-induced release of basic fibroblast growth factor from endothelial cells

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Although bFGF is an important factor in the control of vascular growth, the mechanisms of its release from endothelial cells are still not well understood. We have shown before that shear stress induces bFGF release and that the integrin $\alpha(v)\beta_3$ plays an important role in this process. Here, we investigated whether proteases could be involved in shear stress induced $\alpha(v)\beta_3$ stimulation and which signaling cascade was activated by $\alpha(v)\beta_3$. Porcine aortic endothelial cells (PAEC, 1st or second passage) were subjected to shear stress (16 dyn/cm^2) in a cone and plate apparatus. Compared to resting cells, shear stress induced a significant increase of bFGF release (84 ± 9 vs $482 \pm 66 \text{ pg/mL}$; $n=9$). This increase went along with an enhanced proteolytic activity (3fold, $n=6$) in the supernatant of the cells. This proteolytic activity was due to an elastase as assessed by the substrate MeOSuc-Ala-Ala-Pro-Val-pNA. Inhibition of this proteolytic activity by aprotinin prevented the shear induced increase of bFGF release by $32 \pm 12\%$ ($n=9$). Likewise, treatment with exogenous elastase ($0.1-2 \text{ U/mL}$) resulted in an up to threefold increase of bFGF ($n=9$). The bFGF release induced by exogenous elastase was dependent on integrin $\alpha(v)\beta_3$ since the a medium dose of Abxiximab ($0.5 \text{ } \mu\text{g/mL}$) significantly reduced it by $32 \pm 12\%$ ($n=7$). Immunohistochemistry using specific bFGF antibodies revealed a translocation of (peri-)nuclear distributed bFGF towards the cellular membrane after stimulation of PAEC with elastase or shear stress. In contrast, no translocation was observed when cells in suspension were treated with elastase. The bFGF translocation coincided with translocation of HSP27 to the cell membrane. Moreover, western blots demonstrated that elastase treatment resulted in HSP27 phosphorylation ($n=4$, each). This phosphorylation was due to an activation of p38 MAP kinase as assessed by the inhibitory effects (by 67%) of SB202190 ($1 \text{ } \mu\text{M}$). Phosphorylated HSP27 and bFGF could be co-precipitated, indicating a close physical interaction between these two proteins ($n=3$). We conclude that shear stress treatment activates an elastase, which in turn initiates integrin $\alpha(v)\beta_3$ dependent signaling cascades stimulating the release of bFGF. We further propose that this new model could help to understand the translation of mechanical stress into biological growth signals in the vascular wall.

Gloe T et al., (2002) *J Biol Chem* 277, 23453-8

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PC378

Liver Growth Factor treatment of SHR reduces intrinsic stiffness in resistance through improvement of wall composition

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Background. Liver Growth Factor (LGF) is a liver mitogen with antifibrotic and regenerative properties in several biological systems, including liver, nervous, reproductive and respiratory system (1, 2). Our previous study in spontaneously hypertensive rats (SHR) demonstrates that a short treatment with LGF (4.5 micro grams LGF/rat, i.p., twice a week during 2 weeks) exerts antihypertensive effects, improving large artery composition and function (3). Resistance arteries play a key role in the regulation of blood pressure and in SHR resistance artery remodelling and stiffening participates in the development and maintenance of hypertension. Our aim has been to determine if the antihypertensive actions of LGF in SHR are partially due to improvement of the resistance vasculature. **Methods.** 3rd order Mesenteric Resistance Arteries (MRA) from adult male SHR, WKY and SHR treated with LGF were used. MRAs were mounted on a pressure myography to study vascular structure and mechanics. Thereafter, MRAs were pressure-fixed with 4% PFA at 70 mm Hg and stained with the nuclear dye DAPI. Intact arteries were mounted on a slide provided with a well to avoid vessel compression and visualized with confocal microscopy at 364nm (DAPI) and 488nm (autofluorescence of elastin) to determine cellular distribution and elastin organization. Images were analyzed with Metamorph Image Analysis Software. **Results.** As previously described SHR MRA showed eutrophic inward remodelling and stiffening, when compared to WKY vessels. LGF treatment did not modify vascular structure but improved distensibility and reduce intrinsic stiffness. Detailed analysis of the structure of the different layers of the vascular wall showed that SHR arteries exhibited a reduction of the volume of the media occupied by Smooth Muscle Cells (SMC) with no change in adventitial cell or endothelial cell density. The relative area occupied by elastin in the internal elastic lamina was significantly larger in SHR arteries compared to WKY, due to a reduction in the size of fenestrae. LGF increased SMC and endothelial cell density, with no change in the adventitia and reduced the area occupied by elastin increasing fenestrae number without modification of size. **Conclusions.** Short LGF treatment of SHR rats improves wall composition by increasing SMC number and reducing extracellular matrix. These changes reduce the vascular stiffening characteristic of SHR resistance vessels and might participate in the hypotensive effects of LGF treatment in SHR rats.

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3. Somoza et al (2006). Cardiovasc Res 69,764-771

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC379

Role of upregulated of K_v1.3 channel in the smooth muscle cell response to injury and neointimal hyperplasia

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In vascular injury, the mechanisms responsible for contractile smooth muscle phenotype are altered, leading to vascular smooth muscle proliferation and vascular diseases such as atherosclerosis and neointimal hyperplasia. We previously reported a change in ion handling when the cells switch phenotype (Cheong et al, 2005; Kumar et al, 2006). This study presents data suggesting a novel role for the voltage-gated potassium channel K_v1.3 in mediating vascular proliferation.

Vascular smooth muscle cells were obtained from aorta dissected post mortem from male eight-week old mice (Fountain et al, 2004) or from discarded human saphenous veins obtained at coronary artery bypass graft surgery with ethical consent. Quantitative real-time RT-PCR analysis of mouse vascular smooth muscle cell mRNA revealed upregulation of KCNA3 gene (encoding K_v1.3) in proliferating cells and loss of other K_v1 mRNA species. As a model of vascular injury, a linear wound was made in the culture, removing cells from a predefined region. Cell response to the injury was inhibited by the K_v1.3 blockers margatoxin and correolide compound C in mouse and human vascular smooth muscle cells. Ionic currents measured by whole-cell patch-clamp in human smooth muscle cells were consistent with the presence of active K_v1.3 channels. Margatoxin and correolide compound C, as well as Psora-4 (a third KV1.3 blocker) inhibited a component of the outward current elicited by a 500ms depolarising step to +40mV from a holding potential of -60mV. The threshold of activation of the current was near -40mV. To assess more directly the potential relevance to smooth muscle adaptation *in situ*, we grew neointimal formations within segments of human saphenous vein as previously described (Cheong et al, 2005). The new cells in the neointimal formations are almost exclusively alpha-actin positive smooth muscle cells and expressed K_v1.3 protein at higher levels than cells in pre-existing vein. Furthermore, using paired vein segments from the same patient, we reveal that treatment with margatoxin or correolide compound C significantly reduced the neointimal growth compared to vehicle control.

Together, these data indicate that K_v1.3 is upregulated in proliferating smooth muscle cells and that blockers of K_v1.3 may have therapeutic potential as agents acting against diseases caused or exacerbated by smooth muscle cell proliferation.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC380

The role of nitric oxide and vascular function in vein graft failureJ. Wu¹, R.M. Wadsworth¹ and S. Kennedy²¹*Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, Glasgow, UK* and ²*Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK*

Background: Vein grafting is the final solution for occlusive vascular diseases. However, clinically the 10-year-patent rate is less than 60%. Intimal hyperplasia and subsequent accelerated atherosclerosis are major causes of vein graft failure.

Methods: The inferior vena cava from the donor mouse was implanted into the right carotid artery of the recipient mouse, which was anaesthetized by sodium pentobarbital (60mg/kg body weight). Vein grafts were harvested at 1, 3, 7, 14 and 28 days after operation, and were fixed by 10% formalin for histology (H&E) and immunohistochemistry (eNOS and iNOS) study. Fresh vein grafts were also harvested at 28 days for vascular reactivity study using myograph technique. Circulating leukocytes were depleted by injection of anti-CD45/LCA antibody at the end of the 2nd week after surgery to investigate the influence of chronic inflammation and circulating leukocyte on neointima remodeling.

Results: In the vein grafts, neointima was present at 1 week after operation, and was very thick by 4 weeks. The endothelium was denuded within 3 days after surgery. In days 1-3 there was intense inflammation, which resolved when endothelial cells re-grew reaching confluence 2 weeks after surgery (stained for anti-eNOS). There was positive staining for iNOS in neointima and endothelium at 2 and 4 weeks, associated with leukocyte adhesion. Vein grafts contracted and possessed endothelial-dependent relaxation, although much smaller than normal carotid artery. Relaxation to a nitric oxide donor was also reduced. Depletion of circulating leukocytes to 30% of normal level, with recovery to 70% within one week, had no effect on vein graft vasomotor function at 4 weeks.

Conclusion: Injuries including surgical trauma and endothelial damage initiated an acute inflammation accompanied by a rapid formation of neointima. The intense inflammatory process produces a very thick neointima with abnormal expression of iNOS. The neointima is capable of contraction and relaxation though these processes are severely disrupted compared to normal artery. The remodeling process was not significantly affected by depletion of circulating leukocytes during the first few days.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC381

Resistin exerts pro-angiogenic effects in murine endothelial cells

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Angiogenesis, the formation of new blood capillaries from existing vessels, is required for physiological processes such as embryonic development, wound healing, and female reproduction, but

when dysregulated can contribute to tumour growth and atherosclerosis. Endothelial cell migration and proliferation, in response to angiogenic stimuli, such as hypoxia, plays an essential role in this process. Here, we examine the effect of the novel, cysteine-rich secretory protein, resistin, on the angiogenic responses of murine b.End.5 endothelioma cells. Resistin causes insulin resistance in hepatic and peripheral murine tissues, is present within human and murine atherosclerotic lesions, and induces inflammatory, proliferative and angiogenic responses in vascular cells.

Using a model in which endothelial cells remodel an induced 'wound' in a confluent cell monolayer, we show that treatment with murine recombinant resistin (50ng/ml) increases growth and migration of endothelial cells by 59% and 33% ($p < 0.001$) over 24 and 48h, respectively, compared with the control incubation. Under hypoxic conditions murine resistin and VEGF stimulated comparable degrees of growth and migration, possibly via the same pathway as no additive response was noted when both resistin and VEGF were added together. These effects of resistin were accompanied by an apparent increase in network formation, on a MatrigelTM substrate. Resistin induces a significant degree of endothelial cell migration, as judged by apical to basolateral migration (5h) across TransWell culture dishes (8µm pore), and in response to treatment in the presence or absence of murine resistin (50ng/ml), VEGF (10ng/ml) or both resistin and VEGF. Resistin and VEGF increased endothelial cell migration by 48% and 23%, respectively, ($p < 0.01$), compared with the control incubation; cellular migration increased by 66% ($p < 0.01$) in the presence of both resistin and VEGF. Finally, the presence of resistin (30-100ng/ml) also induced a mild proliferative effect (14-15%; $p < 0.05$) in b.End.5 cells, as judged by conversion of MTT to formazan.

These data suggest that resistin may promote angiogenesis by increasing endothelial cell proliferation and migration, and may have an important role in pathological processes such as atherosclerosis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC382

The PPAR γ -independent effects of thiazolidinediones on human cardiac fibroblast function

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Background: Cardiac fibroblasts (CF) account for two-thirds of the total number of cells in the heart and are fundamental in the maintenance of its structural integrity. Thiazolidinediones (TZDs) are insulin sensitising agents used in the treatment of type 2 diabetes and are agonists for PPAR γ (peroxisome proliferator activated receptor- γ). Previous studies suggest that TZDs may have beneficial effects on adverse cardiac remodelling, a key feature of which is increased CF migration, proliferation and secretion of matrix degrading metalloproteinases (MMPs). No previous studies have investigated the effects of TZDs on these aspects of human CF function.

Aims: The aims of this project were two-fold. Firstly, to determine any functional effects of TZDs on human CF and secondly, to determine whether these effects occur via activation of PPAR γ . **Methods and Results:** Human CF were cultured from right atrial appendage of patients undergoing coronary artery bypass surgery. TZDs (ciglitazone, rosiglitazone, troglitazone) and 15-deoxy- δ 12,14-prostaglandin J2 (15d-PGJ2, endogenous ligand for PPAR γ) inhibited CF proliferation in a concentration dependent manner, with similar efficacy. GW9662 and T0070907 (selective PPAR γ antagonists) failed to reverse the inhibitory effects of TZDs on proliferation and unexpectedly had an inhibitory effect themselves. TZDs and 15d-PGJ2 had no marked effect on CF migration and invasion, as determined using modified Boyden chamber assays, in the absence or presence respectively, of a Matrigel basement membrane barrier. Gelatin zymography revealed that TZDs and 15d-PGJ2 did not inhibit IL-1 α -induced MMP-9 secretion.

Conclusion: TZDs clearly have pleiotropic effects and may have potential to attenuate adverse myocardial remodelling, by modulating CF proliferation, but not invasion or MMP-9 secretion. The anti-proliferative effects of TZDs occurred independently of PPAR γ activation. Since type 2 diabetic patients are at increased risk of coronary heart disease, treatment with TZDs may not only increase insulin sensitivity, but also have cardioprotective effects.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC383

The poly(ADP-ribose) polymerase inhibitor PJ-34 inhibits neovascularization and vascular endothelial growth factor signalling

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Angiogenesis is a tightly regulated process known to be essential for normal embryonic development that is implicated in physiological, as well as, pathological phenomena in fully developed organisms. Although stimulation of new blood vessel growth has been proposed as a therapeutic approach to treat certain disorders, an excessive and deregulated angiogenic response is thought to contribute to cancer, diabetic retinopathy, arthritis and psoriasis. Therefore, several anti-angiogenic agents have been developed to be used as effective treatments in angiogenesis-related disorders. In the present study, we have investigated the effects of poly (ADP-ribose) polymerase (PARP) inhibition on angiogenesis. PARP is a DNA-repair enzyme that also regulates transcription by affecting histones, DNA methylation and enhancer/promoter regions. In order to investigate potential effects of PARP on angiogenesis we have utilised the selective PARP inhibitor, PJ-34 [N-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide], which is currently evaluated in human clinical trials for neurodegenerative diseases and ischemia

reperfusion-induced tissue injury. Treatment of chicken chorioallantoic membranes (CAM) with PJ-34 reduced vascular length in these tissues; paradoxically, lower doses of PJ-34 (0.03 or 0.3 nmol/egg) were more effective in inhibiting neovascularization than higher doses (3 or 30 nmol/egg). In vitro, incubation of endothelial cells (EC) with PJ-34 (300 nM to 10 μ M concentration) inhibited their proliferation in a concentration-dependent manner; maximal inhibition of 22.3 % was observed at 10 μ M. Capillary morphogenesis on matrigel was also negatively affected by PJ-34. Moreover, PJ-34 (300 nM) abolished the migratory response to the prototype angiogenic factor, vascular endothelial growth factor (VEGF). In conclusion, we provide evidence that PARP inhibition reduces EC proliferation, migration and organization leading to decreased angiogenesis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC384

Increased vascular contractility in isolated vessels from cigarette smoking rats is mediated by basal endothelin release

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The effect of chronic cigarette smoking on endothelin modulation of vascular contraction, and CYP enzyme levels was studied in 20 male Sprague-Dawley rats. The animals were divided equally into smoking and non-smoking groups. The smoking group was exposed to 6 research cigarettes per rat per day 5 days a week for 16 weeks. The control group was sham smoked. Functional contractile studies were performed in aortas and carotid arteries to determine the regulation of vascular tone by basal release of endothelin. Liver samples were analyzed for CYP1A1 and CYP1A2 gene expression by RT-PCR. Plasma samples were assessed for endothelin-1 (ET-1) level by enzyme immuno assay (EIA). Treatment of aortas and carotid arteries with bosentan, the dual endothelin receptor antagonist, caused a significant reduction in constrictor responses of smoking rats, indicating, increase greater regulation of tone by endothelin in smoker rats compared to controls. There was a greater expression of the cytochrome P450-liver enzymes (CYP1A1 and CYP1A2) in smoker rats. Body weight gain was also significantly decreased in smoker rats. We conclude that increased endothelin release in smoker rats significantly contributes to increased arterial tone and so contribute to the cardiovascular pathophysiology associated with cigarette smoking, such as increased vascular muscularization, increased contraction, decreased dilation and possibly vasospasm.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC385

Additive effects of collagen and adrenaline on platelet aggregation in 96-well plates

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Platelet aggregation is routinely measured by light transmission techniques¹ and this has been developed for use in 96-well plates². This method allows testing of many aggregatory responses over an identical short time period. When two or more platelet agonists are added together in low concentrations to platelet-rich plasma the individual effects of each other are enhanced³. We have evaluated the effects of the combined addition of collagen and adrenaline on aggregation of platelet-rich plasma using 96-well plate method.

Human blood was collected by venepuncture into tri-sodium citrate (3.8% w/v final) and centrifuged to obtain platelet-rich plasma (PRP). The PRP was then added to the wells of 96 well plates in the presence or absence of collagen, adrenaline or combinations of collagen and adrenaline. Plates were then immediately placed in a 96-well plate reader and absorbance determined at 595nm every 15s for 16min with vigorous shaking. Changes in absorbance were converted to % aggregation by reference to the absorbance of PRP and platelet-poor plasma.

Aggregatory responses to combined agonists were enhanced compared to collagen or adrenaline alone. For example at 4min, the aggregation induced by combination of 1µg/ml collagen and 10⁻⁷ M adrenaline was 56±16%, compared to 1µg/ml collagen alone, 27±12%, or 10⁻⁷ M adrenaline alone, 22±5% (n=4 for each). At 8min, combination of 10⁻⁷ M adrenaline with 0.1, 0.3 or 1µg/ml collagen increased aggregation to 61±12%, 64±14% and 79±9% respectively compared to collagen alone (0.1µg/ml, 34±10%; 0.3µg/ml, 42±14%; 1µg/ml, 57±7%) or 10⁻⁷ M adrenaline, 46±9% (n=4 for each).

Using 96-well plates we demonstrated the additive effects of combinations of low concentrations of collagen and adrenaline on platelet aggregation in PRP. This approach could be useful since low concentrations of several agonists may better mimic the conditions under which thrombosis occur *in vivo* than single agonists.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC386

Statin treatment for endothelial dysfunction in thoracic aortae following maternal protein restriction in pregnancy

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Epidemiological studies demonstrate that low birth weight is associated with an increased risk of cardiovascular disease in adult life (Osmond *et al.*, 1993). In the rat, the restriction of dietary protein during gestation leads to raised systolic blood pressure and endothelial dysfunction in the offspring, possibly due to defects in the nitric oxide (NO) pathway (Lamireau *et al.*, 2002; Torrens *et al.*, 2006). Statins (HMG-CoA reductase inhibitors) are used in the management of dyslipidaemia, but have been shown to have other pleiotropic actions, including increasing NO bioavailability and reducing oxidative damage (Davidson *et al.*, 2005). The aim of this study was to assess the effect of statin therapy on endothelial function in offspring of protein restricted dams, in a vascular bed which was predominantly NO dependent.

Pregnant Wistar rats were fed a control (C; 18% casein) or protein restricted (PR; 9% casein) diet throughout pregnancy and returned to standard chow postpartum. At weaning a subset of the PR group were given atorvastatin (PRS, 10 mg/kg/day) in the drinking water. At 145 days of age male offspring were sacrificed by CO₂ inhalation and cervical dislocation. To assess vascular function 2 mm aortic segments were mounted in an organ bath. Concentration response curves were conducted to phenylephrine (PE), acetylcholine (ACh) and sodium nitroprusside (SNP). Responses to ACh were repeated in the presence of L-NAME (100 µM). Data is given as mean ± S.E.M. and differences were assessed by one-way ANOVA with Bonferroni *post hoc* correction. Significance was accepted at *p* < 0.05.

In male aortae the contractile responses to PE were increased in the PRS group compared to all other groups (% max response: C, 138.3 ± 4.0, n = 7; PR, 160.6 ± 5.4, n = 8; PRS, 190.3 ± 12.7, n = 5, *p* < 0.05). Vasodilation to ACh was significantly blunted in both the PR and PRS groups compared to controls (% max response: C, 79.4 ± 3.6, n = 6; PR, 48.9 ± 7.3, n = 8; PRS, 45.5 ± 3.1, n = 5; *p* < 0.05). Incubation with L-NAME completely abolished the ACh response in all groups. No differences were seen in the response to SNP (*p* = ns).

The data demonstrates that protein restriction during gestation leads to vascular dysfunction in isolated thoracic aorta segments from the male adult offspring. Results show relaxation to ACh

is entirely NO dependent and indicates that abnormalities in the NO pathway may be responsible for the attenuated responses in the PR groups. Despite the reported pleiotropic effects of statins, and what we previously have reported in the small mesenteric arteries (Torrens *et al.*, 2007), statin treatment did not restore vascular function in the thoracic aortae of the PR group.

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This work was supported by the British Heart Foundation and Pfizer.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC387

Cardiovascular functioning during different phases of menstrual cycle

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The menstrual cycle is an integral part of life in woman and associated with pronounced physiological changes. There is widespread agreement about the hormonal changes that occur during the menstrual cycle, but studies on cardiovascular functioning during different phases of menstrual cycle are more controversial.

The purpose of the present study was to evaluate the influence of the normal menstrual cycle on heart rate (HR) and heart rate variability (HRV). Fourteen healthy regularly cycling woman (age 19-24 years) were studied during the follicular phase and luteal phase over three month. The time schedule for the luteal phase was from days 3 to 7 before the first day of bleeding, and for the follicular phase was from days 7 to 11 after the first day of bleeding [1]. HRV was recorded after 10-minute supine rest for 5 minutes while subjects were resting in a supine position [2]. The values were compared by Mann-Whitney test (U).

Mean values of HR, VLF, LF, HF and LF/HF for the different phases are presented in Table 1. We found pronounced changes in HR during the menstrual cycle with a minimum in the follicular phase and maximum in the luteal phase ($U=1008.00$; $p<0.0001$). Power spectral analysis of HRV showed that the HF component of HRV, which reflects only parasympathetic activity, decreased more during the luteal phase than follicular phase ($U=1557.00$; $p=0.0016$), suggesting that parasympathetic nerve activity increases in this phase. The LF/HF ratio, which reflects the balance of autonomic nerve activities, in the luteal phase was significantly higher than that in the follicular phase ($U=1818.50$; $p=0.04$), indicating that sympathetic nerve activities in the luteal phase became predominant. VLF and LF did not show any significant change during the different phases of menstrual cycle.

This findings indicate that sympathetic nervous activity in the luteal phase is significantly greater than in the follicular phase whereas parasympathetic nervous activity is predominant in the follicular phase. Although the mechanism by which the menstrual cycle affects the autonomic nervous system is unclear, some endogenous female sex hormones may be responsible for these changes in the cardiac autonomic innervation.

Table 1. Heart Rate Variability Parameters During Different Phases of Menstrual Cycle

Parameter	Follicular Phase	Luteal Phase
HR	64.14±0.77	72.79±1.13*
VLF	1581.98±148.19	1839.90±171.09
LF	1537.56±191.18	1087.52±194.47
HF	1204.41±114.48	689.82±53.36*
LF/HF	1.41±0.11	2.10±0.22*

Data presented as mean ± SE.

* – Significant difference between follicular phase and luteal phase ($p<0.05$).

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC388

Linear relationship between collagen-induced platelet aggregation and thromboxane A₂ production

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There has long been discussion concerning the relationship between platelet activation and thromboxane (Tx) A₂ formation¹. Adapting the Born platelet aggregometry method for use in 96-well plate readers^{2,3} has allowed us to demonstrate a linear relationship between platelet aggregation and thromboxane A₂ production when arachidonic acid is used as an agonist⁴. Here we have continued to study whether the same relationship exists when collagen is used as an agonist, taking into account that thromboxane (Tx) signalling is only necessary for aggregation when lower concentrations of collagen are used⁵.

Human blood was collected by venepuncture into tri-sodium citrate (3.8% w/v final) and centrifuged to obtain platelet rich plasma (PRP). The PRP was then added to the wells of 96 well plates in the presence of increasing concentrations of aspirin (0.01-1000µM) or vehicle and incubated for 30min at 37°C before the addition of collagen (1 or 3µg/ml) or vehicle. Plates were then immediately placed in a 96-well plate reader and absorbance determined at 595nm every 15s for 16min between vigorous shaking. Changes in absorbance were converted to % aggregation by reference to the absorbances of PRP and platelet

free plasma. At the end of the aggregatory responses plasma samples were removed and retained for analysis by radioimmunoassay of the content of TxA_2 , as a measure of platelet TxA_2 formation.

Platelet aggregation ($72 \pm 1\%$ of maximum and $84 \pm 1\%$ of maximum in response to collagen $1 \mu\text{g}/\text{ml}$ and $3 \mu\text{g}/\text{ml}$, respectively) and TxA_2 production were both inhibited in concentration-dependent manners by aspirin. The log M IC_{50} values for aspirin as an inhibitor of collagen-induced platelet aggregation (-5.5 ± 0.3 and -4.7 ± 0.3 , respectively) and TxA_2 production (-5.3 ± 0.5 and -5.2 ± 0.7 , respectively) were not significantly different ($n=8$ for all). Furthermore, under all conditions there was a direct linear relationship between concentrations of TxA_2 and platelet aggregation.

Here, therefore, we demonstrate a linear relationship between platelet aggregation and TxA_2 production in this model of aspirin and collagen activation of platelets. Such a relationship has important implications for our understanding of aspirin and NSAID therapy.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC389

Generation of clonal populations of prostaglandin I_2 producing rat vascular smooth muscle cells: potential utility for production of bypass vessels

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Objectives - Atherosclerosis is a chronic disease of the vasculature that involves a complex interplay between numerous components and products of the blood and the arterial wall. One important anti-atherosclerotic and anti-thrombotic product of the arterial wall is prostaglandin I_2 (PGI_2)¹.

Currently, vein grafts remain an important choice of conduit for coronary artery bypass surgery in atherosclerosis, but because of poor long-term patency² and the lack of reliable interventions to prevent vein graft failure the idea of "designer" bypass vessels is attractive.

Here we have investigated, using rat cells, the possibility of selecting populations of vascular smooth muscle cells (VSMCs) with

characteristics favourable for the production of vascular media, focusing upon the biosynthesis of PGI_2 .

Methods - Multiple monoclonal populations of VSMCs were derived from WKY 12-22 rat VSMCs by dilute plating and then characterised for their morphological characteristics and their production of PGI_2 . Selected clonal cell types were then further investigated for their influences on platelet aggregation, using light transmission aggregometry³, and adhesion, using the conversion of p-nitrophenol phosphate to p-nitrophenol by platelet acid phosphatase⁴, in a 96-well plate reader.

Results - Morphologically distinct spindle-type VSMC clones produced greater amounts of PGI_2 than epithelioid-type clones (Figure 1). Spindle clone 7 was also found to inhibit both platelet aggregation (Figure 2) and platelet adhesion (data not shown) in response to adenosine diphosphate ($0.1\text{--}30 \mu\text{M}$), arachidonic acid ($10\text{--}1000 \mu\text{M}$), collagen ($0.1\text{--}30 \mu\text{g}/\text{ml}$), adrenaline ($0.001\text{--}100 \mu\text{M}$), ristocetin ($0.2\text{--}3.0 \text{mg}/\text{ml}$), TRAP-6 ($0.1\text{--}30 \mu\text{M}$), and U46619 ($0.1\text{--}30 \mu\text{M}$).

Conclusions - These results demonstrate that clonal VSMC populations with favourable characteristics can be isolated. Such defined, pure VSMC populations could potentially be used to produce vascular grafting media.

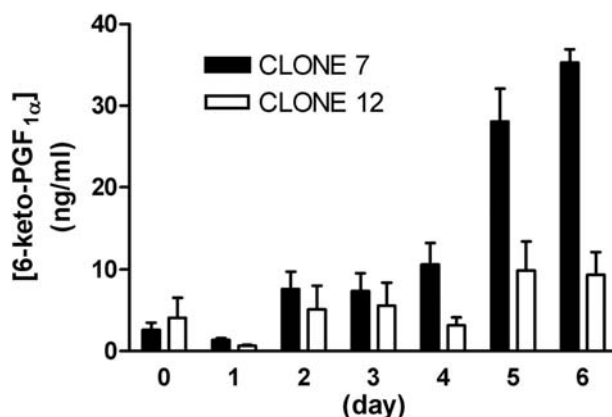


Figure 1. Clonal spindle cells (clone 7) produce more PGI_2 than clonal epithelioid cells (clone 12) when cultured in the presence of 15% serum. Data represents mean $\pm$ s.e.m from $n=9$ observations for each. ($P<0.01$ between clonal types, 2-way Anova)

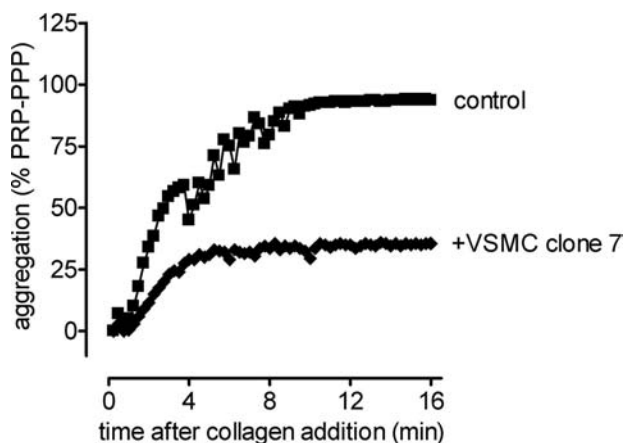


Figure 2. Representative trace of platelet aggregation in response to collagen ($10 \mu\text{g}/\text{ml}$) in the presence or absence (control) of spindle VSMC clone 7

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC390

Use of a 96-well plate method for the determination of ristocetin cofactor activity in patient plasma samples

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Von Willebrand Factor (vWF) mediates platelet adhesion and thrombus formation at sites of vascular injury, and also acts as a carrier of procoagulant factor VIII in circulating blood; both are essential for haemostasis. Von Willebrand disease (vWD) is a common inherited human disorder of haemostasis, where the underlying cause of bleeding is related to a quantitative or qualitative abnormality of vWF¹. Plasma vWF activity can be determined by the ability of patient plasma to support a ristocetin-induced aggregation of formalin-fixed platelets². Patients with a vWD have a reduced activity in ristocetin cofactor assays and these are used routinely for diagnostic purposes³. Here we have developed a 96-well plate method for the rapid, small volume determination of vWF activity.

Reference control and patient plasma samples were diluted to six concentrations, and plated in duplicate volumes of 10µl into the individual wells of a 96-well plate. Lyophilised formalin-fixed platelets were reconstituted in 5ml tris-buffered saline and incubated in volumes of 80µl with the plasma samples for 3 minutes. Ristocetin (10µl; 1mg/ml final concentration) was then added to each well to stimulate aggregation, and the plate was agitated on a heated plate shaker at 37°C. The plate was read on a plate reader at 595nm at 4, 8, 12 and 16min. The absorbance at each time point was then converted to percentage aggregation, using absorbances of vehicle-treated wells as control values. As the reference control plasma samples were supplied with known vWF activities, the activities of the patient samples could be determined by direct comparison. Table 1 shows the activity for each plasma sample from our 96-well plate assay compared to their activities determined by The Royal London Hospital Haematology Department in their standard vWF ristocetin cofactor assay. Normal physiological activity falls between 50% and 150%. We have determined that a 96-well plate based method provides a quick and accurate method for calculating vWF activity in patients. This provides an opportunity for the improvement of diagnostics by providing a higher throughput assay which maintains the necessary accuracy.

Table 1. Activity of the plasma samples determined by 96-well aggregometry compared to activity determined by traditional Born aggregometry

	Plasma activity by 96-well plate method	Plasma activity by traditional method
Patient A	24%	20%
Patient B	48%	37%
Patient C	100%	123%
Patient D	88%	119%
Patient E	85%	95%

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PC391

The cytochrome P450 epoxygenase inhibitor, SKF525A, induces COX-2 in monocytes: a role for PPAR-α?

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Cytochrome P450 (CYP)2J2 is an epoxygenase that has anti-inflammatory effects in the cardiovascular system (Node et al., 1999). We have recently shown that CYP2J2 activates peroxisome proliferator-activated receptor (PPAR)-α, a nuclear receptor that also has anti-inflammatory properties (Bishop-Bailey, 2000; Wray et al., 2003). As one anti-inflammatory action of PPARα is to reduce COX-2 expression (Staels et al., 1998), we investigated whether COX-2 expression in monocytes is regulated by endogenous epoxygenases.

Murine monocytes cells (J774.2) were maintained in DMEM supplemented with antibiotics/antimycotics and 10% FCS (37°C, 5% CO₂, 95% air). Expression of murine CYP2J isoforms were measured by RT-PCR. Cells were incubated (24h) with vehicle, IL-1β (10ng/ml) and PMA (1nM), the epoxygenase inhibitor SKF525A (30µM), or the selective synthetic PPARα agonist GW7647 (10nM), either alone or in combination. Cells were then lysed, and protein samples (10µg) subjected to Western blot analysis for COX-2, β-actin or PPARα (Bishop-Bailey et al., 2002). J774.2 macrophages contain PPARα and mRNA for CYP2J5, CYP2J6 and CYP2J13. IL-1β and PMA induced COX-2 expression; an affect which was completely inhibited by co-incubation with GW7647. The addition of SKF525A alone also strongly induced COX-2. Addition of IL-1β and PMA did not significantly affect SKF525A induced COX-2. The PPARα ligand GW7647 also significantly decreased SKF525A-induced COX-2 expression (Figure 1).

These results suggest that monocytes contain active epoxygenases that tonically suppress inflammation by activating PPARα.

Epoxygenases, by producing PPAR α ligands may therefore represent an important novel anti-inflammatory pathway.

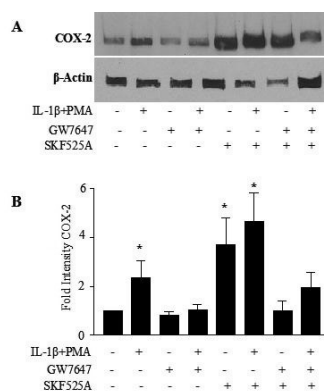


Figure 1. PPAR α activation inhibits IL-1 β and PMA, and SKF525A induced COX-2 expression. COX-2 expression was measured in J774.2 cells treated with IL-1 β (10ng/ml) and PMA (1nM) for 24h. Cells were pretreated with SKF525A (30 μ M) and/or GW7647 (10nM) for 2h and 1h respectively. (A) A typical western blot and (B) corresponding analysis by densitometry normalized to β -actin. Data represents n=3 from 3 separate experiments. * $p < 0.01$ One-way ANOVA with Bonferroni post test, significant difference from control.

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PC392

Farnesoid X receptor activation inhibits rat aortic smooth muscle cell migration

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The farnesoid X receptor (FXR; NR1H4) is a ligand-activated transcription factor that regulates bile acid and lipid homeostasis, and is expressed in the vasculature (Bishop-Bailey et al., 2004). The migration of vascular smooth muscle cells from the tunica media to the sub-endothelial region is a key event in the development and progression of atherosclerosis. Matrix metalloproteinases (MMP) play a critical role in these processes of SMC migration. We have recently showed that

FXR ligands inhibit MMP-2 and -9 expression and activities in rat aortic smooth muscle cells (RASM; Li et al., 2006). We have therefore investigated whether FXR could regulate platelet-derived growth factor (PDGF)-BB or IL-1 β -mediated RASM migration.

RASM culture was as previously described (Bishop-Bailey et al., 2004). Cell migration assays were performed over 72h using growth factor reduced Matrigel-coated (diluted 1:2 in serum-free DMEM) polycarbonate filters (8 μ m pore size, Transwell; Corning). Cells invading to the lower chamber were trypsinized and counted on a Casy 1 counter (Sharfe System GmbH Retlingen, Germany). The FXR ligand, 6 α -ethyl-chenodeoxycholic acid (6ECDCA; 30 μ M) or vehicle was added to RASM for 1h before plating and were present throughout the experiment (Thomas et al., 2001). 25ng/ml PDGF-BB or 10ng/ml IL-1 β was placed in the lower chamber to induce migration.

PDGF-BB but not IL-1 β induced RASM migration. 6ECDCA reduced both the basal and the PDGF-BB induced migration of RASM (Figure 1).

FXR activation blocks smooth muscle cell migration. FXR may therefore be a novel regulator of vascular remodelling.

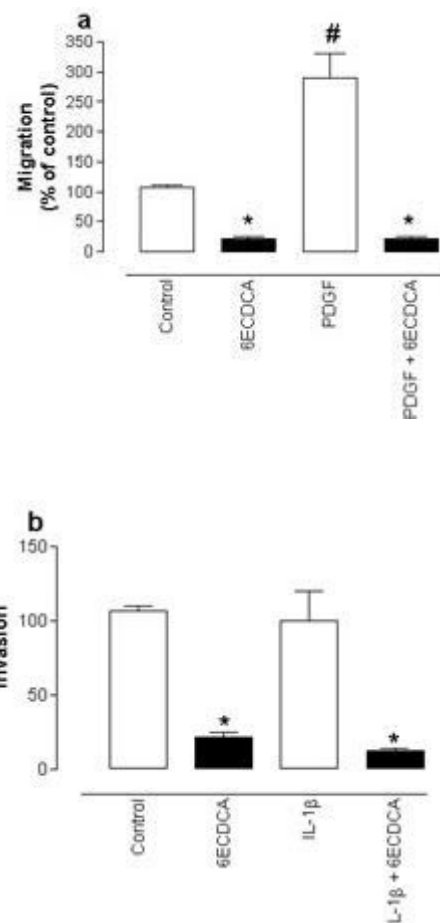


Figure 1: The FXR ligand 6ECDCA inhibits RASM migration. Transwell migration of RASM was enhanced by (a) PDGF-BB, but not (b) IL-1 β . All data represents mean $\pm$ S.E.M n=12 from 4 experiments. * & # indicate $p < 0.05$ by one-way ANOVA (Bonferroni's post-test); control vs 6ECDCA treatment or PDGF-BB treatment.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC394

Use of super ecliptic pHluorin tagged GluR2 to study DHPG-induced LTD

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A distinct form of hippocampal long term depression (LTD) is induced by activation of group I metabotropic glutamate receptors (mGluRs) by the specific agonist dihydroxyphenylglycine (DHPG)¹.

AMPA receptor redistribution has been implicated in this form of LTD. Also the AMPA receptor subunit GluR2 is transiently dephosphorylated in response to DHPG via a protein tyrosine phosphatase (PTP)². The mechanism of AMPA receptor redistribution is not fully understood however. Here we have used a super ecliptic pHluorin tagged version of GluR2 (SEP-GluR2) to study this phenomenon³. 14 day old postnatal hippocampal cultured neurones were infected with SEP-GluR2 using the sindbis virus. 17-24 hours later these neurones were used in experiments.

SEP-GluR2 infected neurones show a reduction in mEPSC frequency upon DHPG application that is comparable to that seen in non-infected neurones (76±2% of pre-DHPG frequency). The mEPSC amplitude was unaffected. In addition we found that 30 minutes after DHPG application the fluorescence from surface expressed SEP-GluR2 was significantly decreased to 83±6% of pre-DHPG values. The DHPG induced decrease in surface SEP-GluR2 was prevented when DHPG was applied in the presence of either the mGluR5 antagonist MPEP (93±10% of control) or the PTP inhibitor orthovanadate (100±7% of control). In summary, by using live cell imaging we have found that DHPG causes a reduction in surface SEP-GluR2 containing AMPA receptors.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC395

KHG21834 reduces lipopolysaccharide-induced microglia activation

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Benzothiazole derivatives are highly interesting molecules for drug development, because they already have been shown to be useful for treating various diseases including neurodegenerative disorders. An inflammation response in the central nervous system mediated by activation of microglia is a key event in the early stages of the development neurodegenerative diseases. We have synthesized and examined various benzothiazole derivatives against to the neuroinflammation. In this study, we have investigated the neuroprotective effects of KHG21834, a benzothiazole derivative, on lipopolysaccharide (LPS)-stimulated murine BV-2 microglia. KHG21834 was a selective inhibitor of proinflammatory cytokine production by activated glia. KHG21834 significantly suppressed the LPS-induced upregulation of tumor necrosis factor- α and interleukin-1 β in a dose-dependent manner. The production of inducible nitric oxide synthase (iNOS) was also studied in LPS-stimulated BV-2 cells as a model of microglia activation. KHG21834 dose dependently attenuated nitrite production and iNOS protein expression in LPS-stimulated murine BV-2 microglia. In addition, the neuroprotective effect of KHG21834 was similarly observed in vivo. These results demonstrate a potent suppressive effect of KHG21834 on proinflammatory responses of microglia, suggesting a therapeutic potential for this compound in neurodegenerative diseases accompanied by microglial activation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC396

Metabotropic glutamate receptor-mediated long-term depression involves AMPA receptor redistribution triggered by protein tyrosine phosphatases

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Long-term depression (LTD) can be induced at hippocampal CA1 synapses, by activation of either N-methyl-D-aspartate receptors (NMDARs) or metabotropic glutamate receptors (mGluRs). Although NMDAR-LTD has been relatively well characterised, the molecular mechanisms underlying mGluR-LTD are still unknown. However, these can be investigated by bath application of the group 1 mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG). Previous studies have indicated that

DHPG-LTD is calcium independent and does not require activation of CaMKII, protein kinases A or C or serine/threonine phosphatases. It does however require protein tyrosine phosphatases (PTPs), G α_q activation and involve internalisation of AMPA receptors (AMPA receptors) and NMDARs. In this study, 400 μ m thick acute hippocampal slices were obtained from female Wistar rats (10-12 weeks of age), and incubated in 100 μ M DHPG for 10 minutes, followed by immunoprecipitation of AMPARs and quantitative immunoblot analysis using an anti-phosphotyrosine antibody at different time-points. We have established that DHPG-LTD involves a transient tyrosine dephosphorylation of the GluR2 AMPAR subunit, and that this is blocked by prior application of the PTP inhibitor, orthovanadate. The total AMPAR content in the slices is unaffected in DHPG-LTD. AMPAR phosphotyrosine levels do not change significantly upon activation of NMDARs implying that the mechanism is specific to mGluR-LTD. The combination of cell surface biotin-labelling and immunoprecipitation revealed that tyrosine dephosphorylation is required for the trafficking of AMPARs away from the synapse. This study has therefore established that AMPAR tyrosine dephosphorylation by PTPs is an important postsynaptic component of mGluR-LTD which does not seem to be involved in NMDAR-LTD.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC397

Mechanisms of SERT functional regulation by interacting proteins

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The serotonin transporter (SERT) is a transmembrane protein that belongs to the family of Na⁺/Cl⁻ dependent monoamine transporter. SERT is expressed at presynaptic sites of serotonergic neurons where it is responsible for the high-affinity re-uptake of the released neurotransmitter into nerve terminals, thereby determining the intensity and duration of serotonin (5-hydroxytryptamine, 5-HT) signalling (1). SERT plays an important role in brain function because of its association with complex behaviours like emotion, mood and reward. Furthermore, it is linked to severe disorders including attention-deficit/hyperactivity disorder, schizophrenia, autism and depression (2). It is a crucial pharmacological target for psychostimulants such as cocaine, amphetamines and antidepressants (3). SERT function is regulated by various mechanisms, including several protein kinase-dependent pathways such as the activation of p38 MAPK by cytokine treatment as well as by a number of interacting proteins. We have previously identified several such SERT-interacting proteins using the yeast two-hybrid approach (4, 5). The physical interaction with SERT and the functional relevance of these interactions have been verified for a few of these proteins. Based on our studies we hypothesized that SERT exists within multi-protein complexes which are crucial in regulating SERT function. In order to characterize such complexes and to study their functional consequences we have now employed an alternative proteomic approach, in which we isolated SERT-protein

complexes from rat brain tissue using an antidepressant coupled to magnetic beads (Dynabeads, Invitrogen). Purified samples were separated by 1D gel electrophoresis and subjected to analysis by mass spectrometry (performed in collaboration with Dr. P. Maguire, Clinical Proteomics Group Leader, Conway Institute, UCD). This approach enabled us to isolate large complexes from different cellular compartments which corroborate with known SERT localizations, e.g. at presynaptic sites, axons and in glial cells. Currently, SERT-protein interactions are verified in detail using biochemical and cell biological techniques such as co-immunoprecipitations and co-localization studies in heterologous expression systems as well as in primary neuronal cultures. Furthermore, the role of candidate proteins in the regulation of SERT gene expression and protein interaction by pro-inflammatory cytokines is assessed using reverse transcriptase and/or real-time PCR. Our analysis will significantly contribute to the understanding of SERT functional regulation by interacting proteins.

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PC398

NMDA receptor subtypes in neurons of rat subthalamic nucleus

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NMDA glutamate receptors (NMDARs) in subthalamic nucleus cells are of potential importance in changes in the behaviour of the basal ganglia circuitry that occur in Parkinson's disease and are therefore potential therapeutic targets (Hallett & Standaert, 2004). We have used patch-clamp recording methods to investigate the subtypes of NMDAR present in outside-out membrane patches from subthalamic nucleus cells in 300 μ m coronal brain slices from humanely killed 7 days old rats.

Single channel recordings of NMDAR channels activated by 200 μ M NMDA and 10 μ M glycine were made. Distributions of channel amplitudes identified four conductance levels (19 ± 1.1 , 41 ± 2.0 , 52 ± 1.5 and 67 ± 1.0 pS). The presence of the two lower conductance states indicates that NR2D or NR2C subunits contribute to these receptors (Cull-Candy et al., 2001) while unusually large conductance openings (67 pS) were also observed in 3 out of 6 patches. The NR2B antagonist, ifenprodil (10 μ M) reduced channel open probability by $62.6 \pm 12.5\%$ (mean $\pm$ S.E., $n = 6$ patches) confirming the presence of NR2B receptors. These data suggest that functional NMDARs in subthalamic nucleus in 1 week old rats are heterogeneous and include both low and high conductance receptors.

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PC399

Targeting of exogenous poly(A) binding protein (PABP) to human mitochondria

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Mitochondria are subcellular organelles present in eukaryotic cells that play a central role in the conversion of metabolic fuels to a readily utilisable source of energy in form of ATP. One of the most distinct features of mitochondria from the other organelles of metazoan cells is the possession of their own genome. The mammalian mitochondrial genome encodes only 13 peptides all of which are involved in respiratory chain in addition to the RNA components of mitochondrial translation machinery. However, the majority of mitochondrial proteins are encoded in the nucleus, synthesized in cytoplasm and then imported into the mitochondria.

In mammals, all mRNA encoded by mitochondrial DNA exhibit stable 50-60 nt poly(A) tails, suggesting the existence of mitochondrial poly(A) binding protein. To date, no such protein has been isolated.

To investigate the function(s) of polyadenylation in human mitochondria we decided to disturb its role(s) by targeting an exogenous poly(A) binding protein (PABP) into the organelles. Anticipating that the exogenous PABP will interact with mt-mRNA poly(A) region, we expect possible changes in mt-mRNA metabolism and translation.

By using this alternative approach we hope to clarify whether or not there is a mitochondrial poly(A) binding protein and whether it plays a role in transcript stability or translation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC400

Mapping alterations in regional brain activity by 2-deoxyglucose imaging following pharmacological manipulation of cholinergic neurotransmission

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The central cholinergic system has been implicated in cognitive function. Pre-clinical research has shown that the anticholinesterase, donepezil, can improve rodent performance in behavioural tasks of learning and memory, reverse behavioural deficits associated with administration of muscarinic antagonists and increase extracellular acetylcholine levels in the cerebral cortex. Furthermore, the muscarinic antagonist scopolamine has been widely reported to impair performance in a variety of rodent tests of learning and memory.

In order to investigate the effects of scopolamine and donepezil on glucose utilisation, two studies were carried out using semi-quantitative ^{14} C-2-deoxyglucose autoradiography on male Hooded Lister rats following scopolamine (1mg/kg & 2mg/kg i.p. or vehicle) or donepezil (0.1mg/kg, 0.5mg/kg & 1mg/kg i.p. or vehicle) treatment. The relative glucose uptake values were calculated for discrete brain regions and analysed by one-way ANOVA; if significant post-hoc Dunnett's tests were performed ($*p < 0.05$; $**p < 0.01$). The relative glucose uptake values for selected brain regions are summarised in table 1 below, with mean glucose uptake for each treatment expressed as a percentage of the mean glucose uptake of the vehicle treatment groups for comparison.

These findings were in accordance with previously published studies of scopolamine-mediated alteration in glucose utilisation using the fully quantitative 2-deoxyglucose method (e.g., Helen & London, 1984). Donepezil, however, induced no significant changes in glucose uptake in any brain region. It is therefore likely that no changes will be detected in response to donepezil without a challenge to the cholinergic system through pharmacological manipulation or lesioning. However, it is possible that cognitive enhancing muscarinic agonists that act directly on the receptors could produce significant alteration in glucose utilisation.

Table 1

	Relative Glucose Uptake (as % of vehicle treated)			
	0.5mg/kg donepezil	1mg/kg donepezil	1mg/kg scopolamine	2mg/kg scopolamine
caudate putamen	100	100.9	107.4**	109**
sensorimotor cortex	102.8	102.8	82.7**	85**
lateral septum	98.7	100	107.9	111.2*
diagonal band of Broca (horizontal limb)	92.2	91.1	109.1	113.6**
anteroventral thalamus	100.7	105.9	76.1**	75.4**
anteromedial thalamus	100	105.4	74.6**	76.3**
medial nucleus amygdala	97	106	116.4	119.5**
ventrolateral nucleus thalamus	102.1	103.6	85*	85.7*
dentate gyrus	98.8	100	107.3	119.5**
retrosplenial granular cortex	100.8	103.3	85.9**	85.2**
auditory cortex	100	104.9	84.4*	81.6**
medial geniculate nuclei	100.9	103.5	86**	83.5**

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PC401

Urocortin restores striatal extracellular DA in the 6-hydroxydopamine hemiparkinsonian rat

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Parkinson's disease (PD) is a neurodegenerative disorder with no cure and treatments with limited efficacy. We have recently observed that the CRF-like peptide urocortin (UCN) reverses key features of nigrostriatal neurodegeneration in the 6-hydroxydopamine (6-OHDA) hemiparkinsonian rat, (Biggs et al., 2006; Abuirmeileh et al. 2007). While these data strongly indicate restoration of nigrostriatal integrity we are unsure if they reflect a restoration of striatal extracellular DA levels. To address this we have measured basal and evoked extracellular striatal DA using microdialysis in freely moving rats.

Male Wistar rats (250-280g-1; n = 4-6 per group) were anaesthetised with isofluorane and stereotactically injected with 6-OHDA or UCN (T0) as previously described (Biggs et al. 2006). In some experiments UCN was given 7 days after 6-OHDA (T+7). 14 days after 6-OHDA, rats were given 0.5 mg/kg of apomorphine to evaluate ipsilateral circling, after which they were implanted with dialysis probes (under isofluorane anaesthesia) as previously described (Whitton et al., 1992), and 24h later perfused with artificial cerebrospinal fluid (Whitton et al., 1992) at 1.0 µl/min. After 1 h, 4 x 30 min samples were collected prior to infusion of 100mM K⁺ (substituted for equimolar Na⁺) for 30 min, and collection two further 30 min samples. DA was estimated as previously described (Biggs et al., 2006). Data were subjected to two way ANOVA and Dunnett's post hoc test.

Compared to rats given 6-OHDA alone, apomorphine induced circling behaviour was greatly attenuated by injection of UCN (25.6 ± 3.77 vs 6.51 ± 1.2 and 8.43 ± 2.2 turns/120 s for 6-OHDA, 6-OHDA + UCN (T0) and 6-OHDA + UCN (T+7) respectively, $p < 0.05$). Striatal dialysis of shams showed basal extracellular DA at 28 ± 2.7 fmol/10 µl (mean $\pm$ s.e.mean, n = 10) and this was increased to around 1000 fmol/10 ml by K⁺ infusion. 6-OHDA reduced both basal and stimulated DA levels by between 80-90 %. However, when UCN was given either concomitantly with the toxin (T0) or seven days later (T+7) basal and evoked extracellular DA was comparable to Sham treated controls.

These data provide further evidence for a potential neuroprotective role for UCN in models of PD. Two key points arise from these findings. Firstly, since UCN can restore striatal extracellular DA concentrations this indicates that local DA receptors will be engaged and we presume this underlies the restoration of motor deficits. Secondly, the observation that UCN reverses motor and neurochemical deficits when given at T+7 indicates that it is capable of effecting restoration once the lesion has become established (Abuirmeileh et al., 2007). These observations suggest that UCN or similarly acting molecules offer therapeutic promise in PD.

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PC402

Study of the role of central GABAB on pain sensitivity during the estrous cycle in rats

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Both baseline pain sensitivity and the response to antinociceptive treatment are sensitive to the animal's sex and estrous cycle stage. On the other hand, the role of GABA due to GABAB receptor in nociception was investigated. So the aim of present study was to investigate the effect of inter cerebroventricular injection of GABAB receptor agonist (baclofen) and GABAB receptor antagonist (CGP35348) on pain sensitivity during different stages of estrous cycle.

Twenty four adult female rats weighting 200-220g were used. Rats were maintained on 12h reversed light/dark cycle, and standard temperature 20 ± 4 . Food and water were available ad libitum. Pain sensitivity was evaluated by formalin test. Formalin test was performed by subcutaneous injection 50 µl of formalin (2.5%) solution into the hind paw in all stages of estrous cycle. Animals were divided in 4 groups: 1- control (intact animal), 2- sham (received 2 µl Artificial cerebrospinal fluid-ACSF), 3- experiment1 (received 2 µl of baclofen 4.25 and 8.5 µg/rat), 4- experiment2 (received 2 µl of CGP35348 3 and 5 µg/rat). Data was analyzed by two ways ANOVA measuring. The level of significant was $p < 0.05$.

Our data showed that baclofen decrease pain sensitivity in all phases of estrous cycle, but this analgesic effect was high during metestrus and diestrus phase of estrous cycle and low during proestrus and estrus. CGP35348 increased pain sensitivity in all phases of estrous cycle, but this Hyperalgesic effect was low during metestrus and diestrus phase of estrous cycle and high during proestrus and estrus.

According to our data GABAergic system can modulate pain sensitivity during estrous cycle.

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PC403

Selective adenoviral vector mediated inhibition of pontospinal noradrenergic neurones causes hyperalgesia in rats

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The pontospinal noradrenergic (NA) neurones are thought to form an important anti-nociceptive control system. These neurones are distributed across three areas, locus coeruleus, A5 and A7. Because of the diffuse distribution of NA somata (especially A5) and their widespread projections it has been difficult to study the pontospinal NA system in isolation. To investigate this role in nociception, we have used 2 adenoviral vectors (AVV), Ad-PRS-EGFP (control) and Ad-PRS-Kir2.1-EGFP (Kir), both containing a synthetic promoter element (Hwang et al., 2001) that is specifically active in catecholaminergic neurones to retrogradely target NA neurones that project to the lumbar dorsal horn (Howorth et al., 2005). By expressing a potassium channel (Kir2.1) we have aimed to inhibit these neurones and examine their role in modulating responses to noxious stimuli.

Wistar rats (n=36) had injections of control or Kir AVV (tester blinded) under ketamine (5mg/100g) and medetomidine (30µg/100g i.m.) recovery anaesthesia into the lumbar dorsal horn (L4-5). After 7-14 days, the formalin assay was performed (50µl of 5% formalin into the plantar surface of the hindpaw). Flinches and foot lifts were scored for 1 hour and after a further 2 hours, animals were killed with an overdose of pentobarbital. A subgroup of animals (n=8) also had Hargreaves testing before surgery and again prior to formalin testing. The expression of Kir2.1 and c-Fos was determined using fluorescence immunocytochemistry. All data are reported as mean ± S.E.M.

In the formalin assay, a typical biphasic behavioural nociceptive response was observed. However, the animals transfected with Kir, showed an increased number of flinches and foot lifts in phase 1 (113±10, n=11 vs control 85±4, n=17, P<0.05), but not phase 2 (130±11 vs control 111±6, P>0.05). c-Fos staining in the spinal cord, was significantly increased in the deep dorsal horn (468±93, n=8 vs control 236±39, n=7, P<0.01) with a similar trend seen in the superficial dorsal horn (310±56 vs control 155±27, P>0.05). Hargreaves testing showed paw withdrawal latency was significantly decreased in the Kir group (pre 15±2s vs post surgery 11±1s, P<0.05, n=4) but not the control group. We have demonstrated the use of an AVV to selectively inhibit noradrenergic neurones projecting to the dorsal horn. Further we have shown that the inhibition of these neurones results in hyperalgesia and increased spinal c-Fos expression. Thus indicating that this small population of pontospinal neurones (~150) projecting to the lumbar dorsal horn play a prominent role in the descending control of nociception.

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PC405

Microglial reaction to spinal cord injury and axonal degeneration. An *in vivo* laser scanning microscopy study in mice

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Regeneration in the central nervous system is assumed to be largely disabled by glial activity. We applied *in vivo* two-photon laser-scanning microscopy to investigate acute and subacute reactions of microglia and their interaction with lesioned axonal fibre tracts in the dorsal column of transgenic mice with cell-type specific fluorescent protein (FP) expression.

In crossbreedings of TgN(Thy1-EYFP) and TgH(CX3CR1-EGFP) mice neurons and microglia could be distinguished by selective expression of yellow or green FP expression, respectively. In fully anaesthetized mice (for details see below) a laminectomy was done and small lesions were performed with a fine needle or with infrared laser pulses of different intensity in the dorsal columns at spinal cord segment L4. Subsequently, cellular responses were either continuously recorded for up to 11 h after lesion or were repeatedly observed over 2-5 days. Between the long-term experiments the dorsal wound was closed and mouse behaviour was closely followed for signs of infection or pain. Anaesthesia/analgesia: (1) single acute experiments: initially pentobarbital 80 mg/kg i.p., followed by methohexital-Na 50 mg/kg, i.v. (2) repeated experiments: during the experiments pentobarbital initially 80 mg/kg i.p., 20 mg/kg i.p. added about every hour as required; during the periods between the experimental inspections these mice received buprenorphin 0.05 mg/kg i.p. every 8-12 hours.

Within 1-2 min after the lesion the microglia started to extend processes with protrusions at their end to the direction of the lesion. Subsequently, also some microglial somata moved into the same direction. After a few minutes lesioned axonal fibres generated bulbous endings. These bulbs were partly engulfed by microglia and removed. Microglia activity continued for the next days. The process was almost symmetric to the lesion site in the early acute state but was mainly rostrally orientated i.e. to the distal part of the axons in the subacute state. While microglial responses within the first hours after injury were mainly restricted to process extension towards the lesion site, long-term recordings over several days revealed a significant accumulation of

numerous microglial cells starting at about 10 to 15 h post lesion and continuing for at least 5 days.

The results show that the microglial cells play a continuous role in spinal cord injury, starting already within a few min and continuing for several days. Microglial responses can be dissected in distinct phases for which the molecular control mechanisms still have to be identified.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC406

Structural perspectives of RTN4/Nogo proteins: implications for their functions and molecule design of therapeutic application

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Recently RTN4/Nogo proteins have received intense attentions because they have been implicated in a variety of critical cellular processes including CNS neuronal regeneration, vascular remodeling, apoptosis, interaction with beta-amyloid protein converting enzyme (BACE) and formation/maintenance of the tubular network of the endoplasmic reticulum (ER). Therefore, several years ago we initiated a systematic NMR characterization of Nogo proteins.

Firstly, the successful determination of the solution structure of the buffer-insoluble Nogo-60 offered us rationales to successfully design structured and buffer-soluble Nogo-54, which can be used as a template to further design novel NgR antagonists to enhance CNS neuronal regeneration. Secondly, bioinformatics, CD and NMR characterization revealed that except for the Nogo66 loop, both N- and C-termini of Nogos were intrinsically-unstructured. We speculate that being intrinsically unstructured may allow Nogos to serve as double-faceted functional players, with one set of functions involved in cellular signaling processes and with another in forming/maintaining membrane-related structures. Integration of the two sets of functions in one intrinsically-unstructured protein may represent a general mechanism to place membrane-associated machineries under direct control by cellular signaling.

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PC407

Potassium cyanide and 3-nitropropionic acid neurotoxicity is NMDA receptor-dependent

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The neuronal toxicity of potassium cyanide (KCN) and 3-nitropropionic acid (3-NPA) has been shown to involve the NMDA receptor (1, 2), but it is not clear whether the same downstream effectors of NMDA-induced neurotoxicity are also involved in the toxicity of both compounds. Excessive stimulation of the NMDA receptor elevates intracellular calcium levels, resulting in the generation of reactive oxygen or nitrogen species such as superoxide anion, hydrogen peroxide, and nitric oxide, which are known to mediate neuronal death (3). We hypothesized that similar downstream effectors of NMDA receptor-mediated neuronal damage may be involved in the induction of neurotoxicity by both poisons. Cerebellar granule neuronal cultures from 7- or 8-day old Sprague-Dawley rat pups were prepared as earlier described (4), and maintained in Minimum Essential Medium supplemented with 10% Foetal Calf Serum, 2mM glutamine, 25mM KCl, and 50µg/ml gentamicin, at 37°C in a humidified atmosphere of 5% CO₂/95% O₂. Cells were seeded at 1 x 10⁶ cells/ml into 96-well plates pre-coated with poly-D-lysine. Cytosine arabinoside (10µM) was added to the cultures 24 h after plating to inhibit non-neuronal growth. Treatments were applied at 8 or 9 days in vitro and cultures allowed to recover for at least 18 h before viability was determined using the Alamar Blue reduction assay (5) (incubation period of 6 h). Exposure to the various agents was for 1 or 6 h, and in the case of NMDA receptor antagonists, a pre-treatment period of 15 min was included. Morphological effects were monitored using the Olympus DP50 inverted phase-contrast microscope. Potassium cyanide (10µM – 1mM) lowered neuronal viability in a concentration-dependent manner, and 3-NPA (10µM – 1mM) in a concentration- and time-dependent manner, compared to the controls. The non-competitive and competitive NMDA receptor antagonists, MK-801 (10µM) and D-AP5 (50µM), respectively, blocked completely the reductions in viability induced by both poisons, whereas the glycine-site blocker kynurenic acid (up to 1mM) had no effect. The non-NMDA receptor blocker CNQX (10µM) also failed to

modify the toxicity elicited by the poisons. The nitric oxide synthase inhibitor L-NAME (1mM), and the antioxidant enzymes catalase and superoxide dismutase (each at 100U/ml) partially but significantly protected against the neurotoxicity. Morphological effects correlated with results obtained from viability assays. Taken together, these results show that the mechanisms of toxicity of potassium cyanide and 3-nitro-propionic acid involve similar pathways downstream of NMDA receptor activation, and also suggest that activation of the glycine-sensitive site on the receptor may not be obligatory for the induction of neurotoxicity by both mitochondrial poisons.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC408

Comparison of petal of *Crocus sativus* L. and fluoxetine in the treatment of depressed outpatients: A pilot double-blind randomized trial

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Depression is one of the most common neuropsychiatric conditions, with a lifetime prevalence approaching 17%(1). Although a variety of pharmaceutical agents are available for the treatment of depression, psychiatrists find that many patients cannot tolerate the side effects, do not respond adequately, or finally lose their response. On the other hand, many herbs with psychotropic effects have far fewer side effects. They can provide an alternative treatment or be used to enhance the effect of conventional antidepressants. A number of recent preclinical and clinical studies indicate that stigma and petal of *Crocus sativus* have antidepressant effect (2-5). Our objective was to compare the efficacy of petal of *Crocus sativus* with fluoxetine in the treatment of depressed outpatients in an 8-week pilot double-blind randomized trial. Forty adult outpatients who met the DSM- IV criteria for major depression based on the structured clinical interview for DSM- IV participated in the trial. Patients have a baseline Hamilton Rating Scale for Depression score of at least 18. In this double-blind and randomized trial, patients were randomly assigned to receive capsule of petal of *Crocus sativus* 15 mg bid (morning and evening) (Group 1) and fluoxetine 10 mg bid (morning and evening) (Group 2) for a 8-week study. At the end of trial, petal of *Crocus sativus* was found to be effective similar to fluoxetine in the treatment of mild to moderate depression ($F = 0.03$, d.f. = 1, $P = 0.84$). In addition, in the both treatments, the remission rate was 25%. There were no significant differences in the two groups in terms of observed side effects. The present study is supportive of other studies which show antidepressant effect of *Crocus sativus*.

Table 1. Baseline data

	Petal of <i>Crocus sativus</i> Group	Fluoxetine Group
Women	10	11
Man	10	9
Age (Mean $\pm$ SD)	35.55 $\pm$ 8.05 (year)	34.09 $\pm$ 5.50 (year)
Age of onset	31.75 $\pm$ 7.16 (year)	32.85 $\pm$ 5.24 (year)
Weight (Mean $\pm$ SD)	72.45 $\pm$ 10.44	70.35 $\pm$ 10.33
Height (cm)	175.45 $\pm$ 8.21 (cm)	177.65 $\pm$ 7.61 (cm)
Duration of recent episode (Mean $\pm$ SD)	2.30 $\pm$ 1.70 (month)	2.40 $\pm$ 1.80 (month)
Number of previous episodes	3.42 $\pm$ 0.76	3.63 $\pm$ 0.89
Medications history	Fluoxetine: 15; Nortriptyline: 3; Sertraline: 2	Fluoxetine: 13; Nortriptyline: 4; Sertraline: 3

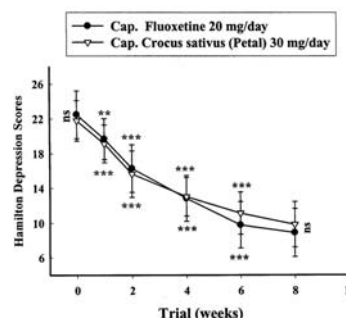


Figure1. Mean $\pm$ S.E.M. scores of two groups of patients on the Hamilton Depression Rating Scale. ns=non-significant, ** = $P < 0.01$ and *** = $P < 0.001$. The horizontal symbols (** and ***) were used to express statistical significance versus their respective baseline value and vertical symbols (ns) were used for between group comparisons.

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PC410

Different sites on xanthine oxidase may contribute to the production of neurotoxic reactive oxygen species

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Production of toxic reactive oxygen species (ROS) due to over activity of xanthine oxidase (XO) is thought to be involved in many neuronal and vascular pathologies. A combination of xanthine (X) and XO is used as an in vitro model to produce mainly superoxide, but it has also been reported to generate hydrogen

peroxide. Interestingly, it was reported that the cell death resulting from applying this combination to cultures of cerebellar granule neurons (CGNs) was prevented by catalase but not by superoxide dismutase, suggesting the predominant role of hydrogen peroxide over superoxide in this toxicity. Deferoxamine, an iron chelator that hinders the conversion of hydrogen peroxide to the highly toxic hydroxyl radical prevented the cell damage, confirming the predominant role of hydrogen peroxide and its subsequent conversion to hydroxyl radical. In the light of these findings we have examined the effect of blocking different sites on XO on cell viability in a model of X/XO toxicity in cultured CGNs to help determine which site on the enzyme is responsible for the generation of ROS (especially hydrogen peroxide). A concentration of X(100 μ M)/ XO (0.02 units/ml) applied to cultured CGNs for one hour resulted in consistent and significant damage. Catalase at 300 units/ml completely prevented the damage, whereas superoxide dismutase (50-1500 units/ml) failed to provide protection. Allopurinol, an inhibitor of the molybdenum site on XO, provided significant protection at 100 μ M. Diphenyleneiodonium (DPI), a known NAD(P)H-oxidase inhibitor, which can also inhibit the flavin adenine dinucleotide (FAD) site on XO (a site that is not blocked by allopurinol), also provided significant protection at 1000 nM. These results suggest that more than one site on XO is responsible for the production of toxic reactive oxygen species (especially hydrogen peroxide) and the subsequent cell death.

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PC411

Novel antioxidant agents to combat oxidative stress in Alzheimer's disease

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Oxidative stress, mediated by reactive oxygen species (ROS), is well recognised to be integral to the disease processes in Alzheimer's disease.[1-2] The major source of ROS production is the electron transport chain from where electrons leak to react with oxygen to produce ROS. Unregulated, ROS undergo a wide array of reactions with critical biomolecules thus imparting serious structural and functional cell damage. A high prevalence of oxidised fats and proteins has indeed been demonstrated in murine models of AD.[3]

The superoxide dismutase-catalase (SOD-CAT) enzyme system is a vital defence against ROS, converting superoxide to water and oxygen.[4] This study investigated the use of SOD-CAT mimetics as novel antioxidant agents to treat AD.

A novel SOD-CAT mimetic, NMC-X, was prepared and tested against EUK-134, a known SOD-CAT mimetic. An assay consisting of the agent, an ascorbate probe and the ROS H₂O₂ was prepared. Antioxidant activity was assessed by comparing the protection afforded by each agent to the ascorbate probe, a molecule highly sensitive to oxidative attack. Both agents were also tested for their stability in water and in the presence of the ROS H₂O₂. Results revealed intriguing differences in antioxidant properties between EUK-134 and NMC-X. EUK-134 exhibited excel-

lent antioxidant activity over a short 2 hr time period allowing only a 3.1 % oxidation rate of ascorbate. Conversely, NMC-X in fact exhibited pro-oxidant activity over 2 hrs, with the ascorbate having a 64.3 % oxidation rate.

Stability testing showed that both agents underwent structural change in a peroxide environment over the 2 hr period that was detrimental to antioxidant activity. The changes appeared to be twofold in nature. There was initial alteration of the oxidation state of each agent that allowed the reduction of ROS to water and oxygen. This was however accompanied by a more gradual change to the agent that accounted for the reduction in antioxidant activity. Further investigation is required to determine the exact nature of this change but it is likely to be due to a non-enzymatic reaction between the agent and the ROS.

This research outlines the importance of the structure function relationship in drug development. We describe how the identification of structural features that can reverse antioxidant activity is vital to the increased understanding of the structure-function relationship. Furthermore, our results show that with increased emphasis on improving the stability of the SOD-CAT mimetic class of antioxidant agents, they are well worth pursuing as the next generation of drugs to treat diseases in which ROS play an integral role.



Figure 1: NMC-X Key: Black: manganese Grey: hydrogen Yellow: chlorine Red: oxygen Blue: nitrogen Green: carbon

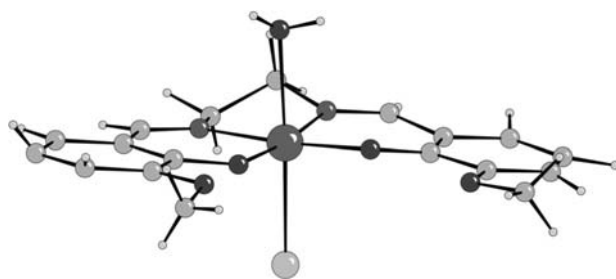


Figure 2: EUK-134 Key: Red: oxygen Blue: nitrogen Green: carbon Black: manganese Grey: hydrogen Yellow: chlorine

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PC412

CB₁ receptor antagonists increase inhibitory neurotransmission in the mouse cerebellum

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Within the cerebellum, a major component of presynaptic inhibition is mediated by G-protein coupled cannabinoid CB₁ receptors. In particular, CB₁ is prominently expressed in inhibitory synapses between interneurons and Purkinje cells, the latter representing the sole output of the cerebellum. Here, we combine single and multi-electrode electrophysiological recording in acute brain slices with GTPγS binding assays in isolated membranes to investigate the effects of synthetic agents and the phytocannabinoid Δ⁹-tetrahydrocannabinol (Δ⁹-THCV) on CB₁ receptors in the mouse (male TO strain, 3-5 weeks old) cerebellum.

Using whole-cell patch clamp recordings from Purkinje cells in mouse cerebellar brain slices, we have recently shown that the CB₁ agonist WIN55,212-2 caused a reduction in mean miniature inhibitory post-synaptic current (mIPSC) frequency and that this reduction was increased to levels above control by subsequent application of AM251 or Δ⁹-THCV (Ma et al. BPS Oxford Meeting 2006 abstract). Moreover, when applied alone, AM251, Δ⁹-THCV or SR141617A all acted to increase mIPSC frequency. We have now made extracellular multi-electrode array (8x8) recordings of spontaneous excitatory activity in the same preparation. Recording electrodes were positioned proximal to the Purkinje cell layer and the effects of AM251 (2μM) or Δ⁹-THCV (5μM) examined (bath-applied for a minimum of 15 min, n=6 slices with a minimum 6 electrodes' data each slice, significance by Mann-Whitney U test). Both agents reduced spontaneous excitatory spike firing frequency (Δ⁹-THCV: 25±12% of control; AM251: 71±9% of control; both P<0.05).

In parallel studies, we measured the effects of synthetic CB₁-selective agents and Δ⁹-THCV on [³⁵S]-GTPγS binding to mouse cerebellar membranes. Log concentration-response curves (10pM-10μM) for agonists WIN55,212-2 and CP-55,940 showed a dose-dependent increase in [³⁵S]-GTPγS binding, whilst AM251 or Δ⁹-THCV alone had little effect. In contrast, log concentration-response curves for WIN55,212-2 were shifted rightward with no clear decrease in E_{max} by AM251 (100pM-10nM) and Δ⁹-THCV (100nM-5μM), consistent with a CB₁ antagonist action. Preliminary Schild analysis gave apparent K_B values of 63 pM (AM251) and 68 nM (Δ⁹-THCV).

Overall, we provide functional evidence that AM251 and Δ⁹-THCV act to increase GABA release onto Purkinje cells and that this action correlates with a reduction in spontaneous excitatory activity in the cerebellum. AM251 and Δ⁹-THCV act as CB₁ receptor antagonists suggesting that the increased GABA release is due to removal of an endocannabinoid tone. Moreover, the potent effect of Δ⁹-THCV on spontaneous activity may suggest a differential effect on CB₁ receptors at inhibitory vs excitatory synapses.

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PC413

Synaptic activation of GABAB receptors modulates gamma band network oscillations in the rat hippocampusJ.T. Brown¹ and A. Randall^{1,2}

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In the mammalian CNS, GABAB receptors mediate slow pre- and postsynaptic inhibition. Using extracellular recording from area CA3 of adult rat hippocampal slices and we have investigated the role of synaptic GABAB receptors in regulating kainate-induced subthreshold neuronal network oscillations in the gamma frequency range (25-80 Hz; mean frequency 30.9 ± 1.1 Hz; n=6). The GABAB receptor agonist baclofen largely eliminated gamma oscillations (n=3). The GABAB receptor antagonist, CGP55845, reversed this action of baclofen, but alone did not alter the power or frequency of ongoing oscillations. Thus, following addition of CGP55845 (1 μM), the power of kainate-driven gamma oscillations was 2.14 ± 0.81 μV² cf. a baseline power of 1.72 ± 0.64 μV², whilst the frequency was 29.2 ± 1.0 Hz (P>0.05, n=6). To examine the role of synaptically released GABA on network activity, we electrically stimulated stratum radiatum of CA3, whilst recording gamma oscillations from stratum pyramidale. Single stimuli produced a pronounced, transient (up to 1 s in duration), inhibition of gamma frequency oscillations. The gamma oscillations which re-emerged 300-500 ms after synaptic stimulation had a significantly slower period than those prior to stimulation (34.6 ± 1.4 Hz after stimulation cf. 38.0 ± 1.8 Hz prior to stimulation; P<0.01, n=13). This slowing of the gamma oscillations was transient, such that 4 s after stimulation, the gamma period had returned to baseline levels (37.3 ± 2.1 Hz; P=0.45, n=13). The stimulus-induced shutdown of network activity was significantly enhanced by the GABA uptake inhibitor tiagabine and largely inhibited by CGP55845. Multiple stimuli delivered at frequencies of 1-3 Hz resulted in an activity-dependent fatigue of the inhibition of gamma activity, such that, after a number of stimuli, oscillations could be detected tens of milliseconds after the stimulus. Interestingly, this activity-dependent fatigue of inhibition uncovered a stimulus-dependent temporal entrainment of the gamma oscillations. Furthermore, the amount of repetitive synaptic input that was required to cause this entrainment was dramatically reduced by GABAB receptor antagonism such that it was evident within just a few stimuli. These data suggest that convergent afferent synaptic activity can alter the precise temporal arrangement of neuronal network activity. Furthermore, the flow of such information into a functioning neuronal network is highly regulated by GABAB-receptor-mediated synaptic inhibition.

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PC414

Physiological characterisation of the human microglial cell line C-13NJ

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Microglia are the resident immuno-competent cells of the CNS. They are involved in host defence and repair and the generation of neuro-inflammatory states. This latter aspect role has made them the target of potential therapies for diseases that generate CNS inflammation, e.g. Alzheimer's disease. There has been a steady growth in research dedicated to the physiology of microglia, much of which has relied cultured rodent cells. Although a small number of physiological studies of human microglia exist such work is reliant on a supply of donor tissue. Here we present our initial data on a little studied human microglial cell-line C13-NJ.

C13-NJ cells were cultured in DMEM supplemented with 10% FCS. Experiments were performed at room temperature on cells adhered to glass coverslips. Cell-attached and whole-cell patch clamp measurements were made with a range of bath and electrode solutions designed to characterise various channel types. Ratiometric Ca²⁺ imaging was performed on Fura-2 loaded cells using standard methods.

In >95% of cell-attached recordings at a pipette potential of -70 mV, large outward-going channel openings were observed. The opening of these channels was greatly enhanced by the Ca²⁺-ionophore A-23187. These channel openings were absent from all patches recorded with paxilline (2 µM) in the pipette. This pharmacology, combined with gating and conductance measurements, indicates these are BK potassium channels.

In current clamp recordings under approximately physiological ionic conditions C13-NJ cells had a resting potential of -49 ± 2 mV; more depolarized resting potentials were seen with Cs⁺-containing pipettes. The membrane time constant measured with a 20 pA current injection was 4.9 ± 1.0 ms. Mean membrane capacitance measured in voltage clamp was 20.8 ± 0.8 pF (n=184). Strong hyperpolarizing current injections from rest lead to an initial charging of the membrane which was followed, in a number of cells, by a slow hyperpolarizing drift, which appears to result from the slow closure of a depolarising conductance tonically open at rest. Voltage-clamp experiments indicate that this conductance is sensitive to flufenamic acid and can exhibit spontaneous time-dependent changes in activity.

Step depolarization from negative voltage clamp potentials resulted in a small transient inward current followed by a larger outward current. Much of the latter was paxilline-sensitive and thus seems to arise from the BK channels invariably observed in cell-attached recordings. The former current has kinetics and pharmacology commensurate with activation of the cardiac Na⁺-channel isoform.

In Ca²⁺ imaging ADP, ATP, histamine and LPS triggered acute [Ca²⁺]_i elevations whereas β-amyloid42 and carbachol were ineffective.

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PC415

Comparison of the electrophysiological properties of neonatal mouse and rat sympathetic preganglionic neurones *in vitro*

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Sympathetic preganglionic neurones in both the cat *in vivo* (1) and rat *in vitro* (2) possess distinctive electrophysiology with large amplitude, long duration action potentials, a biphasic after-hyperpolarisation, pronounced transient rectification and anomalous rectification. We are interested in taking advantage of the genetic tractability of mice to examine the functional role of genes expressed in SPN, however to date no recording from mouse has been reported, thus we have sought to compare the properties of mouse and rat SPN in spinal cord slices *in vitro*.

Mice (n=6, p8-12, C57/Bl) and rats (n=7, p8-12, Wistar) were deeply anaesthetised with halothane, decapitated and the spinal cord was removed. Transverse slices (300µm) of mid-thoracic spinal cord were cut in aCSF at 4°C. After recovering for an hour at 36°C, visualised whole-cell patch clamp recordings were made at room temperature (18-20°C) from neurones in the lateral horn. The pipette solution contained KGluconate-130; KCl-10; NaCl-10; MgCl₂-2; HEPES-10; ATP-2; GTP-0.2 with Lucifer yellow (1mg/ml) to enable morphological identification. Voltage responses to injected current were used to study the active and passive membrane properties. The membrane time constant was calculated by fitting multiple exponentials to small hyperpolarising responses to current steps (<5mV). Data reported as mean±SE and statistical significance determined using unpaired t-test (at P<0.05).

Recordings from mouse (n=10) and rat (n=12) SPN showed similar properties both qualitatively (presence of transient rectification, anomalous rectification, action potential shape) and quantitatively in their active and passive membrane properties (table 1). There were no significant differences between the species in any of the measured parameters.

Mouse SPN exhibit the same characteristic profile of active and passive membrane properties that have been reported previously for cat (1) and rat (2). Thus the mouse should make a useful model to profile the molecular basis of these characteristic membrane properties and examine their functional role in determining the patterning of the sympathetic outflow.

Table 1

	Mice (n=10)	Rats (n=12)
Resting potential (mV)	-67.8±2.4	-65.0±2.5
Input resistance (MΩ)	481±83	324±62
Time constant (ms)	59.8±6.8	62.3±7.8
Spike amplitude (mV)	76.8±4.2	73.7±4.1
Spike duration (ms)	4.3±0.4	4.3±0.6
AHP duration (ms)	280±105	307±75
Max firing frequency (Hz)	7.1±1.5	6.9±1.5

Resting potential corrected for 10mV junction potential. Maximum firing frequency evoked in response to a depolarising current pulse.

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PC416

Remodelling of calcium homeostasis: effects of hypoxia and soluble amyloid β peptide oligomers

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Hypoxic episodes, induced by chronic respiratory disease or stroke, increase the probability of the sufferer developing Alzheimer's disease (AD) as they age. Although the mechanisms underlying this observation are unknown, we have previously shown that hypoxia causes dramatic remodelling of cellular Ca^{2+} homeostasis by increasing Ca^{2+} influx through voltage-sensitive and insensitive ion channels and by modifying cellular Ca^{2+} buffering of store-mediated Ca^{2+} release (Scragg et al, 2005; Smith et al, 2005). Additionally, Ca^{2+} dyshomeostasis has been implicated in the neurodegeneration of AD, and hypoxic disruption of Ca^{2+} signalling has been linked to pro-amyloidogenic processing. Investigations of the role of A β peptides (A β) in these changes using synthetic monomers have produced contradictory results. A possible explanation for this has been the recent observations that many of the cytotoxic effects of A β are due to soluble oligomers of A β (Walsh and Selkoe, 2004). Therefore, we investigated the effects of hypoxia and soluble oligomers of A β on bradykinin (BK)-induced Ca^{2+} release in primary cultures of astrocytes and a neuroblastoma cell line, SH-SY5Y loaded with fura-2 AM.

Chronic hypoxia (1% O_2 , 24hr) caused a significant (>2fold) increase in the size of the BK-induced Ca^{2+} response relative to normoxic controls in both cell types. O_2 concentrations closer to physiological (9%) than atmospheric (21%) had no effect on BK-induced rises in $[\text{Ca}^{2+}]_i$. Conditioned media (CM) from CHO cells stably expressing human APP751 containing the V717F mutation (7PA2 cells; a gift from D. Walsh) which secrete A β was tested to ensure that it contained A β monomers and oligomers and then applied to cells for 24hr. Treatment with 7PA2 CM caused a large (~2fold) increase in the BK-evoked Ca^{2+} transient in both astrocytes and SH-SY5Y cells. Treatment with CHO CM had no effect. Neither hypoxia nor soluble A β oligomers had any effect on capacitative calcium entry.

These results confirm the ability of chronic hypoxia to modulate Ca^{2+} signalling in different cell types, supporting previous studies. The modulation of Ca^{2+} homeostasis by A β oligomers contributes to growing evidence suggesting this aggregation state to be important in neuronal dysfunction in the early stages of Alzheimer's disease. These results also suggest Ca^{2+} dyshomeostasis as a possible mechanism by which A β oligomers exert their toxicity. The observation that CCE is unaltered by either treatment suggests that, as with hypoxia, A β oligomers may act on Ca^{2+} extrusion and buffering mechanisms rather than directly on the Ca^{2+} stores.

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PC417

Activation of metabotropic glutamate receptors 4 and 7 in the substantia nigra pars reticulata reverses akinesia in the reserpine-treated rat

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Increased glutamate release in the substantia nigra pars reticulata (SNr), from overactive subthalamic nucleus efferents, contributes to the akinesia seen in Parkinson's disease. Our previous studies indicate that activation of two types of group III metabotropic glutamate (mGlu) receptor, mGlu4 and mGlu7, can inhibit glutamate release in slices of rat SN (Austin et al. 2006). The aim of the present study was to establish whether modulation of these same receptors *in vivo*, using N-phenyl-7-(hydroxyamino) cyclopropa[b]chromen-1a-carboxamide (PHCCC), the positive allosteric modulator of mGlu4, and N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), the selective mGlu7 allosteric agonist, could reverse akinesia in the reserpine-treated rat.

Male Sprague-Dawley rats were stereotactically cannulated (under isoflurane inhalation: 4% in 95% O_2 -5% CO_2 ; maintained during surgery with 2% isoflurane in the same O_2 - CO_2 mix) 2 mm above the SNr. 7 days post-surgery they were rendered akinetic using reserpine (4mg kg⁻¹ s.c.). Once akinetic (~18 h later), rats received a single unilateral intranigral injection (2.5 μl) of either PHCCC (3-75 nmol), AMN082 (3-100 nmol) or vehicle (40% DMSO in PBS). In some cases, the ability of the group III mGlu receptor antagonist, (RS)-cyclopropyl-4-phosphonophenylglycine (CPPG), to reverse these effects was tested by intranigral injection of 75 nmol CPPG 30 min prior to injection of PHCCC (75 nmol) or AMN082 (100nmol). Locomotor activity was recorded for 60 min post-injection and the number of 360° contraversive rotations quantified as a measure of relief of akinesia. Treatment groups were compared using either a 1-way ANOVA with student Newman-Keuls post hoc test or, for the antagonist study alone, an unpaired t-test (5% level).

PHCCC (3-75nmol) produced a dose-dependent reversal of akinesia reaching 141±28 rotations 60 min⁻¹ (75 nmol PHCCC) compared with vehicle (11±8 rotations 60 min⁻¹; P<0.01). AMN082 (3-100 nmol) also produced a dose-dependent reversal of akinesia reaching 226±75 rotations 60 min⁻¹ (100 nmol AMN082) compared with vehicle (17±9 rotations 60 min⁻¹; P<0.05). For both PHCCC (75nmol) and AMN082 (100nmol), pre-treatment with the group III antagonist, CPPG (75nmol), inhibited the response by 71% and 70%, respectively.

Both PHCCC and AMN082 were able to reverse reserpine-induced akinesia, an effect blocked by the group III mGlu antagonist, CPPG. These data support a role for both mGlu4 and mGlu7 receptor sub-types in mediating this response. On the basis of our previous *in vitro* data (Austin *et al.* 2006), these anti-akinetic effects most likely reflect inhibition of abnormally-elevated glutamate release within the SNr of the reserpine-treated rat.

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PC418

The phytocannabinoid Δ^9 -tetrahydrocannabinarin acts as a CB₁ receptor antagonist in [³⁵S]GTP γ S binding assays in mouse cerebellar membranes

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The G-protein coupled cannabinoid receptors CB₁ and CB₂ are molecular targets for endocannabinoids and are also modulated by exogenous synthetic agents and phytocannabinoids derived from *Cannabis sativa*. We have recently shown that the phytocannabinoid Δ^9 -tetrahydrocannabinarin (Δ^9 -THCV) acts to increase inhibitory neurotransmission in synapses between interneurons and Purkinje cells (IN-PC) in the mouse cerebellum, suggesting either an antagonist or inverse agonist effect at CB₁ receptors (Ma *et al.*, BPS Oxford Meeting 2006 abstract). Additionally, Δ^9 -THCV has recently been reported to act as a CB₁ receptor antagonist in membranes from whole mouse brain (Thomas *et al.*, 2005). In the current study, the effect of Δ^9 -THCV on [³⁵S]GTP γ S binding in mouse cerebellar membranes was investigated and compared with synthetic CB₁-selective agents, to complement electrophysiological findings and investigate mode of action in a specific brain area.

Cerebellar membranes were prepared from male TO mice (3-5 weeks old). [³⁵S]GTP γ S binding assays were performed to establish log concentration-response curves (10pM-10 μ M) for the synthetic CB₁ receptor agonists WIN55212-2 and CP55940, the antagonist/inverse agonist AM251, and Δ^9 -THCV. The effects of AM251 (100pM-10nM) or Δ^9 -THCV (100nM-5 μ M) on log concentration-response curves for WIN55212-2 were also investigated.

AM251 and Δ^9 -THCV (Figure 1) alone showed no concentration dependent reduction of [³⁵S]GTP γ S binding. AM251 and Δ^9 -THCV (Figure 1) produced rightward shifts in the log concentration-response curve for WIN55212-2. Preliminary Schild analysis gave apparent K_B values of 63 pM (AM251) and 68 nM (Δ^9 -THCV).

These results suggest that AM251 and Δ^9 -THCV act as antagonists rather than inverse agonists at CB₁ receptors in the cerebellum. Therefore, the observed increase in inhibitory neurotransmission at IN-PC synapses by AM251 and Δ^9 -THCV may

be due to antagonism of CB₁ receptors (which will block endocannabinoid-induced decreases in neurotransmission) in the cerebellum.

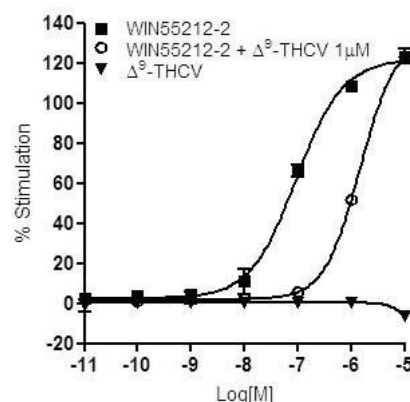


Figure 1. Log concentration-response curves for [³⁵S]GTP γ S binding with WIN55212-2 and Δ^9 -THCV in cerebellar membranes

Thomas *et al.* (2005). *Br J Pharmacol.* 146, 917-926.

Δ^9 -THCV was generously supplied by GW Pharmaceuticals. Work supported by University of Reading RETF Award to I.D.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC419

Comparison of spontaneous and reflex-evoked sympathetic nerve activity in neonate and juvenile rats *in situ*

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Many *in vitro* studies of sympathetic neuronal activity (including cellular and nerve recordings) have been done in preparations from neonatal rats. However, it has been difficult to extrapolate these findings to mature animals, because there have been no recordings of sympathetic nerve activity (SNA) in functionally intact, neonatal rats. Hence we have compared spontaneous and reflex-evoked SNA in neonate (p10-12) and juvenile (4-5 weeks) rats using the working heart brainstem preparation that has eupnoeic respiratory drive and preserved cardiorespiratory reflex function (Paton 1996).

Neonatal (n=6, 15g) and juvenile (n=23, 70-80g) Wistar rats were deeply anaesthetised with halothane, bisected below the diaphragm and decerebrated. Perfusion was reinstituted through a double lumen catheter in the aorta (allowing perfusion pressure (PP) monitoring). Perfusate flow was adjusted (neonates~7ml/min; juveniles~17ml/min), to obtain a rhythmic incrementing eupnoeic phrenic discharge pattern. Thoracic SNA (T6-10) was recorded using a bipolar suction electrode. All data are mean $\pm$ SE and unpaired t-test was used to assess significance (P<0.05).

The PP in the neonate was lower than in the juvenile (22 $\pm$ 1 vs 63 $\pm$ 2mmHg, P<0.001). The pattern of tSNA was similar at both

ages with prominent respiratory modulation, bursts peaking during the inspiratory and post-inspiratory periods. Power spectral analysis showed the frequency distribution of tSNA was similar to that seen previously in juvenile rats, with a dominant peak at the respiratory frequency.

Baroreflex challenges (phenylephrine, 5µg, i.a.) evoked equivalent bradycardia but the sympathetic baroreflex gain was increased in the neonates (Table 1). Activation of the peripheral chemoreflex (NaCN, 100µl, 0.05%, i.a.) evoked similar increases in respiratory frequency, SNA and PP but a smaller bradycardia was found in the neonates. Engagement of the diving response (10°C saline to the snout) evoked equivalent degrees of sympathoexcitation in both groups.

Thus we have shown that rats as young as 10 days postnatal have respiratory modulated sympathetic activity that is functionally regulated by peripheral somatic and cardiorespiratory afferents in a manner similar to that seen in more mature animals. In particular our findings extend previous work on the maturation of the heart rate baroreflex in neonates (1, 2) and indicate that the gain of the sympathetic limb is increased.

Table 1

		Neonate (n=6)	Juvenile (n=23 baro, n=8 chemo)	P Value
Baroreflex	Pressor challenge (mmHg)	20±5	24±2	NS
	Heart rate gain (bpm/mmHg)	1.6±1	1.7±0.4	NS
	Sympathetic gain (%/mmHg)	3.4±0.3	1.5±0.2	P<0.001
Chemoreflex	Increase in resp. rate (%)	194±47	167±21	NS
	Increase in tSNA (%)	137±29	168±30	NS
	Bradycardia (bpm)	72±12	164±19	P<0.01
	Pressor effect (mmHg)	6±1	7±2	NS

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Quigley KS, Myers MM & Shair HN. (2005) Auton Neurosci 121,26-32

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PC420

Local dendritic activity sets release probability at hippocampal synapses

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The release of neurotransmitter at a synapse is a stochastic process such that the arrival of an action potential triggers vesicle fusion with a limited probability (pr). Although pr is a fundamental parameter in defining synaptic efficacy, it is not uniform across all synapses, and the mechanisms by which a given synapse sets its basal release probability are unknown. In this study we employed FM-dye imaging, quantal analysis of paired whole-cell recordings and serial ultrastructural analysis with 3D reconstruction to address this question. By using small networks of cultured hippocampal neurons we were able to make optical measurements of pr at single synapses and show that release probability is set locally by the dendrite through a negative feedback mechanism. In synaptically connected pairs of neurons, a high resolution spatial analysis revealed that neighboring synapses on the same dendritic branch show very similar release

probabilities, even though the connection overall has a broad distribution of pr. Moreover, pr is negatively correlated with the number of synapses made between the axon and the dendritic branch. Increasing network activity elicits a homeostatic decrease in pr that is dependent on dendritic depolarization, and imposing a spatially uniform input to the dendrite significantly reduces the variability in pr. Furthermore, manipulating the activity of a single dendritic branch leads to a spatially selective decrease in pr. Our results indicate that local dendritic activity is the major determinant of basal release probability, and theoretical simulations suggest that this retrograde regulation provides a means to maintain synchronously activated synapses in their operational range.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC421

Is there any relationship between the hypothalamic noradrenergic neurotransmission and kisspeptin in the central regulation of gonadotropin release in female rat?

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It has recently been shown that kisspeptin plays a critical role in the central regulation of the hypothalamo-pituitary gonadal axis in both male and female (Smith et al. 2006). It potently stimulates luteinising hormone (LH) release from the anterior pituitary, and this effect appears to be mediated via the hypothalamic gonadotrophin releasing hormone (GnRH) system. We have investigated effects of kisspeptin on serum LH and follicle stimulating hormone (FSH) levels and concomitant hypothalamic noradrenaline concentrations in pro-oestrous rats. In addition, an α -1 adrenergic receptor antagonist (prazosin) was used to challenge kisspeptin by blockade of the central noradrenergic neurotransmission. Adult female Sprague-Dawley rats were used in this study. Daily vaginal smear was performed, and only pro-oestrous animals were chosen for experimentation. Pro-oestrous rats were intraperitoneally injected with kisspeptin-10 (50 nM/kg), prazosin (2 mg/kg) or prazosin + kisspeptin at 14.00h. Controls received saline alone. All animals were decapitated at 15.00h and blood samples obtained. The brains were rapidly removed and frozen on dry ice. Medial preoptic area (MPOA), arcuate nucleus (ARN) and median eminence (ME) were surgically isolated by micropunch technique. Concentrations of noradrenaline in these samples were determined by high performance liquid chromatography with electrochemical detection. Protein content in the hypothalamic samples was measured by spectrophotometry, and the results were expressed as pg amine/µg protein. Serum LH levels were measured by enzyme immunoassay. This study was approved by the local ethics committee. Mann-Whitney U test was utilized for statistical analysis of the data. Kisspeptin significantly increased noradrenaline

content in the hypothalamic areas examined ($p < 0.05$). Although prazosin lowered noradrenaline levels, these changes were significant in only the ARN ($p < 0.05$). Co-administration of kisspeptin with prazosin resulted in increased noradrenaline concentrations in the MPOA and ARN. Kisspeptin caused a significant increase in serum LH and FSH levels ($p < 0.05$). Prazosin brought about a slight and dramatic decrease in LH and FSH values, respectively ($p > 0.05$). Combination of kisspeptin with prazosin elevated serum LH levels compared to prazosin-treated group ($p < 0.05$). These findings suggest that kisspeptin stimulates both LH and FSH in the pro-oestrous rat. This is the first attempt to show that stimulatory effect of kisspeptin on GnRH and thus gonadotropin release may involve increased noradrenergic activity in the hypothalamus.

Smith JT et al. (2006). *Reproduction* 131: 623-30.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC422

Enteral intake of Aluminium Sulphate in acidic medium enhances absorption and alters tissue content of other trace elements in male albino rats

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Introduction:

Aluminium (Al) intoxication was recognised as a causal agent in patients undergoing haemodialysis and then was linked to Alzheimer disease. Nevertheless, environmental pollution with Al is mainly via drinking water particularly when acidic rain falls on Al containing soil and Bedrock. Aluminium utensils are widely used for cooking and some concern exists regarding the levels of aluminium released through different cooking processes in these vessels. Therefore, increasing concern is emerging for the role of pH in the absorption of aluminium from ingested food. Aim and Methods:

The objective of this study was to establish the effect of acidification on the absorption of aluminium from a 5 mM aluminium sulphate solution (Al) taken orally in albino rats. Levels of Al, Ca, Cu, and Fe in plasma, brain, kidney, and liver were measured. Results:

The results indicate that acidification of Al salt solution enhances its absorption. Increased plasma Al level ($p < 0.05$) were associated with increased deposition in tissues and a reduced overall body mass ($p < 0.05$) and mass of cerebral hemispheres ($p < 0.01$) relative to controls ingesting the non acidified Al salt solution. The increased plasma levels of Al correlated positively with increased Al deposited in the kidney ($r = 0.790$), liver ($r = 0.967$), and brain ($r = 0.955$) despite the blood brain barrier (BBB). Increased Al also correlated negatively with levels of Ca, Cu, and Fe in all tissues except in brain in which there was a positive correlation with Ca deposition. Thus, we conclude that there is increased absorption of aluminium from ingested acidified solutions and that there is also increased deposition of metal

ions in various tissues including the brain where aluminium and calcium are associated with degenerative states. The rising trends of Al deposition in the brain, which correlated, negatively with Cu and Fe levels in experimental animals have not been attempted earlier. Thus, an intelligent interpretation cannot be based on solid ground rather than assuming that the excessive accumulation of Al as positively charged heavy earth metal will remove out similarly charged particles of the same family that normal present.

Conclusion:

Thus, we conclude that, there is increased absorption and deposition of Al in all tissues from rats ingested acidified Al solution. This increased absorption of Al was associated with altered tissue contents of other trace elements.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC424

The role of beta-adrenergic system in the enhancement of spatial learning induced by epinephrine injection in young male rats

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Background: There are controversial reports about memory improving and memory impairing effects of epinephrine and propranolol (1, 2). Talley (2000) reported that epinephrine attenuated performance in rats that were deprived of food for 24 hours (1). On the other hand, there are evidence that propranolol didn't impair performance in the water maze (2). Also, adrenergic systems have consistently been shown to be involved in memory processing and storage (3).

Methods: Young male wistar rats were given daily injection of epinephrine (0.1 mg/kg, sc) (4) or propranolol (20 mg/kg, sc) (5) 30 min before training and co-administration of epinephrine (0.1 mg/kg) and propranolol (20 mg/kg). sham groups were received saline at the same volumes and conditions. A Y-maze apparatus was used for training.

Results: The results showed that epinephrine enhances spatial learning in first ($p < 0.05$), second ($p < 0.01$), third ($p < 0.05$) and fourth ($p < 0.05$) days. Also propranolol impair memory in fifth day ($p < 0.001$). On the other hand, Comparison between co-administered and epinephrine groups significantly decrease spatial learning in first ($p < 0.05$), second ($p < 0.01$) and fourth ($p < 0.05$) days. But there wasn't any significant difference between co-administered and propranolol groups.

Conclusion: These findings indicate that propranolol impairs improvement of spatial learning caused by epinephrine via blockade of beta-adrenergic receptors and it seems that epinephrine exerts its spatial memory enhancing effects via beta-adrenergic receptors.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC425

Modulation of human swallowing function by a virtual lesion to healthy motor cortex

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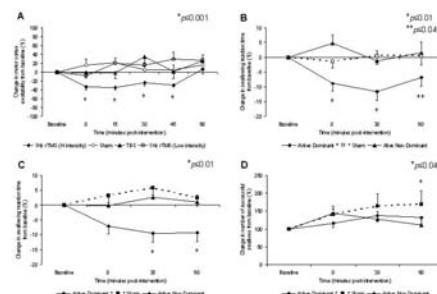
Background: Cortical control of human swallowing is bilateral with hemispheric asymmetry in motor cortex. However, the physiological relevance of this asymmetry remains unclear. Repetitive transcranial magnetic stimulation (rTMS) can be used to induce targeted 'virtual lesions' of brain regions (1, 2). We applied inhibitory rTMS to the swallowing motor cortices ('dominant' (D) and 'non-dominant' (ND)) to establish whether such inhibition could result in behavioural changes in swallowing function.

Methods: Single pulse TMS combined with pharyngeal motor evoked potentials (PMEPs) (recorded via an intraluminal catheter) were used to assess swallowing excitability. The hemisphere evoking the largest PMEPs was termed D, and contralateral ND. Effects on PMEPs were measured before and up to 1 hour after two inhibitory rTMS paradigms: 1Hz rTMS (n=9; 6M/3F, mean age 34) and theta burst stimulation (TBS) (n=9; 6M/3F, mean age 37) delivered in a randomised, double blind order on separate days). In a separate set of studies (n=8; 5M/3F, average age 36), a swallowing reaction time task was used to assess the effects of inhibition (to each hemisphere) on swallowing function and comprised 3 cued tasks: normal, fast and challenged swallows (within a 150ms time window). Measurements were taken before and up to 1 hour after 10 minutes of 1Hz rTMS (active stimulation to D and ND hemispheres and sham, randomised, double blind, separate days). Baseline vs. hemispheric intervention data were analysed using repeated measures ANOVA.

Results: (Fig A) High intensity 1Hz rTMS to D dramatically suppressed excitability immediately and up to 45 minutes ($-34 \pm 14\%$, $p \leq 0.001$). Ipsilateral hand motor cortex showed a transient but non-significant decrease in responses ($-15 \pm 12\%$, $p=0.14$) post-rTMS whereas contralateral hemisphere (ND) showed no change ($15 \pm 12\%$, $p=0.45$). By comparison, low intensity 1Hz, TBS and sham rTMS interventions had no effect. Moreover, active 1Hz rTMS to D demonstrated a reduction in both normal (Fig B, $-12 \pm 3\%$, $p \leq 0.01$) and fast (Fig C, $-9 \pm 4\%$, $p \leq 0.018$) swallow reaction times compared to baseline whereas

active rTMS to ND and sham had no effect. In comparison, challenge swallows (Fig D) showed a (predicted) rise in successful trials following sham ($p < 0.041$) but no change following active rTMS of either hemisphere.

Conclusions: Our data show clear differences in swallowing motor cortex excitability and behaviour to a virtual lesion of each hemisphere, providing novel evidence for functionally relevant asymmetry in the cortical swallowing motor system.



Huang YZ et al. (2005). Neuron 45, 201-206.

Muellerbacher W et al. (2000). Clin Neurophysiol 111, 1002-1007.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC426

A novel technology to detect CYP2C9*3 Alleles based on DNA-assembled exciplexes

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A novel technique was developed and verified for single nucleotide polymorphisms of human cytochrome P450 2C9 (*3 alleles) that affect the metabolism and clinical toxicity of drugs such as phenytoin, warfarin (Romkes et al)1. The detection system is split at a molecular level into signal-silent components. On hybridisation to their complementary target sequence, these components are assembled into a specific 3-dimensional structure, and can give an exciplex (Figure 1), which is detected by fluorescence spectroscopy (Bichenkova et al)2. Eight pairs of 12-mer oligonucleotide probes, labelled with pyrenyl or naphthyl exciplex partners at their 5'- or 3'- termini, respectively, were evaluated. Target DNA used in this study was in the form of short synthetic oligonucleotides (47 mer). The probes annealed to a complementary Wild Type oligonucleotide target and showed a characteristic exciplex fluorescence at 480 nm after excitation at 350 nm. This was accompanied by a quenching and slight red shift in the pyrene signal. With *3 SNP target oligonucleotide, exciplex signals of lower fluorescence intensity were detected, and there was a decrease in the Tm of the hybrid duplex by 1-2 °C. These results show that it is possible to use exciplex probes for detection of cytochrome P450 SNPs, with promise for future applications in genetic testing and molecular diagnostics.

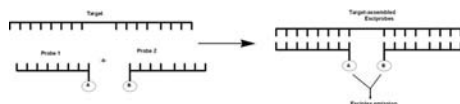


Figure 1. Target assembly of pyrenyl- and naphthalenyl- labelled exciproses in tandem.

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PC427

Creatine supplementation does not affect muscle glycogen accumulation in non-exercising human muscle

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There is wide variation in muscle TCr accumulation following supplementation, which can be attenuated by ingestion of a large amount of simple sugars (Green et al, 1996). We demonstrated that muscle glycogen concentration also increases with Cr and carbohydrate (CHO) supplementation and that post-exercise glycogen supercompensation can be enhanced by Cr + CHO supplementation in exercising muscle (Robinson et al, 1999). In this study, we investigated whether Cr supplementation alone can influence muscle glycogen synthesis and storage, and whether any elevated glycogen concentrations were sufficient to improve endurance exercise performance.

Six healthy male volunteers participated in the study, which had ethical committee approval. Participants reported to the laboratory on 4 occasions to exercise on a cycle ergometer to the point of volitional exhaustion, after 3 days of a controlled diet. After a familiarisation visit, participants cycled to exhaustion after no supplementation, then two weeks later again cycled to exhaustion after 5 days of CHO supplementation, then 2 weeks later again cycled to exhaustion after 5 days of Cr supplementation. During the experimental visits, muscle and blood samples were obtained before, during and after exercise.

Resting muscle glycogen concentrations were significantly elevated above those of the normal dietary condition following CHO supplementation but not by Cr supplementation. At the time of exhaustion of the normal diet without supplementation (TEXnormal), muscle glycogen concentrations were not significantly different at the same time point after CHO or Cr supplementation. Glycogen concentrations at the time of exhaustion after CHO or Cr supplementation (EXHCHO and EXHCr respectively) were also not significantly different from those at TEXnormal. No significant improvements in exercise duration

compared with Normal were observed following either CHO or Cr supplementation (Mean [+ SEM]; Normal 92 [10]; CHO 95 [18]; Cr 102 [14] min, $p > 0.05$).

The principal finding of this investigation is that 5 days of Cr supplementation and a normal, controlled diet had no effect on muscle glycogen storage. It would appear that Cr supplementation alone is not sufficient to increase muscle glycogen concentration and that Cr-associated increases in muscle glycogen are as a result of an interaction between the supplementation and other mediators of muscle glucose transport, such as glucose-stimulated insulin release, muscle contraction, or both.

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The authors thank Liz Simpson, John Fox and Julie Lambourne for their assistance.

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PC428

Characterisation of the muscle-specific ubiquitin-binding 'atrogene' ZNF216

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Increased activity of the ubiquitin-proteasome system (UPS), resulting in an imbalance between protein degradation and protein synthesis, is thought to be central to the loss of mass of skeletal muscle undergoing atrophy. Both animal and human studies consistently demonstrate that, as part of the muscle atrophy programme, expression of the E3 ubiquitin-ligase enzymes MAFbx and MuRF1 (gene products of so-called 'atrogenes') is significantly increased. Further, *MAFbx* and *MuRF1* knockout mice preserve muscle mass and fibre-size when subject to denervation or disuse atrophy, indicating they are required for atrophy to occur. This upregulation of both *MAFbx* and *MuRF1* is likely to result in increased ubiquitylation of specific muscle proteins, and presumably, their subsequent degradation by the 26S proteasome complex. Recently, a new 'atrogene' designated *Znf216* has been identified [Hishiya et al., 2006]. The ZNF216 protein possesses Znf-A20 and Znf-AN1 domains at its N- and C-termini, respectively, both of which can be linked to the UPS. In order to further understand its role in muscle homeostasis, we have initiated an *in vitro* functional characterisation of the rat ZNF216 protein. The coding region of *Znf216* was cloned from mRNA isolated from rat skeletal muscle, which enabled recombinant expression (in *E. coli*) of a soluble glutathione S-transferase tagged protein (GST-rZNF216); milligram quantities of GST-rZNF216 were successfully purified using glutathione-Sepharose affinity chromatography. Initial work

indicated that rZNF216 can self-associate and form homo-oligomers. Despite the presence of the Znf-A20 domain previously found in some deubiquitylating (DUB) enzymes, rZNF216 displayed no apparent DUB activity *in vitro*. However, rZNF216 clearly interacted with immobilised monoubiquitin (ubiquitin-Sepharose) in addition to unanchored K48- and K63-linked polyubiquitin chains, in a linkage-independent manner. Furthermore, affinity chromatography experiments have shown that rZNF216 can efficiently precipitate ubiquitylated proteins from skeletal muscle extracts, whilst at the same time, revealing other proteins which may interact directly with rZNF216. Since ZNF216 is thought to interact with both the 26S proteasome and ubiquitylated proteins, we propose that its role may be to act downstream of MAFbx/MuRF1 in the atrophy programme, delivering ubiquitylated substrates of MAFbx/MuRF1 to the proteasome. Thus, identification of ZNF216-interacting proteins from skeletal muscle could provide further insights into the molecular mechanisms that regulate the muscle atrophy programme, and may also help uncover the elusive *in vivo* substrates of MAFbx and/or MuRF1.

Hishiya *et al.* (2006). *EMBO J* 25, 554-64.

We would like to thank the Medical Research Council for financial support.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC429

Cyclic changes in S6K1 activity are required for C2C12 myoblast differentiation and fusion

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How myoblasts differentiate and fuse to form myotubes has important implications for skeletal muscle development, regeneration and hypertrophy. The majority of muscle cell lines, *in vitro*, are grown to confluence in high serum media and are differentiated by reducing the amount of growth factors present by serum withdrawal. The combination of high cell density and low serum are necessary to induce differentiation and fusion. However, despite the wide spread use of the procedure, the mechanisms by which this induces differentiation are not fully understood. One would expect that serum withdrawal would decrease growth factor receptor mediated signalling. However, during the time course of differentiation the opposite occurs. PKB activity as measured by GSK3 α/β phosphorylation increases by 2.5 ± 1.5 , 3.1 ± 0.3 and 3.38 ± 1.3 fold at day 1, 3 and 5 respectively. The increase in PKB activity at day 1 occurs concomitant with a 2.2 ± 0.4 fold increase in IRS-1 expression and a 2.4 ± 0.5 fold decrease in S6K-1 activity, as measured by S6 phosphorylation. Considering the role of S6K1 in IRS-1 regulation this decrease in S6K1 activity may initiate the fusion process. To resolve the role of S6K1 in differentiation we utilized two separate expression systems; one under the control of the myosin light chain 2-fast (MLC2-F) promoter, and the other under the control of the RheoSwitch inducer to overexpress various mutants of S6K1.

When kinase dead S6K1 is overexpressed just before differentiation using the MLC2-F promoter, myoblast fusion is enhanced, as is myosin heavy chain (MHC) expression (a marker of terminal differentiation). However when wild type S6K1 is overexpressed with the same system, fusion and myosin heavy chain expression are inhibited. When the expression of these genes is induced at the same time as differentiation, fusion appears to be normal. However, myosin heavy chain expression is enhanced in the cells overexpressing the kinase dead S6K1 and inhibited in the cells overexpressing wild type S6K1 suggesting that high S6K1 activity inhibits differentiation. The more widely recognised role of S6K1 in cell size regulation is also observed in these cells. The cells overexpressing kinase dead S6K1 did not achieve the same diameter as transfection controls in spite of greater differentiation and MHC expression. In conclusion, the dynamic changes in S6K1 activity during differentiation are required for normal myoblast differentiation likely through its regulation of IRS-1 levels. Post fusion, the increase of S6K1 activity is required for the attainment of normal cell size.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC430

Simvastatin treatment causes a change in fuel metabolism and upregulation of MAFbx and MuRF-1 mRNA in rat skeletal muscle

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Statins inhibit synthesis of mevalonate by HMG-CoA reductase, the rate-limiting step in hepatic cholesterol synthesis, and are used clinically for cholesterol reduction. However statin therapy is also associated with myopathic changes (Thompson *et al.*, 2003). The ubiquitin-dependent proteolytic system is central to muscle atrophy, and is mainly regulated by two muscle specific ubiquitin (E3) ligases, i.e., MAFbx and MuRF-1 (Glass, 2003). Furthermore, the transcription factor, FOXO, which activates MAFbx and MuRF-1, also activates pyruvate dehydrogenase kinase (PDK) transcription, thereby potentially inhibiting muscle carbohydrate oxidation (Furuyama *et al.*, 2003). Therefore, we investigated the effect of chronic treatment with Simvastatin on muscle glycogen concentration, the protein:DNA ratio and on the transcription of MAFbx, MuRF-1 and PDK4 to determine its impact, if any, on muscle fuel metabolism and atrophy-related signalling. Twenty nine, female Wistar Hannover rats were given Simvastatin (80mg/kg/day) for 4 (n=6), 8 (n=6), 10 (n=6) and 12 days (n=5), or vehicle (0.5% w/v hydroxypropyl methylcellulose and 0.1% w/v polysorbate 80) (Control, n=6) for 12 days by oral gavage, after which muscle was harvested under terminal anaesthesia. Animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986. Muscle glycogen concentration, the protein:DNA ratio and the expression of MAFbx, MuRF-1 and PDK4 mRNA were measured in Biceps Femoris (Table 1). One-way ANOVA was used to

identify the effects of Simvastatin administration (treatment effects). The increase in glycogen concentration 12 days after Simvastatin administration suggests a shift in muscle fuel utilisation towards increased fat oxidation occurred, possibly through increased PDK4 mRNA expression (observed at day 4 and 12) inhibiting PDC activation, thereby reducing glycogen utilisation. Simvastatin administration also caused marked upregulation in the expression of MAFbx and MuRF-1 at Day 12, but without any change in the alkaline soluble protein:DNA ratio. It is possible, therefore, that the changes in MAFbx and MuRF-1 were secondary to FOXO-linked changes in fuel metabolism, rather than reflecting the induction of a muscle atrophy programme.

Table 1: Glycogen concentration and alkaline soluble protein:DNA ratio (absolute values) and mRNA expression (fold changes relative to control) in Biceps Femoris muscle after Simvastatin administration.

	Control	Day 12	Day 4	Day 8	Day 10	Day 12
Glycogen (mmol/kg DM)	137 ± 14	153 ± 19	162 ± 24	135 ± 20	178 ± 20 [†]	
Protein:DNA (g/kg DM)	377.5 ± 64.1	261.7 ± 64.1	350.8 ± 58.5	467.6 ± 71.7	332.3 ± 71.7	
MAFbx	1	6.7 ± 2.9	2.7 ± 2.9	2.5 ± 2.9	10.7 ± 3.1 ^{††}	
MuRF-1	1	5.4 ± 2.3	1.4 ± 2.3	1.5 ± 2.3	8.8 ± 2.4 ^{††}	
PDK4	1	13.6 ± 4.2 [†]	3.1 ± 4.2	4.2 ± 4.2	14.7 ± 4.4 ^{††}	

Values expressed as mean ± SEM, [†]P<0.05, ^{††}P<0.01 compared to Control

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC431

L-Glutamine transporter SNAT2 controls signalling to protein synthesis through mammalian target of rapamycin in skeletal muscle cells

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Muscle protein wasting (cachexia) arising from metabolic acidosis is a serious problem in illnesses such as renal failure, and may be reversed by therapeutic exercise. It is therefore important to understand how physiological stimuli such as acidosis and exercise signal to protein metabolism in muscle. We have shown previously that acidosis inhibits the SNAT2 L-glutamine (L-Gln) transporter, the dominant determinant of amino acid levels in skeletal muscle cells. This transporter may also be up-regulated by exercise (King 1994). Intracellular amino acid concentration is sensed by mammalian target of Rapamycin (mTOR), a key regulator of protein synthesis.

Aims: To investigate the effect of SNAT2 inhibition during acidosis on Rapamycin-sensitive signalling through mTOR to protein synthesis in L6 skeletal muscle cells.

Methods: Cells were cultured with 2mM L-Gln and 2% serum or 100nM insulin. Protein synthesis was determined from incorporation of ³H-L-Phe. Phosphorylation of targets of mTOR which regulate translation was determined by immunoblotting with phosphospecific antibodies.

Results: Lowering extracellular pH to 7.1 to model acidosis led to a sustained (48h) impairment of protein synthesis (13.7 ± 0.6 nmol ³H-L-Phe/mg protein/4h v 16.0 ± 0.9 in control cultures at pH 7.4 (P<0.05)). Blockade of mTOR signalling with 10nM Rapamycin abolished this effect (12.3 ± 0.6 at pH 7.1 v 13.0 ± 0.6 at pH 7.4, NS), as did removal of extracellular L-Gln (11.6 ± 0.7 at pH 7.1 v 11.9 ± 0.6 at pH 7.4, NS). Competitive inhibition of SNAT2 at pH 7.4 with its selective substrate MeAIB also impaired protein synthesis (12.9 ± 0.3 v 15.4 ± 0.4 in controls, P<0.05) as did selective silencing of SNAT2 expression with siRNA (11.9 ± 0.9 v 13.9 ± 0.3 in controls, P<0.05). Within 2h acidosis or MeAIB impaired signalling through mTOR, leading to reduced phosphorylation of ribosomal protein (rp)S6, rpS6 kinase and 4E-BP1, assessed by densitometry of immunoblots (e.g. 52 ± 8 % inhibition of rpS6 phosphorylation with MeAIB, P<0.05). Similar impairment of rpS6 phosphorylation (78 ± 9 % inhibition, P<0.05) was induced by silencing SNAT2 with siRNA.

Conclusions: Acidosis signals to protein synthesis by inhibiting SNAT2 and depleting intracellular amino acids thereby inhibiting mTOR. SNAT2 is therefore a key regulator of mTOR and, in view of its strong responsiveness to factors such as acidosis and exercise, is of central importance in regulating protein metabolism and muscle cell growth.

King PA (1994). *Am J Physiol* 266, C524-C530.

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PC432

The influence of alpha actinin 3 R/X genotype upon human muscle function and contractile properties

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The alpha actinin3 gene (ACTN3) encodes a protein of the Z disk of myofibrils, a polymorphism of ACTN3 results in a complete loss of the protein. ACTN3 is a fast-twitch-specific isoform, expressed only in type II skeletal muscle fibres (North and Beggs, 1996), which are very important for rapid and powerful contractions. Therefore it is possible that ACTN3 genotype may influence muscle function and performance particularly during high velocity movements. Some preliminary evidence suggests that ACTN3 RR genotype is associated with elite sprint performance (Yang et al, 2003) and higher elbow flexor strength in untrained women (Clarkson et al, 2005). The aim of this study was to conduct a thorough investigation of the influence of ACTN3 genotype on human muscle function and contractile properties.

Following ethical approval a range of quadriceps muscle function measurements were taken from 79 recreationally active but non strength trained White British males on two occasions. A conventional strength testing chair was used to measure maximum isometric strength. Maximum twitches (Digitimer DS7AH,

UK) were electrically evoked to assess time to peak tension (TPT) and half relaxation time (HRT). Isokinetic peak torque was measured at 3 velocities ($30^{\circ}.\text{s}^{-1}$, $90^{\circ}.\text{s}^{-1}$, $240^{\circ}.\text{s}^{-1}$) using isokinetic dynamometry (Cybex Norm, USA). Relative strength at high velocities ($240^{\circ}.\text{s}^{-1}$; $30^{\circ}.\text{s}^{-1}$, torque ratio) was also calculated and is recognised as a functional indicator of fibre type composition (Gur et al, 2002). Maximum angular velocity during unloaded knee extension was determined with goniometry. ACTN3 R/X genotype was determined from whole blood samples using polymerase chain reaction.

ACTN3 genotype distribution was in Hardy Weinberg Equilibrium (XX 15, RX 37, RR, 27). Physical characteristics were independent of genotype. Isometric and isokinetic strength measures, and relative strength at high velocities were not influenced by genotype. There was a trend for a difference in TPT between genotypes (XX, 70 ± 11 ms; RX, 64 ± 8 ms; RR, 68 ± 12 ms; ANOVA $P = 0.06$). No other differences were found between the ACTN3 genotypes for measures of HRT and maximum unloaded knee extension. Whilst there was a trend for a difference in TPT between genotype groups, overall there was no clear effect of ACTN3 polymorphism on muscle function and contractile properties even during high velocity movements. Either there was no influence of ACTN3 genotype on fibre type composition or the effect was too small to show an influence on the functional and contractile properties of muscle.

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Yang et al. (2003). *American Journal of Human Genetics*, 73, 627-631.

Clarkson et al. (2005). *Journal of Applied Physiology*, 99, 154-163.

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PC433

Effects of a carbohydrate, protein and creatine supplement on exercise training adaptations in humans

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Nutritional supplements containing carbohydrate and protein and/or creatine are widely consumed by athletes in training. There have been few chronic nutritional intervention studies evaluating the physiological effects of such supplements for athletes in training using controlled laboratory measures. Past studies include those that have involved untrained subjects completing a resistance-training programme (e.g. Antonio et al, 2000), so cannot be generalised to highly trained athletes. We hypothesised that ingestion of carbohydrate, protein and creatine over 10 weeks of primarily endurance type training would increase the rate of adaptation to training compared with carbohydrate alone.

Twelve swimmers and tri-athletes completed the study out of fifteen volunteers. The study was a repeated measures double blind design with two groups CHO-PRO-Cr and CHO

(matched based on aerobic capacity). The CHO-PRO-Cr group consumed a carbohydrate, protein and creatine supplement while the CHO group consumed a carbohydrate supplement following training (up to 11 sessions per week) for 10 weeks. Body composition (air displacement plethysmography), muscle function (Biodex dynamometer) and aerobic capacity ($\text{VO}_{2\text{peak}}$; on-line metabolic cart) were assessed at baseline, 5 weeks and 10 weeks supplementation. A 7-day food diary was completed using weighed food portions at baseline. Muscle function was assessed during bilateral knee and shoulder isometric and isokinetic tests at 90 degrees/s and 180 degrees/s. $\text{VO}_{2\text{peak}}$ was assessed during an incremental cycle test to exhaustion. Data was analyzed using repeated measures ANOVA at $p < 0.05$. Significant effects were followed up using post-hoc tests.

There were no significant differences between the CHO-PRO-Cr or CHO group at baseline for fat-free mass (63 ± 4 and 62 ± 13 kg), isometric knee extension (225 ± 18 and 246 ± 75 Nm) or $\text{VO}_{2\text{peak}}$ (4.76 ± 0.3 and 4.55 ± 0.6 L.min⁻¹). There was a significant main effect of time on isometric force between baseline and 10 weeks, but not for fat-free mass or $\text{VO}_{2\text{peak}}$. There was no main effect of supplement on fat-free mass, isometric force or $\text{VO}_{2\text{max}}$ ($p > 0.05$) and no interaction effect present.

There were no significant differences in the rate of improvement over 10 weeks in body composition, muscle function and $\text{VO}_{2\text{peak}}$ when consuming CHO-PRO-Cr supplement regularly after training compared with a CHO supplement, suggesting that this supplement did not increase the rate of adaptation to training compared with CHO supplementation.

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PC434

The effects of stimulated or voluntary exercise on different contralateral skin regions in young men and women

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Under normothermic conditions, skin vascular conductance in humans is unchanged at the onset of voluntary isometric exercise despite significant increases in skin sympathetic nerve activity (Ray & Wilson, 2004), except when exercise intensity is relatively high (Cotzias & Marshall, 1992). One study, however, reported that skin blood flow increased in the non-active arm during muscle contractions elicited by contralateral neuromuscular electrical stimulation (Rendell et al. 1997). This could occur through afferent-mediated antidromic vasodilation (Wu et al. 2006). We investigated whether two types of stimulation (low

frequency, isometric tetanic) that are typically used for calf muscle treatment would induce changes in skin vascular conductance at different sites on the body.

With ethical approval, twenty healthy subjects, 11 male, 9 female, (age 20.4 ± 0.4 yrs, body mass index 23.2 ± 2.8 , means $\pm$ SD) participated in three calf exercise protocols whilst seated (room temperature 21 ± 0.1 °C):- voluntary plantarflexion contraction at 30% maximal (Vol, 2 mins), electrically evoked (20 Hz) plantarflexion contraction at 30% maximal (Stim, 2 mins), and low frequency (8 Hz) at maximum tolerated intensity (8 mA, 5 mins). Exercise was performed by one leg while skin perfusion was measured by Laser Doppler flowmetry from forehead, forearm, shin and toe on the contralateral side. Mean arterial pressure (MAP) was used to calculate skin vascular conductance changes from baseline during exercise. Skin electrodermal activity was monitored as galvanic response (GR) from electrodes on the fingers. Resting MAPs and heart rates were 87 ± 2 mmHg and 67 ± 3 bpm (mean $\pm$ S.E.M.). Stim exercise elicited significant increases ($P < 0.05$ v. baseline) in MAP (5 ± 2 mmHg) and GR (2.2 ± 0.6 μ S) but not heart rate. Skin conductance did not change significantly at forehead or forearm but increased by 95 ± 36 % at the shin ($P < 0.05$) and 152 ± 34 % at the toe (NS). Low frequency stimulation did not alter MAP, heart rate or GR but increased conductance at the forehead (58 ± 13 %, $P < 0.05$), shin (130 ± 45 %, $P < 0.05$) and toe (236 ± 74 %, $P = 0.07$). Vol exercise increased MAP (8 ± 2 mmHg) and heart rate (5 ± 1 bpm) but not GR and the only site to show a significant increase in conductance was the forehead (33 ± 9 % increase, $P < 0.05$).

Increases in skin vascular conductance during neuromuscular electrical stimulation suggest that, in comparison with voluntary exercise, afferent-mediated vasodilation may prevail during involuntary exercise in normothermic conditions whether there is evidence of sympathoexcitation (MAP or heart rate rise) or not. This could in part explain the beneficial effects of stimulation in patients with ischaemic limb disease (Debrececi et al. 1995).

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Cotzias C & Marshall JM (1992) Clin Auton Res 2, 235-241

Rendell MS et al. (1997) Clin Physiol 17, 213-224

Wu M et al. (2006) Brain Res 1107, 177-184

Debrececi L et al. (1995) Angiology 46, 613-618

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PC435

Quadriceps femoris muscle recovery after total knee arthroplasty

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Following total knee arthroplasty (TKA) patients exhibit significant impairment in quadriceps strength and function¹. Muscle activation deficits and atrophy contribute to the significant

quadriceps weakness exhibited by these patients². Activation deficits, or the inability to recruit available muscle fibers, can impede the success of traditional strengthening paradigms. This study monitored recovery of the quadriceps after TKA to further understand the morphological and neural mechanisms of long-term quadriceps weakness.

41 patients were tested 9 $\pm$ 5 days before unilateral TKA and 1, 3 and 12 months after TKA. Patients completed 6 weeks of outpatient physical therapy. A burst superimposition test on a maximum voluntary isometric contraction (MVIC) was used to measure quadriceps strength and activation². Magnetic resonance imaging (MRI) determined maximal cross-sectional area (CSA). Differences in quadriceps variables between limbs over time were analyzed using a repeated 2-way ANOVA and paired t-tests for post hoc analysis. Linear regression determined relative contributions to quadriceps strength.

Significant differences between limbs and over time were found in quadriceps MVIC ($F = 138.87$, $p < 0.001$; $F = 51.83$, $p < 0.001$), activation ($F = 20.01$, $p < 0.001$; $F = 8.43$, $p < 0.001$), and CSA ($F = 58.86$, $p < 0.001$; $F = 13.46$, $p < 0.001$). The surgical limb had lower MVIC both before TKA ($t = -5.53$, $p < 0.001$) and 1 ($t = -13.59$, $p < 0.001$) and 3 ($t = -18.57$, $p < 0.001$) months after TKA. The surgical limb had lower CSA both before TKA ($t = -4.77$, $p < 0.001$) and 1 ($t = -8.62$, $p < 0.001$) and 3 ($t = -7.16$, $p < 0.001$) months after TKA. No differences in activation were evident before TKA ($t = -1.27$, $p = 0.211$) or at 3 ($t = -1.85$, $p = 0.07$) months however, the surgical limb had lower activation 1 ($t = -5.54$, $p < 0.001$) month post-TKA. One year after TKA, no significant differences were found between limbs in quadriceps MVIC ($t = -0.33$, $p = 0.747$), CSA ($t = -1.66$, $p = 0.117$), or activation ($t = 0.90$, $p = 0.380$). Together, activation and CSA explained 69% of the variance in involved quadriceps strength ($R^2 = 0.77$, $p < 0.001$) at 3 months. When preoperative strength was added to the equation, 77% of the variance in 3 month involved MVIC was explained. Activation and CSA explained 62% of the variance in involved quadriceps strength 1 year post-TKA ($R^2 = 0.62$, $p < 0.05$) however, preoperative strength was still the single strongest predictor of 1 year involved strength ($R^2 = 0.72$, $p < 0.001$).

Deficits in muscle strength, activation, and CSA persisted 3 months after TKA, when the majority of patients are discharged from formal physical therapy. Complete recovery of quadriceps strength and morphology was evident 1 year postoperatively. Preoperative strength was the best predictor of long-term strength outcomes. Full quadriceps recovery may be limited by delaying surgery until patients are completely debilitated by pain. Preoperative strengthening may optimize benefit from surgery.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC436

The mechanism of statin-induced vacuolation in rat skeletal myofibers

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HMG-CoA reductase inhibitor (statin) induces skeletal muscle injury including myalgia, myositis and rhabdomyolysis. The mechanism of this myotoxicity remains unknown. We examined the effect of statins on single skeletal myofibers enzymatically isolated from rat flexor digitorum brevis muscles. Fluvastatin or pravastatin induced numerous vacuoles in the myofibers on the 3rd day of statin-application in the culture medium. This effect progressed in a time- and concentration-dependent manner followed by cell death on the 5th day. The electron micrographs revealed craters of various sizes along the sarcolemma, the swellings of the sarcoplasmic reticula, and mitochondria, in addition to the intracellular vacuoles. When caffeine was introduced to the myofibers at the 72 hours of fluvastatin treatment, the induced cell-shortening was smaller than that of the control and prominent blebs were formed on the surface of the myofibers, which were not observed in the control fibers. The co-application of geranylgeranylpyrophosphate (GGPP) completely cancelled the morphological changes while farnesylpyrophosphate (FPP) was ineffective. Furthermore, perillyl alcohol, a selective Rab geranylgeranyl transferase inhibitor, mimicked the effect of statins, while a specific geranylgeranyl transferase-I inhibitor, GGTI-298 or a farnesyl transferase inhibitor, FTI-277 failed to do so. Exoenzyme C3, Y27632, or wortmannin did not mimic the statin effect. In addition, the satellite cells and fibroblasts also injured by fluvastatin. GGPP attenuated the damage in both of cells, however FPP was only able to prevent injury in the fibroblasts. These results suggest that Rab GTPase inactivation is therefore a crucial factor in statin-induced-morphological abnormalities in skeletal muscle fibers, and that these morphological changes were accompanied by a vulnerability to the contraction in skeletal myofibers.

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PC437

Mechanosensitive secretion and turnover of the joint lubricant hyaluronan *in vivo*

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Hyaluronan (HA) is a key component of synovial fluid, responsible for hydrodynamic lubrication, synovial fluid conservation

during flexion and cavity formation in embryos. Although HA is selectively retained in the cavity by the synovial lining (Sabaratnam *et al.*, 2007), there is nevertheless a slow trans-synovial loss of HA. This is replaced by synthesis *de novo* by fibroblast-like synoviocytes facing the joint cavity. The aims were a) to measure apparent HA secretion rate and HA loss from rabbit knee joints in order to evaluate the loss-corrected secretion rate; b) to combine measurements of endogenous HA mass with the true secretion rate to evaluate intra-articular (i.a.) HA turnover time *in vivo*; c) to test whether HA secretion is coupled to joint movement.

Knee joints of anaesthetized rabbits were cannulated. Endogenous HA mass was harvested by 18x1ml washes with mixing cycles. Apparent secretion rate q_{HA} was measured by washouts 5h later. In other experiments the apparent loss over 5h was assessed. An order-of-magnitude greater mass of exogenous rooster or rabbit HA (I) was injected i.a. in split doses every 30min for 5h, then the mass remaining at 5h (R) was determined by washout. HA mass and size were analysed by HPLC. Solution of the two simultaneous equations for the effects of loss on q_{HA} and of q_{HA} on apparent loss gave true secretion rate $q_{HA}^{corrected} = I/(R - 5q_{HA})$ and the true loss $L = (I + 5q_{HA}^{corrected}) - R$. HA turnover time was then calculated as $m_{endog}/q_{HA}^{corrected}$. To test whether secretion was stimulated by movement, some of the above studies were repeated on joints cycled passively for 3min in every 15min over 5h.

Apparent q_{HA} was $16.2 \pm 1.3 \mu g h^{-1}$ in static joints (n=34) and $30.9 \pm 2.6 \mu g h^{-1}$ in moved joints (n=25), i.e. movement stimulated HA secretion ($p < 0.05$, unpaired t test). Apparent HA loss (I-R) was $46.4 \pm 6.0\%$ (n=5) in static and $39.5 \pm 4.6\%$ (n=5) in moved joints ($p = 0.38$, unpaired t test). True HA secretion rates corrected for loss ($q_{HA}^{corrected}$) was $30.5 \pm 2.8 \mu g h^{-1}$ (n=34) and $61.9 \pm 5.6 \mu g h^{-1}$ (n=25) in static and moved joints respectively. The mean m_{endog} was $468 \pm 24 \mu g$ (n=59). Turnover time $m_{endog}/q_{HA}^{corrected}$ was $17.1 \pm 1.4 h$ (n=34) in static and $8.4 \pm 0.6 h$ (n=25) in moved joints. Joint movement stimulated HA secretion, indicating that synovial lining cells show mechanosensitive secretion of HA. Turnover time for HA was much longer than the turnover time for intra-articular albumin and water (1-2h). This is in keeping with the known retention of HA by ultrafiltration by synovial lining extracellular matrix proteins (Sabaratnam *et al.*, 2007). The estimates of HA loss and hence $q_{HA}^{corrected}$ may be overestimates for normal joints because the injection of fluid increased fluid outflow, which increases trans-synovial HA flux. Sabaratnam *et al.* (2007) Interstitial matrix proteins determine hyaluronan reflection and fluid retention in rabbit joints; effect of protease. *J Physiol* 578, 291-299.

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PC438

Evaluation of physical exertion required to play the body movement controlled Eyetoy Kinetic video game

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Excessive video game playing has been blamed for the increased prevalence of childhood obesity. Recent developments in the

video games industry have resulted in a variety of novel games controllers which require the use of body movement in order to play the games. More specifically a game entitled "Eyeto Kinetic" has been developed for the Sony Playstation 2 console, providing various games and a personal training programme aimed at improving cardiovascular fitness, muscle tone, balance and coordination. To assess whether or not such a video game would provide a useful adjunct to promoting physical activity and weight control, it is necessary to evaluate the intensity of physical exertion the game demands of the participant.

On the first occasion, 16 subjects (8 male) mean ($\pm$ SD) 20.3 $\pm$ 2.4 years, height 1.76 $\pm$ 0.10 m and body mass 72.7 $\pm$ 12.8 kg undertook an incremental exercise test on a cycle ergometer (Monark 874E) to volitional exhaustion. Respiratory gas analysis was performed using a portable telemetric system (Jaeger Oxycon Mobile) including a heart rate monitor (Polar Electro). Subjects also undertook familiarisation trials with the Minnesota Dexterity test (Two-Hand Turning and Placing test) and a 30 s postural stability test on a balance platform. They also played the two games "Cascade" - 10 mins aerobic workout and "Sidewinder" - 3 mins all out maximal exertion.

On the second occasion, 3 trials of the dexterity and stability tests were performed. Subjects then undertook three 10 mins bouts of the "Cascade" game of ascending difficulty (Easy, Medium & Hard) and then two 3 mins bouts of the "Sidewinder" (Comb1 & Comb2) game. Subjects had 5 mins rest between bouts. Heart rate (HR) and gas exchange variables were monitored telemetrically throughout the exercise periods.

HR and oxygen uptake ($\dot{V}O_2$) normalised for body mass were averaged over the second half of each bout for the "Cascade" game. For the "Sidewinder" game and the $\dot{V}O_{2max}$ test, the highest 30 s average was taken. Statistical analysis was performed using correlation and ANOVA.

Manual dexterity score was significantly correlated with the game score in the second bout of "Sidewinder" ($p < 0.05$). There was no correlation between postural stability and any game scores. Peak $\dot{V}O_2$ in the "Sidewinder" game was significantly higher versus the "Cascade" game but not significantly different from $\dot{V}O_{2max}$ (Fig 1). Peak HR in the "Sidewinder" game was both significantly higher versus the "Cascade" game and lower than that at maximal exercise on the cycle ergometer (Fig 2).

These results indicate that in a group of young adults, the physical exertion required to play the games studied is significant and may provide a novel way to promote increased levels of habitual physical activity.

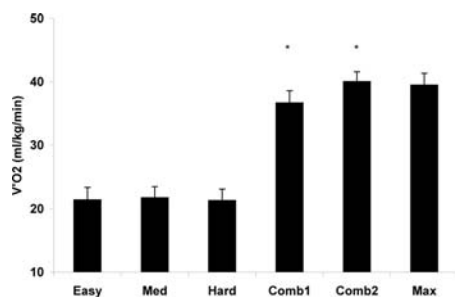


Figure 1. Mean $\dot{V}O_2$ normalised for body mass plotted against exercise condition. Error bars = SEM. * = $p < 0.05$ versus Easy, Medium & Hard.

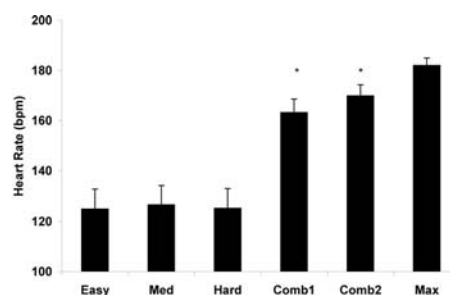


Figure 2. Mean HR plotted against exercise condition. Error bars = SEM. * = $p < 0.05$ versus Easy, Medium, Hard & Max.

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PC439

Comparison of potency and selectivity of β adrenoceptor agonists at human and canine recombinant β_1 and 2 adrenoceptors

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β -adrenergic receptors are G-protein coupled receptors that increase intracellular cAMP on agonist binding (Johnson, 2001). Canine models are often used to study the functional pharmacology of β -adrenoceptor ligands (Badino et al, 2005), however, there is little published information directly comparing the pharmacology between canine and human species. We have compared the potency and selectivity of standard β_2 adrenoceptor agonists at canine and human β_1 and 2 adrenoceptors recombinantly expressed in Chinese Hamster Ovary cells.

Assays were carried out in a 96 well format with 2×10^4 cells per well. Cells were incubated at 37°C in phosphate buffered saline, PH 7.4, containing 0.5mM IBMX (3-isobutyl-1-methylxanthine) for 30-60 minutes. Concentration response curves to salmeterol (Salm), formoterol (Form), salbutamol (Salb) and isoprenaline (ISO) were carried out using an incubation time of 30 minutes. Intracellular cAMP was detected using the DiscoverX HitHunter cAMP II Assay (GE Healthcare).

Potency (represented as geometric mean EC_{50}) is the concentration required to achieve half maximal stimulation for that agonist. Efficacy (represented as arithmetic mean E_{max}) is the maximum stimulation achieved by 10 μ M ISO. Relative Potency (represented as geometric mean RP) is the potency relative to ISO for each n-number. Data is a mean of 4-10 with 95% confidence intervals in brackets. Statistical analysis was conducted using ANOVA in Excel Labstats and compared the mean RP of each agonist between species - see table 1.

The relative potency, selectivity and efficacy of Salm, Form, Salb and ISO were not found to be significantly different between human and canine β -adrenoceptors ($p > 0.05$). This suggests that agonist pharmacology of canine β_1 and 2 adrenoceptors is identical to that of human.

Table 1 – Potency and efficacy of β adrenoceptor agonists at human and canine recombinant β adrenoceptors (c/c = can't calculate).

Human $\beta 1$	Salm	Form	Salb	ISO
EC50 nM	>100,000	124 (79-195)	>100,000	8.6 (7.9-9.5)
E _{max} %	7.5 (4.5-10.5) @100 μ M	88 (60-113)	12 (1.2-22) @100 μ M	109 (88-130)
RP	c/c	16 (7.9-23)	c/c	1.0
Canine $\beta 1$				
EC50 nM	>100,000	94 (59-148)	>100,000	6.5 (5.7-7.4)
E _{max} %	15 (7.4-21) @100 μ M	90 (66-114)	35 (15-55) @100 μ M	100 (74-127)
RP	c/c	14 (8.9-23)	c/c	1.0
Human $\beta 2$				
EC50 nM	1.2 (0.8-2.0)	0.17 (0.1-0.3)	64 (31-130)	3.0 (1.4-6.2)
E _{max} %	88 (85-92)	98 (86-109)	87 (79-96)	93 (81-104)
RP	0.41 (0.3-0.6)	0.058 (0.04-0.08)	21 (18-26)	1.0
Canine $\beta 2$				
EC50 nM	0.65 (0.6-0.8)	0.11 (0.09-0.13)	30 (14-64)	1.9 (1.3-2.7)
E _{max} %	105 (95-115)	110 (100-120)	100 (88-111)	103 (93-112)
RP	0.34 (0.2-0.5)	0.056 (0.05-0.07)	16 (10-23)	1.0

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC440

Extensive retention in the endoplasmic reticulum characterizes the Cys27 variant of the human δ opioid receptor

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The human δ opioid receptor (h δ OR) is one of the three opioid receptors that mediate signals of endogenous opioid peptides as well as exogenous opiate compounds. The exogenous drugs are highly efficient analgesics but they also induce tolerance and addiction. Our previous studies on h δ OR biogenesis have shown that the receptor matures slowly and inefficiently, so that only 40% of synthesized receptors are able to gain stable native conformation and reach the cell surface, while the rest are targeted for degradation (1, 2). Importantly, the receptor cDNA used in these studies represents the less common polymorphic variant of the receptor, carrying 80T→G substitution that leads to exchange of Phe to Cys at position 27. The frequency of the 80G allele in Caucasians is about 9% (3). To compare the biochemical characteristics of the two receptor variants from early folding steps to the functional receptor, the two receptor constructs were expressed as *Myc/Flag* epitope-tagged fusion proteins in stably transfected HEK293 cells under tetracycline induction. Pharmacological characterization revealed no significant difference between the two variants in either expression levels or binding affinities for various opioid ligands. Similarly, both variants induced agonist-mediated downstream signaling with similar efficiency in GTP binding assays. In contrast, clear differences in the relative abundance of receptor mature and precursor forms were detected by Western blot analysis of immunoprecipitated receptors. Namely, the receptor mature to precursor ratio was clearly higher for the Phe27 variant, suggesting that the Cys27 variant accumulates in the endoplasmic reticulum (ER). Metabolic pulse-chase experiments showed that both receptors were

converted to the mature form with similar kinetics and efficiency, but increased turn-over of the Phe27 precursor became clearly evident. This indicates effective clearance of the folding incompetent Phe27 variant from the ER. Prolonged ER retention of the Cys27 variant was not calnexin dependent, as the turn-over of calnexin-bound precursors was significantly faster than that of the total precursor pool in co-immunoprecipitation experiments. On the other hand, one of the two N-glycosylation sites (Asn33) of the Cys27 variant was found to be inefficiently occupied. Given the close proximity of Cys27 to Asn33, it can be hypothesized that the cysteine residue mediates interaction with oxidoreductases within the protein disulfide isomerase family (4). This might prevent efficient N-glycosylation of Asn33 of the h δ OR Cys27 variant and also hinder proper folding and/or efficient retrotranslocation of incompletely folded receptors, subsequently causing accumulation of receptor precursors in the ER.

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PC441

The distribution and desensitisation of μ -opioid receptor (MOR) splice variants

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Morphine is a potent analgesic, exerting its effects through the G protein-coupled μ -opioid receptor (MOR). Since the initial cloning of the MOR, many splice variants, differing only in the intracellular C-terminal tail, have been described in the human, rat and mouse but the literature describing their physiological role, distribution, or even existence, is confused.

To clarify this, we used PCR to look for previously described splice variants throughout rat CNS. MOR1 and MOR1A were readily detectable and present in similar amounts in all tissues tested. MOR1B had a similar distribution but was only detectable using nested PCR and many more cycles. However, we could only detect MOR1C using nested PCR with 30 cycles and 26 cycles in each round respectively. Even so, its detection was erratic even in the same brain region. We did not detect MOR1D. Hence, we conclude that MOR1 and MOR1A are present in similar amounts in the CNS; MOR1B is present in lesser amounts but MOR1C is unlikely to have any physiological role and it is likely that, in this case, we are amplifying sequences from incompletely spliced intermediates.

Given that MOR1, MOR1A and MOR1B differ only in the C terminal region we then studied possible functional differences between them at the level of agonist-induced desensitisation, using whole-cell patch clamp recordings from HEK-293 cells stably transfected with the G protein-coupled inwardly rectifying K⁺ (GIRK) channel subunits Kir3.1 and Kir3.2A and transiently

transfected with receptor constructs¹. Receptor saturating concentrations of either morphine or DAMGO (a peptide MOR agonist) were applied to ensure comparable levels of receptor occupancy. For each splice variant/opioid combination, the evoked GIRK current reached a peak and then declined (desensitised) in the continued presence of the drug. MOR1 and MOR1A desensitised to both morphine and DAMGO to similar extents and with similar kinetics (MOR1, $t_{1/2}$ 1.8 min; MOR1A, $t_{1/2}$ 1.1 min). Although the rate of morphine-induced desensitisation of MOR1B was the same ($t_{1/2}$ 1.7 min) as of MOR1, DAMGO-induced desensitisation ($t_{1/2}$ 4.0 min) was much slower. Interestingly, the rate of DAMGO-induced MOR1B desensitisation was increased when receptor internalisation was blocked by a dominant negative mutant of dynamin, suggesting that DAMGO causes apparently slower desensitisation of MOR1B because the splice variant is able to internalise and recycle rapidly in a resensitised state². Morphine-induced desensitisation does not seem to be influenced by internalisation. In conclusion, we have shown that MOR1, MOR1A and MOR1B splice variants are present throughout the CNS and that the different C-terminal sequences can affect functional properties of the receptor.

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PC442

Cannabinoid Receptor Agonists'-induced effects on neuronal B50 cells in Hypoxia

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Cannabinoid receptor agonists (CB1) have been shown to mediate several central nervous system (CNS) effects and actions, while the action of the G-protein coupled receptors like CB1 are involved in the activation of G-proteins necessary for several neural activities (Breivogel et al 2004; Croxford 2003; Smith 2002). The aim of this study was to investigate the role of Cannabinoid receptor agonists during neuronal hypoxia using cortical B50 cells in culture. The Cortical B50 cells were cultured in normal incubator (21% O₂; 5% CO₂) as the control group and hypoxic incubator (5% O₂; 5% CO₂) as the experimental group and three Cannabinoid agonists namely Win55,212-2 mesylate, Anandamide and 2-Arachidonylglycerol were selected and administered to the cells for 48 hours as treatment against hypoxia after 48 hours of culture at concentration of 10nM, 50nM and 100nM. Neuronal viability, proliferation, differentiation and second messenger activity were assessed using lactate dehydrogenase (LDH) leakage, cellular proliferation assay, second messenger (cAMP) assay, dbcAMP induced differentiation

respectively and the levels of G-protein coupled receptors (CB1), mRNAs were assessed using RT-PCR. The results showed some significant changes ($P < 0.05$) in the level of LDH leakage in normal cultured B50 cells (100%), hypoxic cells (440%) and treated cells with 100nM Anad (69%) and 100nM Arachi (103%). These changes in LDH release, neuronal viability, proliferation and differentiation was shown to be dose dependent between the normal and hypoxic B50 neurons and between treated and untreated B50 neurons in culture, while the RT-PCR mRNA levels showed no appreciable change. These results indicate that Cannabinoid agonists have some positive therapeutic effects in the treatment of hypoxia in neuronal B50 cells in culture.

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PC443

Structure-activity relationships for kisspeptin-10 analogues at human gpr-54

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Metastin (kisspeptin-54), a member of the RF-amide family of peptide hormones was first described as a metastasis' suppressor in melanomas. Kisspeptin peptides and their G-protein coupled receptor (gpr-54) were subsequently shown to be expressed in placenta, where they modulate trophoblast cell migration. More recently, a role for kisspeptins and gpr-54 in the hypothalamic arcuate nucleus, anteroventral periventricular nucleus and median eminence, regions of the brain involved in regulation of fertility, has been established. The kisspeptin system is a major regulator of reproduction via its stimulatory effects on the secretion of gonadotropin releasing hormone (GnRH). Antagonists of kisspeptin action may therefore be therapeutically useful in treatment of conditions affecting the reproductive system. We analysed structure-activity relationships of synthetic kisspeptin-10 analogues using a transfected CHO cell line stably expressing human gpr-54. Effects of analogues on binding of 125I-labelled kisspeptin-10 (YNWNSFGLRF-amide) and on stimulation of 3H-inositol phosphate production were assayed. Results showed that amino acid residues in the C-terminal section of kisspeptin-10 are important for binding to gpr-54, specifically: Phe6, Arg9 and Phe10. These residues may constitute a pharmacophore structure, since substitution with different residues reduced binding affinity. Further studies indicated that modification of these residues resulted in antagonism of inositol phosphate production. Since kisspeptins act upstream relative to GnRH, it is possible that kisspeptin analogues may be developed that are faster-acting and more effective than GnRH antagonists.

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PC444

Tacrine and muscarinic receptors: Unusual, but not exclusive, mechanisms of interaction

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It has been observed repeatedly^{1,2} that the inhibitor of cholinesterases **tacrine** (1,2,3,4-tetrahydroacridin-9-amine) inhibits the binding of orthosteric muscarinic ligands to muscarinic receptors with unusually steep binding curves (high Hill slopes about value 1.5). Definite explanation of this phenomenon is not available. A mechanistical model has been proposed according to which **tacrine** binds to the orthosteric site and to the allosteric site simultaneously with homotropic positive cooperativity³. At the same time, the binding of **tacrine** to the allosteric site has a negative effect on the binding of the commonly used radiolabelled muscarinic ligands (³H]N-methylscopolamine, or ³H]quinuclidinyl benzilate) to the classical site.

Another hypothesis suggests that **tacrine** binds to two spatially separated allosteric sites on muscarinic receptor with positive cooperativity or its binding to the common allosteric site modulates receptor-receptor interactions⁴.

We have discovered recently that structurally closely related compounds **7-methoxytacrine**, **acridin-9-amine**, and **proflavine** (acridine-3,6-amine) also yield characteristically steep binding curves while **quinacrine** (6-chloro-9-([4-diethylamino]-1-methylbutyl)amino-2-methoxyacridine) behaves like a competitor and displaces the orthosteric ligand [³H]N-methylscopolamine yielding the standard curves with Hill slope close to 1. These experimental findings are rather surprising. First, **tacrine** has been synthesized as a three-dimensionally voluminous analogue of **acridin-9-amine** in order to demonstrate that the complete planar acridine nucleus is required for antibacterial activity. Nevertheless, their effects at muscarinic receptors are almost identical. On the other side, allosteric modulators proflavine and acridin-9-amine, **together with "orthosteric" competitor quinacrine belong to the same category of compounds which possess the very special mechanisms of action: intercalation, stacking and self-aggregation**⁵.

We hypothesize that the bulky substitution at position 9 on basic acridine skeleton sterically hinders the simultaneous binding quinacrine into orthosteric and allosteric binding sites while the molecules of tacrine, or 7-methoxytacrine, respectively, can stack themselves, as do molecules of proflavine, or acridin-9-amine, inside the receptor pocket.

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PC445

The calcium-sensing receptor elicits agonist-specific differential Rho and phospholipase C-mediated signalling

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The mechanism by which the calcium-sensing receptor (CaR) regulates parathyroid hormone (PTH) secretion to control calcium homeostasis remains unclear. Orthosteric CaR activation with high extracellular Ca²⁺ (Ca²⁺_o) concentration, or allosteric CaR activation with the calcimimetic NPS R-467, elicits rho/rho kinase-mediated cell process retraction (1). However, the aromatic amino acids (allosteric CaR activators) are without effect despite eliciting intracellular calcium (Ca²⁺_i) mobilisation. To examine the CaR agonist concentration-dependency of actin polymerisation (after 3 hours) relative to acute changes in [Ca²⁺]_i and ERK activation, we examined HEK-293 cells stably transfected with CaR (CaR-HEK) using phalloidin-TRITC, fura-2 and phospho-ERK antibody respectively.

Ca²⁺_o caused a concentration-dependent increase in actin polymerisation with an EC₅₀ of 2.1 mM (peaking at 3mM), but stimulated ERK activation and Ca²⁺_i less potently (EC₅₀, 3.7 and 3.4 mM respectively). Extracellular magnesium also elicited greater potency for actin polymerisation (EC₅₀, 4.1 mM; peaking at 6mM) than for ERK activation (EC₅₀, 8.2 mM). Also, NPS R-467 elicited actin polymerisation, in the presence of 1.2mM Ca²⁺_o (i.e. the plasma free ionised Ca²⁺_i concentration) with an EC₅₀ of 73nM, whereas even 1µM NPS R-467 failed to elicit ERK activation without a further increase in the baseline Ca²⁺_o concentration. In contrast, L-Phe, L-Trp and L-His (10mM) each failed to alter actin polymerisation in the presence of 1.2 mM Ca²⁺_o. The aromatic amino acids have been reported to elicit CaR-induced Ca²⁺_i mobilisation via rho with Ca²⁺_o responses mediated via phospholipase C (2). However, we found that the PLCβ inhibitor U73122 (1µM) substantially inhibited both L-Phe (10mM) and Ca²⁺_o (3mM)-induced Ca²⁺_i responses. Furthermore, C3 exoenzyme pre-treatment had no effect on L-Phe-induced Ca²⁺_i mobilisation despite abolishing high Ca²⁺_o-induced process retraction.

We have proposed a mechanism of CaR-regulated PTH secretion by which Ca²⁺_o concentration may alter the cell cytoskeleton to modulate the trafficking of PTH-bearing secretory vesicles (1). Thus, here we show that the CaR stimulates actin polymerisation more potently than for PLC-mediated Ca²⁺_i mobilisation or ERK activation and over a range of Ca²⁺_o concentrations much closer to those seen physiologically. However, under the conditions tested, L-Phe failed to elicit CaR-induced actin polymerisation. Furthermore, contrary to previous reports (2) L-Phe-induced Ca²⁺_i mobilisation could be attenuated by PLCβ inhibition but not by rho inactivation.

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PC446

Altered pharmacology of chemokine receptors CXCR1 and CXCR2 hetero-oligomers

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CXCR1 and CXCR2 are members of the chemokine receptor family. The receptors are highly homologous and share 78% sequence identity. CXCL8 (IL-8) is an agonist at both CXCR1 and CXCR2 while CXCR2 can bind several other chemokines including CXCL1 (GRO- α). CXCR1 and CXCR2 have been implicated in a number of inflammatory disease states and are of great interest as potential drug targets. Previous studies have determined that CXCR1 and CXCR2 form homo- and hetero-oligomers with equal apparent affinities (Wilson *et al.*, 2005). In order to investigate the functionality of the CXCR1 and CXCR2 homo- and hetero-oligomers, stable cell lines were generated in which the receptors could be both singly and co-expressed. Using these cell lines we observed; (1) when individually expressed CXCR1 and CXCR2 both undergo prominent CXCL8 induced internalisation. (2) Upon stable co-expression of CXCR1 and CXCR2, the internalisation of CXCR1 in response to CXCL8 is significantly inhibited while CXCR2 undergoes prominent CXCL8 mediated internalisation. (3) Exposure to CXCL1 causes a rapid and transient increase in cell surface CXCR1 expression whilst CXCR2 undergoes rapid internalisation.

Wilson S *et al.*, (2005). *J Biol Chem* **280**, 28663 - 28674.

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PC447

Dynamic imaging of D1 dopamine receptor signaling and endocytic trafficking

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Dopamine is a major neurotransmitter within the central nervous system (CNS). The D1 dopamine receptor is a member of a family of seven transmembrane spanning proteins called G-protein coupled receptors (GPCRs) and the principal mediator of excitatory dopaminergic transmission in the CNS. Disorders of dopaminergic signaling underlie a number of disease states including Parkinson's disease, schizophrenia and drug addiction. As such, D1 signaling must be tightly regulated. Membrane trafficking is thought to have important consequences for GPCR

signaling. Ligand-induced endocytosis physically removes GPCRs from the cell surface and contributes to the attenuation of receptor mediated signaling (desensitization). Following endocytosis, rapid recycling of GPCRs is thought to return functional receptors to plasma membrane and restore signaling (resensitization). We are currently investigating membrane trafficking and signaling of D1 on the cellular level in order to better understand how dopaminergic signaling is regulated within the brain.

We have examined membrane trafficking of D1 in HEK 293 cells and in dissociated primary cultures of rat neurons using FLAG-epitope tagged version of the receptor. FLAG-D1 undergoes endocytosis following 10uM dopamine treatment and rapidly recycles to the plasma membrane after agonist washout. We have also investigated D1 mediated signaling using both a biochemical cAMP assay and a FRET-based cAMP reporter. Pretreatment of cells expressing D1 with 10uM dopamine for as little as 1 minute results in a reduced ability of the receptor to stimulate accumulation of cAMP upon subsequent stimulation with dopamine (desensitization). Preliminary results indicate that D1 mediated cAMP production recovers completely within 10 minutes after agonist washout in cells initially challenged with dopamine for 1 minute (resensitization). Cells pretreated with dopamine for 30 minutes do not exhibit this resensitization, even when assayed 60 minutes after agonist washout. This suggests that recovery of D1 signaling is highly sensitive to the duration of initial agonist challenge. We are currently examining the effects that directly inhibiting endocytosis and recycling of D1 have on dopamine stimulated cAMP production. We are also using total internal reflection fluorescence (TIRF) microscopy and a pH-sensitive ecliptic GFP version of the D1 receptor to examine dynamic signaling and trafficking events at the cell surface.

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PC448

Gentamicin-induced renal cell death is ameliorated by calcium-sensing receptor antagonism or lithium cotreatment

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Clinical gentamicin use is associated with proximal tubular toxicity and we have shown that chronic gentamicin treatment elicits dose-dependent, caspase-mediated cell death in proximal tubule-derived opossum kidney (OK) cells and calcium-sensing receptor (CaR)-transfected HEK-293 (CaR-HEK) cells (1). The CaR localises to the apical membrane of the proximal tubule and is sensitive to gentamicin (2,3). Therefore, we investigated whether renal gentamicin toxicity could be attenuated either by CaR antagonism or by the use of the inositol phosphate signalling modulator lithium. OK and CaR-HEK cells were grown for 4-7

days in 1% foetal bovine serum-containing media and gentamicin cellular toxicity was investigated by Trypan Blue uptake or by flow cytometry (FACS).

Chronic treatment of OK and CaR-HEK cells with concentrations of gentamicin (20–50 μ M) similar to those employed clinically, induced cell death after 6–7 days, consistent with the time course of clinical nephrotoxicity. Cotreatment with the calcilytic NPS-89636 (1 μ M; CaR negative allosteric modulator) abolished CaR agonist-induced ERK activation in OK and CaR-HEK cells. NPS-89636 also attenuated 100 μ M gentamicin-induced cell death (–77%, $P < 0.05$ by ANOVA) in OK cells, whilst abolishing 200 μ M gentamicin-induced cell death in CaR-HEK cells, thus implicating the CaR in these responses.

There is evidence that LiCl protects rats from gentamicin nephrotoxicity. To determine whether the LiCl exerts its effect directly on renal cells, we studied gentamicin-induced cell death in both OK cells and CaR-HEK cells in the presence or absence of a therapeutic concentration of LiCl (1 mM; 4 days) and found that it also inhibited gentamicin toxicity in both cell types. The decrease in gentamicin toxicity was not due to decreased cellular uptake as equivalent levels of texas red-conjugated gentamicin were found in OK and CaR-HEK cells in the presence or absence of LiCl. Similarly, lithium failed to significantly alter acute gentamicin-induced Ca^{2+}_i mobilisation. However, the cytoprotective effect of lithium was mimicked by the glycogen synthase kinase-3 inhibitors IX and XI.

Together, these data implicate the CaR in the mediation of gentamicin toxicity in OK and CaR-HEK cells. In addition, under culture conditions, lithium cotreatment ameliorates renal cell aminoglycoside toxicity but without preventing the cellular uptake of the drug.

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CaR-HEK cells and NPS-89636 were a kind gift of NPS Pharmaceuticals.

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PC449

Allosteric and G $\beta\gamma$ -mediated facilitation of ARF-dependent PLD activation by the M $_3$ muscarinic receptor

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The M $_3$ muscarinic receptor in 1321N1 cells and that heterologously expressed in COS7 cells activates phospholipase D (PLD) through an ARF-dependent PLD pathway. Contributions from other mediators such as PKC, Rho A and tyrosine kinases appear relatively minor and both ARF1 and 6 are thought to be involved, displaying co-immunoprecipitation with the M $_3$ receptor and *in vitro* binding to GST-fusion proteins of M $_3$ intracellular domains (Mitchell *et al.* 1998, 2003).

Here we have used cellular signalling assays, co-immunoprecipitation and GST-fusion proteins to investigate two novel mechanisms that cause differential facilitation of M $_3$ signalling through the ARF-dependent PLD pathway relative to its conventional output, G $_{q/11}$ -coupled phospholipase C (PLC).

Transfection of the G $\beta\gamma$ -sequestering construct GRK2-(495–689), GRK-ct, clearly attenuated ARF-dependent PLD responses of M $_3$ and AT $_1$ receptors but not those of 5-HT $_{2A}$ or P $_{2U}$ receptors nor that evoked by phorbol ester, without affecting PLC responses. This suggests that ARF-dependent coupling to PLD significantly involves G $\beta\gamma$ in some but not other GPCRs. In co-immunoprecipitation experiments, GRK-ct reduced ARF1 association with the M $_3$ receptor and in GST-fusion protein experiments purified G $\beta\gamma$ increased ARF1 binding to both i3 and ct domains of the receptor and ARF6 binding to only i3. PLD but not PLC signalling was inhibited by G β constructs with mutations at specific loci within the G β :effector interface.

In conjunction with this work we have been assessing the pathway selectivity of a positive allosteric modulator of the M $_3$ receptor, N-chloromethyl brucine (CMB) and a negative modulator, N-methyl strychnine (NMS) (Birdsall *et al.* 1999). CMB and NMS caused markedly greater increases and decreases respectively in the potency of M $_3$ receptor PLD responses than PLC responses. Current work is addressing the relationship between the allosteric mechanism for facilitation of ARF-dependent PLD activation and the molecular role of G $\beta\gamma$.

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PC450

Palmitoylation of the TP β isoform of the human thromboxane A $_2$ receptor

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Palmitoylation is a reversible and dynamic lipid modification whereby palmitic acid is added via a thioester linkage to carboxyl (C)-terminal cysteine residues. It is a prevalent feature amongst G protein coupled receptors, with up to 80% of GPCRs estimated to contain at least one cysteine within their C-tail domain that may be potentially palmitoylated. We sought to establish whether the TP α and/or TP β isoforms of the human prostanoid thromboxane (TX) A $_2$ receptor (TP) are palmitoylated and to assess the functional consequences thereof. Our studies confirm that consistent with the presence of three cysteines within its unique C-tail TP β , but not TP α , is palmitoylated. Furthermore, site directed mutagenesis and *in vivo* metabolic labeling studies confirmed that TP β is palmitoylated at Cys³⁴⁷ and, to a lesser extent, at Cys^{373/377}. Whilst impairment of palmitoylation did not affect

ligand affinity of TP β , agonist-induced $[Ca^{2+}]_i$ mobilization was significantly reduced relative to the wild type TP β when TP β^{C347} , but not TP β^{C373} or TP $\beta^{C373,377}$, was mutated suggesting that palmitoylation at Cys³⁴⁷ is specifically required for efficient Gq/phospholipase C β effector coupling. As TP β , but not TP α , is reported to undergo both agonist-induced and temperature-dependent tonic internalization, albeit through distinct mechanisms, we sought to determine whether palmitoylation affects TP β internalization. It was found that palmitoylation at Cys^{373,377} is critical for TP β internalization as TP β^{C373S} , TP $\beta^{C373,377S}$ and TP $\beta^{C347,373,377S}$ underwent significantly reduced agonist-induced and temperature-dependent tonic internalization compared to wild type TP β . On the other hand, whilst TP β^{C347S} underwent reduced agonist-induced internalization, it underwent tonic internalization to a similar extent as TP β . The deficiency in agonist-induced internalization by TP β^{C347S} , but not by TP $\beta^{C373,377S}$ nor TP $\beta^{C347,373,377S}$, was overcome by over-expression of either β -arrestin1 or β -arrestin2. Taken together, data herein suggest that whilst palmitoylation of TP β at Cys^{373,377} is critical for both agonist- and tonic-induced internalization, palmitoylation at Cys³⁴⁷ has a role in determining which pathway is followed, be it by the β -arrestin-dependent agonist-induced pathway or by the β -arrestin-independent tonic internalization pathway.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC451

Direct association of phospholipase D with the 5-HT_{2A} receptor and the M₃ muscarinic receptor

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The 5-HT_{2A} receptor is of particular clinical interest due to its suspected involvement in hallucinogenic and antipsychotic drug activity, thus implicating it as a potential therapeutic target for the treatment of various neuropsychiatric disorders (Dean, 2003). It is capable of signalling via various pathways, including the recruitment of the small G-protein ADP-Ribosylation Factor (ARF) and subsequent activation of phospholipase D (PLD) (Robertson et al, 2003). The process by which receptor-associated ARF may induce PLD activity is as yet unknown, however, recent experiments have isolated potential binding sites for both ARF 1 and PLD within the carboxy-terminal tail of the 5-HT_{2A} receptor.

Glutathione S-Transferase (GST)-fusion proteins of 5-HT_{2A} receptor domains of interest were generated and immobilised on glutathione-Sepharose beads as a bait for interacting proteins, which were identified by subsequent Western blot. ARF 1 and to a much lesser extent ARF 6 bound selectively to the carboxy-terminal domain of the receptor. Further GST-fusion protein studies showed that residues 376–384, positioned at the junction

between transmembrane domain 7 and the carboxy-terminal domain of the receptor were necessary for maximal ARF binding, implicating not only the conserved NPxxY motif but also part of the adjacent i4 loop in ARF binding.

In COS7 cells expressing the 5-HT_{2A} receptor, co-transfection of wild type PLD1 facilitated, whilst negative mutant PLD1 inhibited, 5-HT-induced PLD responses without affecting PLC responses. These effects were not reproduced by PLD2 constructs. In addition, co-immunoprecipitation experiments have demonstrated that PLD1 specifically interacts with the 5-HT_{2A} receptor. Subsequent GST-fusion protein studies have further isolated the position of PLD1 binding, again within the carboxy-terminal domain of the 5-HT_{2A} receptor, but in this case, within residues K385 – V471, in a distal region distinct from the ARF 1 binding site.

Further co-immunoprecipitation experiments with the M₃ muscarinic receptor have shown that distal regions of its carboxy-terminal tail also represent a specific PLD binding site but in this case both PLD 1 and PLD 2 are ligands. It has also been reported that the mu opioid receptor binds PLD2 (Koch et al, 2003). Comparison of potential PLD binding sites within these receptors may point to residues involved in PLD binding.

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PC452

Involvement of phosphatidylinositol 3-kinase in platelet signaling and the regulation of thrombus formation

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Phosphoinositide 3-kinase (PI 3-kinase) exists in cells as a family of isoforms. The enzymes are important regulators of fundamental metabolic processes, such as energy utilization, growth, cell proliferation and survival. They are activated by cell surface receptors for hormones, and by G-protein coupled receptors. Enzyme p110 gamma, in particular, catalyzes production of second messengers from inositol phospholipids, including phosphoinositide (3,4,5) triphosphate or Ptdins (3,4,5) P3, Ptdins (3,4) P2 and Ptdins (3) P. The latter mediates specialized responses related to particular functions of cells. The neutrophils, products of PI 3-kinase are thought to be important in chemotaxis motility, phagocytosis and lysosomal breakdown of

microorganisms. Neutrophils cause tissue damage by their capacity to release toxic oxygen radicals (generated by the NADPH oxidase complex), the exocytosis of granules containing highly histotoxic compounds such as elastase and collagenases, and the elaboration and release of additional pro-inflammatory cytokines. PI-3Ks have been shown to be key regulators of both neutrophil recruitment and activation. Neutrophils and platelets were obtained from human blood and macrophages from mouse peritoneal cavity. Reactive oxygen species (ROS) production was measured by a luminol-enhanced chemiluminescence assay; aggregation was measured in platelet-rich plasma (PRP) with a Chronolog Dual Channel Lumi-Aggregometer. Effects of graded concentrations of four enzyme inhibitors (wortmannin, LY-294002, resveratrol and quercetin) were evaluated. All inhibitors caused concentration-dependent depression of ROS generation and human platelet aggregation. They differed only in their potencies as revealed by concentration-response data. Moreover, inhibitors blocked three physiologically important stimulants of aggregation: ADP, collagen and epinephrine. We conclude that inhibition of PI 3-kinase would appear to be a useful therapeutic goal in those conditions where the activities of platelets and/or phagocytes become aberrant.

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PC453

Voltage-dependent activation of G protein-coupled receptors detected by FRET microscopy

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Many important signaling events in excitable cells are regulated by G protein coupled receptors such as α_2 adrenergic receptors. These receptors are exposed to the strong electric field of the membrane. It has been previously hypothesized that activation of certain GPCRs, is affected by voltage (Ben-Chaim 2003; Ben-Chaim 2006; Martinez-Pinna 2005). The development of FRET-based assays to determine receptor activation on the level of the receptor opened the path to study effects of voltage on receptor activity directly. We employed FRET probes for α_{2A} -adrenergic receptors (α_{2A} AR), and downstream events such as receptor / G protein interaction and G protein activation and measured FRET- signals in single cells under conditions of voltage clamp. Non activated receptors did not show any voltage dependent activity. However, at negative membrane potentials norepinephrine (NE) was more potent in activating α_{2A} -AR in comparison to positive potentials. Similarly, negative voltages potentiate agonist induced α_{2A} AR / G protein interactions as well as NE induced G protein activity. At intermediate concentrations of NE (10 μ M) the receptor switched on and off in dependence of the membrane potential. The concentration response curve of NE-evoked α_{2A} AR activation was shifted 2-3 fold by application of positive potential without affecting maximal receptor

activation. These results are in line with the hypothesis that binding of the charged agonist within the electrical field of the membrane represent a major mechanism for voltage dependence of GPCR activation. This concept is further supported by the notion that A_{2A} -adenosine receptors which bind non charged agonists did not show any voltage dependence as detected by means of a similar FRET approach.

This study is the first to prove the existence of voltage dependent activation of a GPCR on the level of the receptor.

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PC454

Transient arrestin interaction with the C-terminus of the human vasopressin receptor V1b is associated with its rapid recycling

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Many external stimuli are translated into a cellular response through activation of G-protein coupled receptors (GPCRs). Once the ligand is bound, a receptor-desensitization is induced, that is an essential step of the regulation process of the state of cell-responsiveness. For most GPCRs it is initiated by an arrestin-receptor interaction leading further to receptor internalization. The endocytosed GPCR can be then either dephosphorylated and recycled back to the plasma membrane or degraded. Receptor-arrestin interaction and trafficking properties allow GPCRs to be ranked into class A or class B receptors. Among the vasopressin family members, all of them GPCRs, there are representatives of both classes. Contrary to V1a, a typical class A receptor characterized with transient arrestin interaction and rapid recycling, the V2 receptor displays properties innate to class B receptors: continuous interaction and a limited recycling.

We investigated the capacity of arrestins to interact with the third vasopressin receptor, the neurohypophyseal V1b receptor, in living cells using the bioluminescence resonance energy transfer (BRET) technique. For this, the C-termini of wild type V1b and V2 were fused to R. luciferase (RLuc) and arrestins to the YFP. Upon agonist stimulation, the two receptors exhibit clearly distinct arrestin interaction patterns: V1b interacts with arrestin in brief and transient manner as opposed to the stable, long-lasting interaction of V2.

It has already been shown that arrestin interaction sites on GPCRs are generally settled in the C-terminal region of the receptor. In order to elucidate the role of the V1b C-tail for such interaction, we created chimeric receptors by exchanging the C-tails of V1b and V2, yielding V1b-CterV2-RLuc and V2-CterV1b-RLuc. In terms of functional properties i.e. ligand binding affinities and second messenger production, these chimeric receptors behave as the wild type receptors whose core they possess. Conversely,

regarding the arrestin-receptor interaction, it is transient within the set of receptors containing the C-tail of V1b (V1bWT and V2-CterV1b) and stable and continuous within receptors containing the C-tail of V2 (V2WT and V1b-CterV2). Interestingly, in binding experiments after agonist removal, one can distinguish the same two sets of receptors: V1bWT and V2-CterV1b exhibit a high recycling rate compared to V2WT and V1b-CterV2 with a limited recycling.

This study highlights that both processes (receptor-arrestin interaction and recycling) involve the receptor C-tail and are strongly related. Moreover, it reinforces the importance of GPCR-arrestin interactions for major cellular events such as signaling and trafficking.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC455

Gonadotropin releasing hormone receptor activation of a β -catenin signalling pathway and inhibition of glycogen synthase kinase-3

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The gonadotropin-releasing hormone (GnRH) receptor is a G-protein-coupled receptor (GPCR), and its ligand GnRH is the central regulator of the reproductive system. GnRH receptors are known to target a wide variety of signal transduction pathways. These include classical Gq / Gs / Gi heterotrimeric G-protein signalling, and activation of the small G-protein Rac1 to promote cytoskeletal reorganization. Several recent studies have shown that activation of GPCRs can impact on β -catenin signalling. β -catenin is the main effector of the Wnt pathway where it acts with the transcription factors TCF/LEF to mediate the transcription of Wnt target genes. We show that GnRH treatment promotes the nuclear accumulation of β -catenin, activation of β -catenin/TCF-dependent transcription and up-regulation of Wnt target genes, c-Jun, Fra-1 and c-Myc. These results are observed in HEK 293/GnRH receptor expressing cells and have been recapitulated using L β T2 and α T3-1 mouse gonadotrope cells. A normal consequence of Wnt signalling is the inhibition of Glycogen Synthase Kinase (GSK-3), allowing the accumulation of β -catenin. Our data shows a time-dependent inhibition of GSK-3 in response to GnRH treatment. Various chemical inhibitors and dominant-negative mutant constructs were employed to elucidate the signal transduction

pathway mediating these events. Our findings extend the number of GPCRs that can target β -catenin signalling through diverse pathways. Furthermore, this is the first demonstration of the regulation of β -catenin/TCF-dependent transcription by a peptide hormone GPCR.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC456

Activation of the MAPK cascades by GnRH: a crucial role for the mammalian GnRH receptor coupling to G_{q/11}

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Gonadotropin-releasing hormone (GnRH) acts on its cognate receptor in pituitary gonadotropes to regulate the biosynthesis and secretion of gonadotropins. This action is thought to be mediated by the receptor coupling to G_{q/11}. GnRH also has extra-pituitary functions including inhibition of cell growth in prostatic, breast and ovarian cancers, in which the GnRH receptor activation of the MAP kinase cascades plays an important role. In extra-pituitary compartments, GnRH receptor signaling is potentially mediated by different G proteins such as G_{i/o} or G_s, although this remains controversial. Here we examined the GnRH receptor coupling to different G proteins and their roles in the activation of the MAP kinases. In MCF-7 and HEK293 cells transiently transfected with GnRH receptor cDNA, pertussis toxin had little effect on ERK activation. In contrast, ERK activation was completely abolished by a G α_q selective inhibitor. In HEK293 cells co-transfected with GnRH receptor cDNA and siRNA against G $\alpha_{q/11}$, GnRH-stimulated ERK activation was markedly reduced. In G $\alpha_{q/11}$ -negative mouse embryonic fibroblasts (MEF) stably expressing the rat GnRH receptor, no GnRH-stimulated ERK or JNK activation was observed. After transient transfection with G α_q cDNA, GnRH stimulated phosphorylation of ERK and JNK. Using specific inhibitors, ERK activation was shown to occur through a classical G $\alpha_{q/11}$ -PLC β -PKC dependent system with consequent activation of c-Src, Raf-1 Kinase and MEK 1/2. Transient transfection of all subtypes of G α_i or G α_{q15} into the MEF stable cells did not facilitate GnRH-stimulated ERK activation. In addition, no GnRH-mediated cAMP response or inhibition of isoproterenol-induced cAMP accumulation in COS-7 and MEF cells was observed. Interestingly, GnRH-stimulated cleavage of poly[ADP-ribose]polymerase (PARP), a substrate of caspase 3, was detected after transfection of G α_q . We therefore propose that the mammalian GnRH receptor does not couple directly to G α_i or G α_s as hypothesised by other studies, that ERK and JNK activation by the GnRH receptor is mainly mediated via the G α_q signalling pathway and that this pathway may play important roles in GnRH analogue-induced antiproliferation and apoptosis in the cell lines tested.

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PC457

Intracellular calcium responses to extracellular calcium sensing receptor activation in explanted vascular endothelial cells

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Small elevations of extracellular calcium cause vasodilation in blood vessels via an endothelium-dependent mechanism. Recently, functional expression of a receptor that senses changes in extracellular calcium, the calcium-sensing receptor (CaSR), has been demonstrated in vascular tissue (Weston *et al.*, 2005). Particularly, functional evidence was presented for the existence of an endothelial CaSR, stimulation of which caused smooth muscle hyperpolarisation. Thus, we investigated the molecular and functional expression of the CaSR in primary isolated vascular endothelial cells. Rat aortic endothelial cells (RAEC) were obtained by an explant technique as previously described (Suh *et al.*, 1999). Cells obtained using this technique displayed characteristic cobblestone morphology when confluent, were positively immunostained for the von Willebrand factor and fluoresced following incubation in DiI-Ac-LDL. Immunocytochemical studies using a monoclonal antibody against the CaSR confirmed the presence of the receptor protein. In functional studies, RAEC were loaded with the calcium indicator dyes FURA-2 or INDO-1 and changes in intracellular calcium concentration examined during application of known CaSR agonists. Raising extracellular calcium (from 0.5 mM to 1.5 and 3 mM) produced concentration-dependent elevations in intracellular calcium. These responses were mimicked by the CaSR activators 0.8 mM Mg^{2+} , 200 μM neomycin, and 500 μM gadolinium. Responses to agonists varied between cells, and included large and sustained increases and high-frequency oscillations, suggesting a heterogeneity of cellular expression and/or function of the receptor. We conclude that the vascular endothelium expresses a functionally active CaSR, activation of which elevates intracellular calcium. Since this further modulates smooth muscle membrane potential to bring about hyperpolarisation (Weston *et al.* 2005) and thus vasodilation, the CaSR may present a useful therapeutic target for the treatment of hypertension.

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PC458

Rapid resensitization of purinergic receptor function in human platelets is mediated by receptor internalization and subsequent recycling to the cell membrane

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ADP a critical regulator of platelet activation, mediates its actions through two G protein-coupled receptors (GPCRs), the P2Y₁ and P2Y₁₂ purinergic. Recently we demonstrated that both receptors desensitize (1) and internalise (2-3) in human platelets by differential kinase-dependent mechanisms. In this study we examined if these responses resensitized following agonist removal and if receptor trafficking played a significant role in this process.

All experiments were undertaken in human platelets or in 1321N1 cells stably transfected with either the P2Y₁ or P2Y₁₂ receptor. P2Y₁ Gq-coupled and P2Y₁₂ Gi-coupled receptor activity was assessed as previously described (1). Receptor surface loss was assessed in 1321N1 cells by ELISA whilst in parallel experiments changes in P2Y₁ and P2Y₁₂ surface receptor levels were measured in fixed platelets using the P2Y radioligand [³H] 2MeSADP in combination with the P2Y₁ receptor antagonist A3P5P and the P2Y₁₂ receptor antagonist AR-C69931MX (2-3). Initial experiments showed that responses to both of these receptors rapidly resensitize following agonist-dependent desensitisation in human platelets or in 1321N1 cells. Further we discovered that in both systems following agonist-induced internalization both the P2Y₁ and P2Y₁₂ receptor rapidly recycle to the cell surface. In 1321N1 cells expression of a dynamin dominant-negative mutant (K44A) which blocks P2Y₁ and P2Y₁₂ receptor internalization or treatment with either the phosphatase inhibitor okadaic acid or with monensin which inhibits receptor recycling attenuated receptor resensitization. Further in human platelets treatment with either monensin or okadaic acid significantly attenuated resensitization of both P2Y₁ and P2Y₁₂ purinergic receptor responses.

In conclusion this study is the first to show that both P2Y₁ and P2Y₁₂ receptor activity is rapidly and reversibly modulated in human platelets and reveals that receptor trafficking is an essential part of this process.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC459

Lysosomal sorting of the beta-2 adrenergic receptor is ubiquitination-independent, but 'ESCRT'-dependent

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Endocytic trafficking of G-protein-coupled receptors (GPCRs) to lysosomes is an important mechanism promoting receptor

downregulation following prolonged agonist stimulation. It has been reported that a number of membrane proteins and some GPCRs require covalent modification by ubiquitin moieties in order to undergo efficient endocytic sorting to lysosomes (1). This sorting process is mediated by ubiquitin-dependent protein interactions directing endocytosed receptors to an endosome-associated sorting machine composed of a conserved set of 'ESCRT' proteins (2). While this mechanism is well supported in studies of receptor sorting in budding yeast, we have previously shown that one mammalian GPCR, the mouse delta opioid receptor, undergoes efficient ESCRT-dependent sorting to lysosomes without requiring receptor ubiquitination (3, 4). Here we demonstrate that the same is true for the human β 2 adrenergic receptor (β 2AR). Using stably transfected HEK 293 cells expressing epitope-tagged receptors we demonstrate that a mutant β 2AR lacking all lysine residues undergoes efficient agonist induced lysosomal proteolysis, in the absence of direct ubiquitination. Furthermore, we establish a critical role of a C-terminal 'recycling sequence' in controlling β 2AR sorting to lysosomes in the absence of receptor ubiquitination. Moreover, we show that ubiquitination-independent lysosomal sorting of the β 2AR, like ubiquitination-directed sorting of other membrane cargo, requires the 'core' ESCRT protein Vps4- as well as the ESCRT-associating protein HRS. Finally, we identify a late stage in the process of β 2AR degradation, which is not dependent on lysosomal sorting or the onset of receptor proteolysis and can reconcile the present results with previous evidence for ubiquitination-dependent lysosomal sorting of the β 2AR. Together these results reveal a fundamental and hitherto unappreciated cellular mechanism of GPCR regulation, which applies to the 'prototypic' β 2AR and thus may play a widespread role in controlling GPCR signaling in mammals.

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PC460

Sympathectomy increases the alpha1D-adrenoceptor component to smooth muscle contraction

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Chemical sympathectomy is known to produce a denervation supersensitivity in smooth muscle in which responsiveness to adrenoceptor agonists is increased, resulting in increased agonist potency. This has long been assumed to be due to a general increase in the number of receptors on the smooth muscle. However, we have recently suggested that denervation results rather in an alteration in the proportion of differing subtypes of alpha-adrenoceptor (Cleary et al. 2004). In rat vas deferens, we found that sympathectomy (by i.p. injection of 6-hydroxydopamine)

resulted in an increase in the proportion of alpha1D-adrenoceptors from undetectable to approximately 40% of the receptor population (the remainder being predominantly alpha1A-adrenoceptors). This increase in the number of alpha1D-adrenoceptors in ligand binding studies was matched by an increase in the potency of noradrenaline and an increase in the alpha1D-adrenoceptor mediated component of the contraction. In this study, we wished to examine whether this effect of sympathectomy can also be demonstrated in mouse vas deferens. In vas deferens from C57 Wild type mice, chemical sympathectomy increased the potency of noradrenaline at producing contractions as compared with vehicle treatment. Hence, denervation supersensitivity occurs in this preparation. However, alpha1A-adrenoceptor antagonism virtually abolished contractions in normal vas deferens, but not in denervated tissues, whereas alpha1D-adrenoceptor antagonism significantly reduced agonist contractions in denervated but not normal tissues. These results suggest that chemical sympathectomy results not in a general increase in the number of alpha-adrenoceptors, but rather in a change from alpha1A to alpha1D adrenoceptors. The increased sensitivity to noradrenaline is then caused not by an increase in number of receptors, but a change to alpha1D-adrenoceptors for which noradrenaline has high affinity.

Cleary L et al. (2004). *Br J Pharmacol* 143, 745-752.

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PC461

Effect of a cannabinoid CB1 receptor agonist on neurite outgrowth in an *in vitro* model of diabetic neuropathy

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The development of a treatment that can delay the onset of diabetic neuropathy and slow its progression is urgently needed. In cell culture models of diabetic neuropathy, elevated glucose attenuates nerve growth factor (NGF)-induced neurite outgrowth (Lelkes *et al.*, 2001). Jordan *et al.* (2005) demonstrated the synthetic cannabinoid CB1 receptor agonist HU210 triggers neurite outgrowth in cultured neurons, implicating a role for CB1 receptors in stimulating neurite outgrowth and regeneration after neuronal injury. In the present study, we investigated the neuroprotective effect of CB1 agonist treatment in a cell culture model of diabetic neuropathy, whereby neurite outgrowth of rat pheochromocytoma (PC12) cells was induced by NGF (50 ng/ml), in the presence of normal and high concentrations of glucose (5.5 and 50mM) to simulate normal and hyperglycaemic conditions *in vivo*. We examined whether impaired neurite outgrowth of PC12 cells cultured in high glucose can be "rescued" with HU210 (0.03-30 μ M).

We observed a reduction in total neurite length induced by NGF in PC12 cells cultured in 50mM glucose on day 6 ($P < 0.01$ versus 5.5mM; $n = 70-79$ from 6 independent cultures). This effect was due to raised glucose levels, used to mimic diabetic conditions, rather than any hyperosmolality effects since mannitol (50mM) produced similar results to the physiological 5.5mM

glucose control ($P=0.79$). When cells were cultured for six days in the presence of combined NGF/CB1 agonist, neurite measurement revealed the addition of HU210 (30nM-3 μ M) had no significant effect on total neurite length in cells cultured in 5.5mM glucose ($P=0.17$ versus the untreated/vehicle DMSO 1:1000; $n=148$ -220 individual cells), whilst increased total neurite lengths were found in cells cultured in 50mM glucose in a HU210 concentration-dependent manner. Total neurite length of PC12 cells treated with 0.3 and 3 μ M HU210 was longer than those in the untreated, 0.03 μ M HU210 and vehicle groups ($P<0.01$; $n=112$ -216 individual cells). Cell viability assays showed there were no effects of glucose or mannitol on cell viability ($P=0.86$). Except for 30 μ M HU210 which was associated with a high occurrence of cell death ($P<0.01$), there was no significant difference in cell survival between the various concentrations of HU210 (30nM-3 μ M) in both normal and high glucose conditions ($P=0.99$ and $P=0.91$ respectively, versus each control, without HU210 and vehicle; $n=4$, in duplicate).

These results demonstrate a neuroprotective role of the CB1 agonist HU210 ($\leq 3\mu$ M) in PC12 cells cultured under conditions mimicking hyperglycaemia, and suggest CB1 receptors may be an appropriate therapeutic target in preventing the neurodegenerative process in diabetes.

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PC462

The pharmacology of agonist responses to pindolol at the β_1 -adrenoceptor

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The β_1 -adrenoceptor is now considered to exist in two different states or conformations. Catecholamines bind to and activate responses via the classical site (site 1) of the receptor that are sensitive to antagonism by classical β -blockers. Agonists responses elicited via the secondary site (e.g. the agonist response to CGP 12177) are relatively resistant to inhibition by classical β -antagonists.

In CHO cells stably expressing the human β_1 -adrenoceptor at 1100fmol/mg protein, pindolol has previously been demonstrated to stimulate both sites of the receptor (Baker *et al.*, 2003). The aim of this study was to examine this pindolol response further using CHO cells stably expressing a lower level of the human β_1 -adrenoceptor (79 fmol/mg protein) and a CRE-luciferase reporter gene. Luciferase gene transcription measurements were carried out as previously reported (Baker *et al.*, 2003).

Pindolol stimulated a mono-phasic classical sigmoidal agonist concentration response curve in these lower expressing cells (log EC₅₀ -5.74 $\pm$ 0.03, $n=8$; 16.4 $\pm$ 0.4% isoprenaline maximum). In the presence of fixed concentrations of the site 1 agonist cimaterol, low concentrations of pindolol inhibited the cimaterol response where as higher concentrations of pindolol were

required to stimulate the agonist response. This is very similar to that seen with CGP 12177 (see Konkar *et al.*, 2000; Baker, 2005) confirming that pindolol binds to site 1 with high affinity but suggest that the agonist response was occurring via a different site. The ability of a range on antagonists to inhibit the pindolol response was then examined (see Table). For some ligands (e.g. nadolol and ICI 118551) the Log KD values obtained were similar to those obtained when CGP 12177 was an agonist, however others (e.g. atenolol) were markedly different. This suggests that the agonist response of pindolol might not be acting through the same secondary site as CGP 12177.

Table 1

antagonist	site 2 agonist			site 1 agonist
	CGP 12177	pindolol	n	cimaterol
carvedilol	-7.29	-7.37 $\pm$ 0.12	5	-9.92
bupranolol	-7.29	-7.55 $\pm$ 0.07	5	-9.22
CGP 20712A	-7.09	-7.55 $\pm$ 0.05	5	-9.61
propranolol	-6.44	-6.30 $\pm$ 0.09	5	-8.65
timolol	-6.28	-6.70 $\pm$ 0.15	5	-9.03
nadolol	-5.93	-6.11 $\pm$ 0.06	5	-7.90
ICI 118551	-5.80	-6.01 $\pm$ 0.06	5	-7.17
betaxolol	-5.73	-6.27 $\pm$ 0.14	5	-8.87
bisoprolol	-5.53	-6.34 $\pm$ 0.08	5	-8.51
metoprolol	-5.26	-5.82 $\pm$ 0.07	5	-7.98
atenolol	-3.82	-5.29 $\pm$ 0.04	5	-7.06
sotalol	-3.66	-4.26 $\pm$ 0.08	5	-6.26

Log KD values for antagonism of pindolol responses. Data in the presence of cimaterol and CGP 12177 taken from Baker 2005.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC463

The putative palmitoylation site (C361) in human proteinase-activated receptor-2 (hPAR2) differentially regulates receptor coupling to calcium and ERK1/2

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PARs are a novel class of GPCRs activated by proteolytic unmasking of a tethered ligand domain within the N-terminus which in turn initiates receptor activation. PAR2, a trypsin and tryptase activated member, has been implicated in cardiovascular disease (Coelho *et al.*, 2003; McGuire, 2004). Previous studies reported that activation of hPAR2 by mast cell tryptase can be regulated by the post-translational modification N-linked glycosylation (Compton *et al.*, 2002). Since palmitoylation is an important post-translational modification that can have profound effects on GPCR function, we investigated the role of the putative palmitoylation site (C361) within hPAR2. Wild-type hPAR2 (wt-hPAR2) and mutant hPAR2C361A were permanently expressed in CHO cells. Intracellular calcium signalling was used to compare the functional activity between wt-hPAR2 and hPAR2C361A

cell lines with matched receptor expression. hPAR2C361A showed a significant reduction in sensitivity towards both trypsin and the selective hPAR2 activating peptide SLIGKV-NH2 (4-fold and 6-fold respectively). In addition, hPAR2C361A displayed a marked decrease in maximal response to both agonists compared to wt-PAR2. ERK1/2 activation was assessed by western blot analysis of cell lysates from agonist treated wt-hPAR2 and hPAR2C361A cell lines. For wt-hPAR2, trypsin and SLIGKV-NH2 stimulated a modest and transient (5min) increase in phosphorylated-ERK1/2 (p-ERK1/2) which declined thereafter to baseline levels. In contrast, for hPAR2C361A trypsin and SLIGKV-NH2 stimulated a robust increase in p-ERK1/2 that remained elevated for up to 20min before declining to baseline. These findings suggest that hPAR2C361 and potentially receptor palmitoylation play an important role in regulating the signalling pathways utilised by hPAR2.

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PC464

Synovial co-expression of protease-activated receptor-2 and -4 in arthritis

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Protease-activated receptors (PARs) are a unique family of G-coupled receptors which are activated by proteolytic cleavage of one of its extracellular domains so as to unmask and create a new N-terminal sequence. Subsequently, it acts as a tethered ligand which can bind to an adjacent extracellular domain of the receptor, resulting in intracellular signaling followed by internalization of the receptor [1]. To date, four PAR subtypes have been identified: PAR-1, PAR-2, PAR-3 and PAR-4. PAR-2 is increasingly recognized to have a pro-inflammatory role, with previous work by our group identifying a key role for this receptor in murine chronic arthritis [2] and in the rheumatoid synovium [3]. There is now emerging evidence suggesting a pro-inflammatory role for PAR-4 [4]. The aim of this study was to compare for the first time the differential expression of all the PAR mRNAs in unstimulated fibroblast-like synoviocytes (FLS) cultured from patients with osteoarthritis (OA) and rheumatoid arthritis (RA).

Synovial samples were obtained with informed consent from 3 OA, and 3 RA patients, and were incubated under standard conditions to obtain cultures of FLS. Total cellular RNA was extracted from FLS using TRIzol Reagent (Life Technologies, UK). To exclude genomic DNA contamination, all RNA samples were treated with DNaseI (Invitrogen) as per the manufacturer's protocol. RT-PCR was performed using the Invitrogen Reverse Transcription System. Aliquots of cDNA were used as templates in PCR using Megamix (Microzone, UK) with the following primer pairs: PAR-1, 5'-TGT GAA CTG ATC ATG TTT ATG -3' and 5'-TTC GTA AGA TAA GAG ATA TGT-3' (708bp); PAR-2, 5'-GAA GCC TTA TTG GTA AGG TTG-3' and 5'-AAC ATC ATG ACA GGT CGT GAT-3' (580bp); PAR-3, 5'-GAA AGC CCT CAT CTT TGC AG-3' and 5'-AGG TGA AAG GAT GGA CGA TG-3' (599bp); and PAR-4, 5'-GGC AAC CTC TAT GGT GCC TA-3' and 5'-TTC GAC CCA GTA CAG CCT TC-3' (243bp). The signal yielded by the PAR primer pairs was normalized to GAPDH. Thermal cycling was performed under the conditions for PCR detection of PAR-2 [2]. PCR products were separated on a 1.5% agarose gel and visualised by ethidium bromide staining. RT-PCR analysis consistently revealed the expression of both PAR-2 and PAR-4 mRNA in unstimulated FLS of all 3 OA and 3 RA samples, whilst no PAR-1 or PAR-3 mRNA was detected in any of the samples. None of the PAR mRNAs were detectable with the omission of reverse transcriptase, confirming absence of genomic DNA contamination. These findings suggest co-expression of PAR-2 and PAR-4 mRNA may independently or inter-actively contribute to the development of synovitis.

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PC465

PAR-2-induced anion conductance in NCL-SG3 human sweat gland cells

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Proteinase-activated receptor (PAR) type 2 (PAR-2)(ref 1), which is activated by the action of trypsin and activating peptides (AP), has been shown to mediate ion secretion in a variety of secretory epithelial cells in the GI tract and airways (2). However, their action on human sweat gland secretory cells is unknown. This

study, investigated the effects of PAR-2 stimulation on ionic secretion in a human sweat gland cell line NCL-SG3 (NCL) (3) determined by Ussing chamber experiments.

NCL cells were grown to confluence on permeable supports, mounted in Ussing chambers, bathed with 95%O₂/5%CO₂-bubbled Krebs-Henseleit solution at 37°C and allowed to equilibrate under open circuit conditions. Transepithelial potential difference was clamped to 0mV using a voltage current amplifier. The current to maintain this potential (short-circuit current, I_{sc}) was recorded. Resistance was calculated by measuring the current in response to a 0.2mV excursion from the holding potential. Positive deflections in I_{sc} were defined as anions moving from the basolateral to the apical side. Results are expressed as means±sem and statistical analysis performed using Student's t-test. P values <0.05 indicate significance. Cells were stimulated by adding compounds to the basolateral baths.

NCL cells exhibited low basal resistances of 65.8±7.5Ω cm⁻², basal I_{sc} levels of 9.3±2.4μA cm⁻², which was unaffected by the presence of the sodium channel blocker amiloride (10μM), demonstrating that NCL cells do not have a basal Na⁺ conductance (n=14). Trypsin (100μM) and AP (100μM; Bachem, Germany) added to the basolateral aspect of the cells caused increases in I_{sc} of 2.54±0.45μA cm⁻², 2.43±0.39μA cm⁻² (n=7; P<0.05, respectively). An inactive PAR-2 peptide (100μM) failed to elicit any response when added to the basolateral side. Both trypsin and the AP failed to elicit any change in I_{sc} in chloride-depleted solutions.

The results of this study demonstrate that PAR-2 receptors are present in this sweat gland cell line and that stimulation of these receptors at the basolateral membrane in these cells can produce chloride secretion.

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PC466

Dual effect of Proteinase activated receptor-2 stimulation on inflammatory signalling

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Proteinase activated receptor-2 (PAR-2) is a novel G-protein coupled receptor, that is activated by means of proteolytic cleavage (Macfarlane et al, 2001) and has both pro and anti-inflammatory actions depending upon the system examined. PAR-2 has been linked to the stress-activated protein kinases, JNK and p38 MAP kinase and also NFκB (Kanke et al, 2001; Sabri et al, 2001),

pathways known to be involved in pro-inflammatory responses in several cell types. The cytokine TNFα is also an important mediator of pro-inflammatory responses, and indeed signals through both the SAPK and NFκB pathways. Since both TNFα and PAR-2 activators are released during inflammation, we examined the possibility of crosstalk/synergy between PAR-2 and TNFα at the level of the SAP kinases and NFκB signalling pathways.

The skin epithelial cell line NCTC2544 stably expressing PAR-2 (Clone G), was generated and incubated with trypsin or PAR-2 activating peptide (AP) and TNFα. The SAPK and NFκB activation was examined in whole cell lysates, which had been resolved by Western blotting, using phospho-specific antibodies.

In Clone G cells trypsin (50nM), SLIGKV-OH (200μM) and TNFα (20ng/ml) caused a significant increase in phospho-p38 MAP kinase and NFκB levels. Preliminary results failed to show evidence of synergy as prolonged TNFα pre-treatment (1-24hrs) failed to modify PAR-2 mediated signalling, whilst PAR-2 activation failed to potentiate SAP kinase and NFκB activation mediated by low concentration of TNFα (1ng/ml). However, surprisingly, activation of PAR-2 using low concentrations of trypsin (1-20nM) or AP (1-30μM) resulted in a reduced/inhibitory effect on the ability of TNFα to signal through p38 and NFκB. The same effect was observed in the EAHy926 cell line (endothelial cells), which endogenously express PAR-2. The phenomenon was not observed for PAR-4 activation. Studies investigating the signalling pathways involved in this response will be presented. These results indicate a potential mechanistic explanation for both the anti and pro-inflammatory actions of PAR-2, dependant upon the occupancy of the receptor.

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PC467

A possible role for protease-activated receptor-2 in modulating the Th1 immunological response

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Immune deviation towards a Th1 response results in tumour/graft rejection involving IgG production, cytotoxic T lymphocytes, natural killer cells and macrophage activation. Conversely, deviation towards a Th2 response facilitates graft tolerance or tumourigenesis. Here, we investigated the involvement

of protease-activated receptor-2 (PAR₂) in Th1/Th2 responses associated with skin allograft rejection, tumourogenesis and autoimmunity.

Skin allografts were performed on C57 Bl/6 mice (anaesthetised with 100mg/kg ketamine and 15mg/kg xylazine) with donor 10mm diameter full skin thickness allografts obtained from Balb/c mice. The mice then received 0.2ml sc injections of either saline (vehicle) or SLIGRL (1.5mg/kg), daily, over a 14 day period. SLIGRL caused fewer animals to reject their grafts at day 10. In addition, SLIGRL-treated animals with accepted skin grafts demonstrated a significant ($p < 0.05$) decrease in plasma IgG levels compared to saline controls whereas there was no difference in plasma IgE levels between treatments, at both days 5 and 10. IFN- γ and IL-10 release from isolated splenocytes were measured as markers of Th1 and Th2 responses, respectively. SLIGRL treatment did not affect IL-10 release, however, IFN- γ release significantly ($p < 0.01$) decreased at day five, compared to control treated animals. Based on this study, we conclude that PAR₂ down-regulates Th1 immunological responses in a skin allograft model. We are currently investigating the role for PAR₂ in tumourogenesis and experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). To date, preliminary data indicates that sc SLIGRL does not affect growth of both melanoma and colon carcinoma *in vivo* as has been suggested to occur in *in vitro* studies (Yada *et al.* 2005, Nishibori *et al.* 2005). No data is available as yet for the EAE study. We predict that PAR₂ dependent inhibition of Th1 phenotype will prevent EAE.

Conclusions to date are difficult, although predictions are sc SLIGRL may down-regulate Th1 immunological responses. This may be beneficial in the situation of a transplant or in autoimmune diseases such as MS.

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PC468

N-linked glycosylation regulates proteinase activation and disarming of human proteinase-activated receptor-1 (hPAR1)

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Proteinase-Activated-Receptors (PARs) are a novel family of G-protein coupled receptors (GPCRs) that mediate many biological effects of proteinases. Thrombin activates PAR1, PAR3 and PAR4, whilst trypsin and mast cell tryptase activate PAR2. We have previously reported that N-linked glycosylation provides a subtle but effective mechanism for regulating PAR2 activation. Since PAR1 contains three putative N-linked glycosylation sites on the N-terminus and two on extracellular loop-2, we hypothesised that glycosylation may regulate both proteinase activation and disarming of PAR1. Wild-type hPAR1 and hPAR1eYFP (wt-hPAR1 and wt-hPAR1eYFP) and the glycosylation-deficient

mutant receptors (hPAR1N35Q, hPAR1N62Q, hPAR1N75Q, hPAR1N62QN75Q, hPAR1N250Q, hPAR1N259Q, hPAR1N250QN259Q, hPAR1N35-N259Q) were permanently expressed in the Kirsten virus sarcoma transformed rat kidney epithelial (KNRK) cell line. Western blot analysis revealed PAR1 to be an N-linked glycosylated receptor. Flow cytometry and confocal microscopy analysis revealed that removal of only one glycosylation sequon decreases hPAR1 cell surface expression. Removal of all glycosylation sequons resulted in near total retention of the receptor within the cytosol. In calcium signalling assays thrombin and trypsin but not the PAR1-AP, TFLLR-NH2 displayed a reduced ability in activating hPAR1N62QN75Q compared to wt-hPAR1. Further, hPAR1N62QN75Q was strikingly more susceptible to disarming by the neutrophil proteinases proteinase-3, elastase and the bacterial proteinase thermolysin when compared to wt-hPAR1. Thus N-linked glycosylation regulates cell surface expression and proteinase activation and disarming of hPAR1.

We would like to thank Chris Crow for his technical support.

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PC469

A novel protease-activated receptor-2 (PAR₂) antagonist ameliorates collagen-induced murine arthritis

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In earlier proof of concept studies, we reported a key role for a G-protein-coupled receptor, PAR₂, in chronic joint inflammation¹ and, demonstrated that a novel small molecule PAR₂ antagonist, ENMD-1068, dose dependently attenuated acute carageenan/kaolin-induced inflammation of the murine knee joint². The aim of the present study was two-fold: to test the hypothesis that antagonism of PAR₂ offered therapeutic potential in the collagen-induced model of rheumatoid arthritis (CIA), and to assess the potency and selectivity of ENMD-1068. All procedures involving animals were performed in strict accordance with the Animals (Scientific Procedures) Act 1986. Initial synovial perfusion studies using a Laser Doppler Imager (LDI; Moor Instruments Ltd) were conducted in adult male C57/6J/Bl mice to assess the potency and selectivity of ENMD-1068. Collagen and Freund's Complete Adjuvant (MD Biosciences) were used to induce CIA in DBA1 mice, with booster (collagen in PBS) given at day 21. From the first day of response, mice were treated once daily for 7 days with vehicle, 4 mg or 16 mg of ENMD-1068. Arthritic index (arbitrary) and paw pad thicknesses were recorded daily and confirmed by blinded observation. Late responders (> day 33) were excluded. ENMD-1068 reduced PAR₂ agonist, but not PAR₁ agonist-induced synovial hyperaemia. Dose response studies also indicated low potency of the compound for PAR₂. Furthermore, ENMD-1068 had no effect on basal synovial perfusion in normal mice. Daily subcutaneous administration of ENMD-1068 significantly reduced the arthritic index

compared to vehicle treatment ($P < 0.0001$, 2-way ANOVA). At day 7 post-response, arthritic indices were: vehicle group 7.1 ± 0.9 ; 4 mg group 4.2 ± 1.0 ; 16 mg group 2.8 ± 0.5 . This study supports ENMD-1068 as a selective but low-potency antagonist for PAR₂, with therapeutic efficacy in ameliorating collagen-induced murine arthritis. PAR₂ may therefore represent a novel target in the treatment of rheumatoid arthritis.

Ferrell WR *et al.* (2003). *J Clin Invest* **111**, 34-41.

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PC470

Agonist-mediated signal trafficking by Proteinase-activated receptor-2 (PAR₂): Differential activation of calcium and MAPkinase signaling by distinct trypsin-revealed tethered ligand sequences

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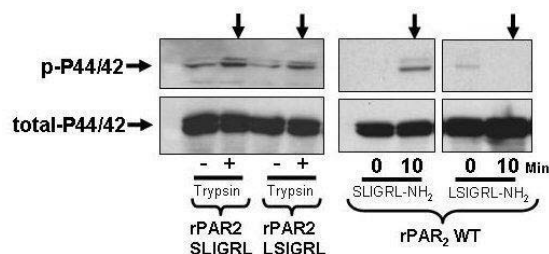
Proteinase activated receptors (PARs) are a family of GPCR's that are activated by serine proteinase cleavage of the receptor N-terminal sequence to reveal a tethered receptor-activating ligand (t_l) (e.g. SLIGRLDTP... for rat PAR₂). Our previous work (Mol. Pharm. 65:149, 2004) employing alanine replacements of the first six amino acids of the trypsin-revealed t_l of rat (r) PAR₂, showed that the t_l sequences, SLIGRL... and SLAAAA... were able to generate a calcium signal, whereas the t_l sequences, AALIGRL... and LSIIGRL... did not. Further, the synthetic peptides SLAAAA-amide, AALIGRL-amide and LSIIGRL-amide were not active. We hypothesized that, while unable to cause a calcium signal, trypsin-revealed t_l sequences like AAIGRL... and LSIIGRL... might still be able to signal via MAPkinase activation.

We utilized Kirsten virus-transformed rat kidney cells transfected with wild-type rPAR₂ and rPAR₂ mutants with N-terminal tethered ligand alanine substitution modifications, to generate receptors with modified t_l sequences: rPAR₂,LSIIGRL, rPAR₂,SLAAAA and rPAR₂,AAIGRL. All receptors were treated with 10nM trypsin to unmask the t_l sequences and both calcium signaling and P42/44 MAPkinase activation at 10 min. were monitored by fluo-3 fluorescence and western blot analysis. Receptor-expressing cells were similarly exposed to synthetic peptides (SLIGRL-NH₂, LSIIGRL-NH₂, SLAAAA-NH₂ and AAIGRL-NH₂; 10μM) corresponding to the wild-type and modified N-terminal t_l sequences. All trypsin-revealed t_l sequences caused MAPkinase activation, but only the t_l sequences SLIGRL... and SLAAAA... triggered a significant calcium signal. All peptides, except for SLIGRL-NH₂, failed to signal via either calcium or MAPkinase.

We conclude that different trypsin-revealed rat PAR₂ t_l sequences can trigger selective signal trafficking by either calcium transients or the activation of MAPkinase, presumably by promoting interactions selectively with either Gq or Gi. Further our data demonstrate dramatic differences in both calcium and

MAPkinase signaling by the PAR₂ activating sequences either as trypsin-revealed t_ls or as synthetic receptor-activating peptides. Table 1: rPAR₂ constructs with their proteolytically revealed tethered ligand and synthetic activating peptides.

Constructs	Tethered Ligand Sequence	Synthetic Activating Peptide
rPAR ₂ WT	R/SLIGRLDTP....	SLIGRL-NH ₂
rPAR ₂ LSIGRL	R/LSIGRLDTP....	LSIGRL-NH ₂
rPAR ₂ SLAAAA	R/SLAAADTP....	SLAAAA-NH ₂
rPAR ₂ AAIGRL	R/AAIGRLDTP....	AAIGRL-NH ₂
rPAR ₂ AAAAAA	R/AAAAADTP....	AAAAAA-NH ₂



Differential MAPK signaling by trypsin-revealed TL sequences and synthetic PAR-activating peptides.

Reference 1: Hollenberg MD & Compton SJ (2002). *Pharmacol Rev.* 54, 203-17.

Reference 2: Ossovskaya VS & Bunnett NW (2004). *Physiol Rev.* 84, 579-621.

Reference 3: Steinhoff M *et al.*, (2005). *Endocr Rev.* 26, 1-43.

Reference 4: Al-Ani B, Hansen KK, & Hollenberg MD (2004) *Mol Pharmacol.* 65:149-56.

Reference 5: Macfarlane SR *et al.*, (2001). *Pharmacol Rev.* 53, 245-82.

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PC471

Agonist-induced trafficking and cellular localization of the putative cannabinoid receptor GPR55

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The CNS actions of cannabis and related compounds (cannabinoids) are thought to be primarily mediated by the CB1 subtype of cannabinoid receptor. However, an increasing number of functional studies have identified novel cannabinoid-like actions in the brain, which point to the involvement of additional receptors. Recently it has been suggested that several cannabinoid receptor ligands bind to the orphan G-protein coupled receptor GPR55. As GPR55 mRNA is expressed within human brain tissue, this receptor may therefore play a role in mediating some of the CNS actions of cannabinoids. In this study we have assessed

a range of cannabinoid ligands in their ability to provoke agonist-induced trafficking and the cellular localization of recombinant GFP, HA and SEP (pH sensitive GFP) fusions of GPR55. The constructs were transfected into neurons (5–14 days in vitro) or cell lines (HEK293 and COS7). In cell lines, treatment with cannabinoid ligands results in a rapid redistribution of HA and GFP fusions into endosomes, however the pharmacology of GPR55 trafficking differs from that for the classical CB1 receptor. When expressed in neurons GPR55-GFP is associated with the somatodendritic plasma membrane and using an antibody to GFP we find that GPR55 is expressed at the cell surface on both axons and soma/dendrites, whereas CB1 is polarized to the axonal surface. This profile was further established following expression of SEP-GPR55 or SEP-CB1, which reveals receptor expression on the cell surface of live neurons. In conclusion, both CB1 and GPR55 internalize following prolonged agonist exposure, which may contribute to cannabinoid tolerance in vivo. The differing cellular distributions of CB1 and GPR55, suggests that they sub-serve distinct functional roles in the CNS, with the potential for both receptors to act presynaptically, but with GPR55 mediating postsynaptic actions of cannabinoids.

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PC472

Membrane diffusion of the β_3 -adrenoceptor is slowed by agonist stimulation

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The β_3 -adrenergic receptor (β_3 -AR) is one of three G-protein coupled receptors which mediate the physiological effects of the catecholamines adrenaline and noradrenaline. In common with the β_1 - and β_2 -adrenoceptors, the β_3 -AR mediates its effects through activation of stimulatory (G_s) G-proteins to increase cellular levels of cAMP (Arch, 2001). However, unlike these closely related receptors, the β_3 -AR is resistant to short term desensitisation and internalisation following agonist exposure (Strosberg, 1997). Here, we have investigated the effect of agonist exposure on the diffusion of the β_3 -AR in the membrane microdomains of individual cells using fluorescence correlation spectroscopy (FCS).

The β_3 -AR was fused via its C-terminus to eGFP in the vector pcDNA3.1. This vector was transfected transiently into Chinese hamster ovary cells (CHO- β_3 GFP cells) using Lipofectamine, according to manufacturer's instructions. Cells were exposed to the β_3 -AR agonist isoprenaline (10^{-5} M) or the antagonist ICI118551 (10^{-5} M) at 37°C as indicated, prior to FCS measurements being taken on the upper cell membrane, as described previously (Briddon et al., 2004). Measurements were taken for 2x15s following a 15s pre-bleach, and autocorrelation data analysed in Zeiss AIM software.

Autocorrelation curves from FCS measurements on the upper cell membrane of CHO- β_3 GFP cells were best fit assuming two diffusion components ($\tau_{D1} = 396 \pm 17 \mu s$ and $\tau_{D2} = 82.6 \pm 6.4 ms$, $n=52$). Of the total diffusing receptor population, approximately 60% was present as the faster-diffusing component (Particle numbers for $\tau_{D1} = 37.1 \pm 4.6$ and for $\tau_{D2} = 53.0 \pm 6.4 \mu m^{-2}$). As shown in Table 1, exposure to isoprenaline for 60 min caused an increase in τ_{D1} , indicating a slowing of receptor diffusion. This was preceded by an increase in the number of receptors showing that slower diffusion time. In contrast, antagonist exposure had no significant effect on receptor diffusion.

These results indicate that, whilst the β_3 -AR may not internalise readily, agonist stimulation causes a decrease in the diffusivity of the receptor within the cell membrane. This slowing could be a result of receptor aggregation, or possibly agonist-induced movement of receptor between differing membrane compartments.

Table 1. Diffusion of the β_3 -AR in CHO cell membranes

	Component 1		Component 2		n
	τ_{D1} (μs)	N (μm^{-2})	τ_{D2} (ms)	N (μm^{-2})	
Control	483 $\pm$ 29	61.1 $\pm$ 10.3	83.0 $\pm$ 11.3	90.6 $\pm$ 12.2	17
Isoprenaline (30 min)	573 $\pm$ 36	95.3 $\pm$ 17.2 [*]	127.9 $\pm$ 18.0	105.3 $\pm$ 15.9	13
Isoprenaline (60 min)	691 $\pm$ 85 ^{**}	74.0 $\pm$ 15.5	132.2 $\pm$ 30.6	78.7 $\pm$ 6.9	12
ICI118551 (60 min)	445 $\pm$ 29	49.5 $\pm$ 7.7	86.9 $\pm$ 9.2	69.3 $\pm$ 13.6	9

Data are shown as mean $\pm$ s.e. mean, with 'n' indicating the number of cells measured. * $P < 0.05$ and ** $P < 0.01$ vs. control (one-way ANOVA with post-hoc Neuman-Keuls)

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC473

Potential role of M3-muscarinic receptor phosphorylation in fear conditioning memory in Mouse

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Almost all G-protein coupled receptors (GPCRs) undergo rapid phosphorylation upon activation by agonists. These phosphorylation events occur at multiple sites on the receptor (mostly on serine and threonine) and can result in receptor G-protein uncoupling, receptor internalization and activation of G-protein independent pathways. The G-protein coupled receptor kinases (GRKs) have classically been involved in this process, but other families of kinases have recently been implicated in this phenomenon. In the case of the M3-muscarinic receptor, the kinases known to be involved in its agonist-dependent phosphorylation include GRK2 and GRK6 but also CK1 and CK2.

In order to investigate the role of phosphorylation in the M3-muscarinic receptor signalling, a knock-in Mouse model was designed, in which the M3 receptor was mutated on 15 serines within its third intracellular loop. These M3 receptor phosphorylation-deficient animals presented a deficiency in fear conditioning memory. When the Mice were confronted to a stressful event announced by a warning tone and then subjected again to the same warning tone 24h later, the M3 knock-in animals did not freeze in anticipation after hearing the second tone as much as the wild type animals did. This finding led us to focus our study on the Hippocampus, a region of the brain known to be involved in formation of new memories and learning, and particularly to further assess the potential differences in M3 receptor signalling between the knock-in and the wild type animals in terms of M3 receptor distribution, phosphorylation pattern, coupling to downstream effectors and potential involvement in the modulation of synaptic transmission in the Hippocampus.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC474

Nuclear β -adrenergic and endothelin receptors regulate RNA synthesis in heart cells

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The β 1-adrenergic receptor (β 1-AR), the β 3-AR, the endothelin A receptor (ETAR) and the ETBR are present on nuclear membranes in adult ventricular myocytes. These nuclear-localized receptors are functional with respect to ligand binding and effector activation. Furthermore, β -AR agonists isoproterenol (β -AR) and BRL37344 (β 3-AR), but not Xamoterol (β 1-AR), stimulated de novo RNA synthesis, which was PTX sensitive. In contrast, ET-1 decreased the amount of RNA synthesis. Employing enriched nuclear preparations, we investigated which signaling pathway(s) might be involved in GPCR-mediated changes in RNA synthesis. Accordingly, inhibitors of p38 (SB203580), Erk (PD98059, U0126), JNK (SP600125), PI3K (LY294002, Wortmannin) and Akt (Triciribine) were used. Inhibition of Akt, in the presence of isoproterenol, converted the activation of the β -AR from stimulatory to inhibitory in regards to RNA synthesis. Inhibition of Erk, p38 or JNK had no appreciable effect. In contrast, the inhibitory effect of ET-1 was abrogated by Erk inhibition. Western blotting of the enriched nuclear preparations revealed the presence of both the total and phosphorylated forms of Erk and Akt. Furthermore, transcription assays and western blots also confirmed that this signaling cascade is under strong phosphatase control. Treatment of isolated nuclei with microcystin LR, a potent inhibitor of type I and IIA protein serine/threonine phosphatases, almost doubled the observed level of RNA synthesis under basal conditions and following β -AR agonist treatment. Additionally, higher levels of Erk phosphorylation were detected in the presence of microcystin. These results demonstrate that two nuclear-localized GPCRs have markedly

different effects upon transcription, and suggest that these two receptor families have distinct roles in the regulation of gene expression in cardiomyocytes.

Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, Trieu P, Allen BG, Hébert TE.

Functional beta-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes.

Cardiovasc Res. 2006 Mar 24;

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PC475

Determining G-protein coupled receptor activation in high and medium throughput screens

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With increasing interest in higher throughput techniques applied to basic research we have demonstrated the ability to detect and characterise activation of GPCRs in an assay which is fully scaleable. Monitoring changes in intracellular calcium with fluorescent dyes using

FLIPRETETRA® is a powerful assay for G-Protein Coupled Receptors and is widely used in primary screens. The imaging of beta arrestin-mediated GPCR desensitization with TransfluoR® using imagers like ImageXpress™ is an enabling tool for the functional analysis of orphan GPCRs and GPCRs with weak calcium signaling.

Using the Angiotensin receptor and a novel red calcium dye, we previously demonstrated that the advantages of both TransfluoR and FLIPR® can be obtained at once in a convenient combination assay using the same cells in the same well. The assay is homogenous and the TransfluoR assay is read on plates fixed immediately following the calcium flux assay. After fixing, the plates can be stored for extended periods of time before imaging therefore uncoupling primary and secondary screen activities. In this study, we looked for agonists and antagonists of the Angiotensin receptor in the LOPAC1280™ library and were able to quickly characterize the FLIPR hits by observing the TransfluoR response in the same cells. For example, non-specific calcium flux caused by compounds targeting endogenous receptors or calcium signalling independent of target receptor could be qualified with TransfluoR; which is independent of calcium signaling and requires over-expression of the target receptor. These experiments also further validate the equivalent pharmacology of the receptor in FLIPR and TransfluoR assays. For high throughput screens, this assay should both help decrease cost and increase speed of the compound characterization process, whilst the imaging of beta arrestin-mediated GPCR desensitization with TransfluoR® provides an ideal first step in smaller screens.

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PC476

Assessment of the functional antagonist potency of tiotropium, atropine and ipratropium in the human isolated bronchus

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The once a day muscarinic antagonist tiotropium bromide (SpirivaTM) is an established bronchodilator therapy for the treatment of COPD. There have been few reports documenting antagonist affinity in functional assays in the human isolated bronchus. In this report we have studied the effect of incubation time on the functional antagonist potency of tiotropium compared with ipratropium and atropine.

Bronchial rings of internal diameter 1-7mm were suspended in 15ml tissue baths under an initial resting tension of 2g and bathed in warmed (37°C), aerated (95%O₂/5%CO₂) Krebs solution. Following a 2h equilibration period two cumulative concentration effect curves (CEC) to carbachol (CCh) were constructed 1h apart. The second CEC served as the control curve. Antagonists were incubated with the tissues for 1h, 2h or 4h before a 3rd CEC to CCh was carried out. Concentration ratios (CR) were calculated and used to calculate pKB values (Gaddum 1957). In some experiments only 2 concentrations of the surmountable antagonists could be tested therefore the average pKB value was quoted. For non-surmountable antagonists a single pKB value was calculated at the lowest effective (CR>2.0) concentration. Data for pKB values are arithmetic means $\pm$ 95% confidence intervals (C.I.). Statistical analysis was performed using unpaired t test.

Atropine and ipratropium bromide produced concentration related rightward shifts in CCh CECs with no suppression of agonist maximum responses at the times and concentrations studied. Atropine gave an average pKB of 9.1(8.7-9.5) n=4 and 9.0 (8.5-9.4) n=5 following 1h and 2h incubation respectively. These values were not significantly different (p>0.05). Ipratropium gave average pKB values of 9.7(9.0-10.4)n=3 at 1h and 9.0(8.4-9.6)n=3 at 4h (p<0.05). Tiotropium bromide exhibited non-surmountable antagonism with suppression of the agonist maximum at all effective concentrations studied. At 1h the average pKB = 10.1(9.2-11.0)n=4; at 2h =10.8(9.5-12.2)n=4 and at 4h =10.2(8.8-11.5)n=3. Incubation time had no influence on functional potency (p>0.05).

Atropine and ipratropium were surmountable antagonists at the concentrations and incubation times studied. Potency was not increased on extended incubation. Tiotropium was a non-surmountable antagonist and its pKB was not increased by extending the incubation time from 1h to 4h. Tiotropium was more potent than atropine at 1h and 2h and ipratropium at 4h (p<0.05). Incubation times of 1h were sufficient for the system to be at equilibrium for tiotropium.

Gaddum, JH. (1957) *Pharmacol. Rev.*, 9: 211-218.

PC477

Novel scorpion probes for nucleic acid sequences based on target-assembled exciplexes

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Scorpions consist of a specific DNA probe sequence held in a hairpin loop with a particular three-dimensional arrangement (1)(2). Scorpion probes were modified to carry the components of an exciplex to allow their use in a new fluorescence-based method for specific detection of DNA sequences (3). The model of Scorpion-probe systems in this study consisted of a Scorpion target bearing one exciplex partner on the 5'-phosphate site, and the second oligonucleotide probe bearing the other exciplex partner on its 3'-phosphate site. The exciplex partners chosen (5'-pyrenyl and 3'-naphthalenyl) were attached to the oligonucleotide via a phosphoramidate link to the terminal phosphate groups (Fig. 1). Hybridisation led to adjacent binding of the 5'-Scorpion-pyrenyl and exci-probe-3'-naphthalenyl exciplex partners registered as an obvious spectral change on excitation from blue (pyrene locally excited state) fluorescence emission (λ_{max} = 378, 397 nm) to green, exciplex emission (λ_{max} = 466 nm) in 10 mM Tris, 0.1 M NaCl, pH 8.4 buffer containing 32% (v/v) TFE at 5 °C. TFE was sequentially added (19.6 %, 32 % and 41 %, v/v) and emission spectra taken after each addition. This addition gave rise to an increase in intensity at 466 nm for the all concentrations, and was largest for 32 % TFE (Fig. 2). This increase in fluorescence intensity can be attributed to exciplex formation by the pyrene-naphthalene exciplex. The shape (broad) and position (466 nm) of the band is typical of pyrene-naphthalene exciplex emission (3); the intensities of the monomer (LES) bands were quenched and the 466 nm band concomitantly increased in intensity. When the oligonucleotide probe bearing the naphthalenyl group was replaced by an identical one but with no naphthalenyl group on its 3'-phosphate site, no exciplex band was detected at 466 nm (Fig. 2). This indicates that the second exciplex partner is needed for exciplex emission at 466nm. This supports the observation that the emission detected in this study results from interaction between the exciplex partners, and the interpretation that the exciplex signals detected in the full systems of this study occur from correct hybridisation of the probe oligonucleotide on the Scorpion target strand and interaction of the exci-partners, rather than pyrene or naphthalene interacting with the nucleotide bases. These results show that it is possible to use Scorpion exciplex probes for detection of oligonucleotide sequences, with potential for future applications in genetic testing and molecular diagnostics.

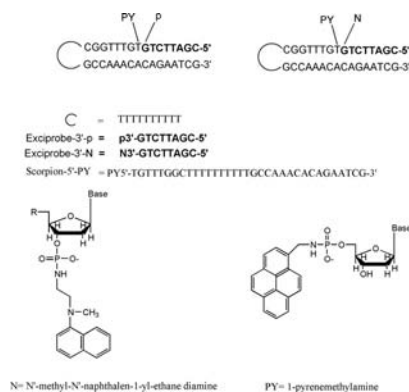


Figure 1.

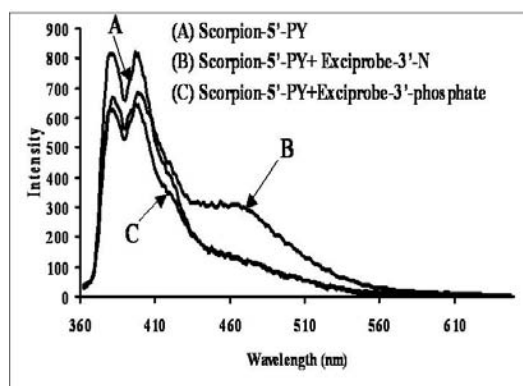


Figure 2.

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PC478

CXCR2 antagonist enhanced DOR agonist function via an allosteric mechanism

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Opioid and chemokine receptor distribution has been described to overlap in white blood cells such as neutrophils and monocytes. Increasing evidence also suggests that opioid ligands can alter immune response by modulating chemotaxis and that chemokine receptors play a role in neurone migration.

In this study we have shown that the DOR and CXCR2 receptors can form heterodimers using techniques including co-immunoprecipitation, FRET and BRET. We then applied the DimerscreenTM technology to investigate DOR/CXCR2 heterodimer pharmacology. This system is based on a complementary pair of mutated fusion proteins, MycCXCR2^{I148E}G₁₂^{C351I} and FlagDOR₁₂^{G204AC351I} that are unable to signal unless the receptors form a dimer. This approach allowed us to investigate selectively CXCR2/DOR heterodimer response to ligands. We observed that the CXCR2 antagonist SB225002 enhanced the function of agonists at DOR when this receptor was co-expressed with CXCR2 but had no effect on DOR when this receptor was expressed separately. This 'off target' allosteric effect was observed for both peptidic and non-peptidic opioid agonists.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC479

Different regulation of serotonin_{1A} receptor signalling by Mn²⁺- and Mg²⁺-ions

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Serotonin (5-hydroxytryptamine) is an important neurotransmitter in organisms, regulating for example mood, thermoregulation, also functioning of muscles and heart. Serotonin_{1A} receptors (5-HT_{1A} receptor) belongs to the superfamily of seven-transmembrane G-protein coupled receptors (GPCR) and is widely distributed in brain, the highest densities are found in hippocampus, septum, amygdala and cortical limbic areas. 5-HT_{1A} receptors couple to G_{i/o}-proteins, resulting in decreased levels of intracellular cyclic adenosine monophosphate. Signalling through 5-HT_{1A} receptor has been linked to mood, learning, memory, eating and sexual behavior.

It has been generally accepted that Mg²⁺-ions participate in signal transduction via GPCR-s, where Mg²⁺-ions are required to achieve high affinity agonist binding and regulate functions of G proteins. Other ions of this group (Mn²⁺, Al³⁺, Zn²⁺ etc) have been considered to act on different biochemical pathways and on toxic doses changing the pattern of reaction via altering, inhibiting or activating different biochemical reactions. The modulatory effect of Mn²⁺ has been found also in the case of 5-HT_{1A} receptors.

Herewith we have found that effects of Mn²⁺ and Mg²⁺-on regulation of agonist binding to 5-HT_{1A} receptors can be very similar, but also different, depending on the region of rat brain studied. These ions did not alter binding properties of 5-HT_{1A} antagonist [³H]WAY-100635, but Mn²⁺-ions enhanced the ability of serotonergic agonists to compete with [³H]WAY-100635 in hippocampal membranes compared to Mg²⁺-ions. Mn²⁺-ions were more effective in enhancing of the binding of 5-HT_{1A} agonist [³H]8-OH-DPAT, but also in changing the abilities of nucleotides to turn the receptors into low-affinity agonist binding state. The biggest differences was found in hippocampal membranes, whereas effects in cortical membranes and in membranes prepared of Sf9-cells (with expressed 5-HT_{1A} recep-

tor and Gi-protein) were considerably smaller. No ion-specific effect was seen in Sf9 membranes expressing 5-HT_{1A} receptor alone or 5-HT_{1A} receptor with Gs-proteins. These results indicate that signal transduction via 5-HT_{1A} receptor in different parts of brain is mediated by different regulation systems, which also vary by their sensitivity towards Mn²⁺- and Mg²⁺-ions.

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PC480

Characterisation of α 1-adrenoceptors mediating relaxation of rat gastric fundus

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Although it is well known that postjunctional α 1-adrenoceptors mediate relaxation in the gastrointestinal tract there is little information on the subtypes involved. The aim of this study was to determine the subtypes of α 1-adrenoceptor mediating smooth muscle relaxation in the rat isolated gastric fundus.

Strips of longitudinal smooth muscle from the fundic region of the rat stomach were prepared via the method of Vane (1957) and suspended in organ baths containing physiological saline solution (PSS) for isometric recording using a Powerlab with Chart 5 software. The PSS was maintained at 37°C and gassed continuously with 95% O₂ and 5% CO₂.

Smooth muscle strips were pre-constricted using barium chloride (1 mM) and cumulative concentration curves (CRC) to the non-selective α 1-adrenoceptor agonist, phenylephrine, obtained. The effects of incubation of the tissue with various α 1-adrenoceptor antagonists on the phenylephrine concentration-response curves were investigated: prazosin (non-selective), 5-methylurapidil (α 1A), BMY 7378 (α 1D) and indoramin (α 1A and α 1L). Shifts of the phenylephrine CRC were calculated for each of the antagonists and Schild plots constructed, where possible. pKB values were obtained from the Schild plots or from the Schild equation.

Prazosin and 5-methylurapidil antagonised phenylephrine-induced relaxation with a pKBs of 8.1 and 8.0 respectively, indicating action of phenylephrine at a low affinity α 1-adrenoceptor (α 1L-). BMY 7378 at concentrations up to 1 μ M failed to inhibit phenylephrine-induced relaxation, consistent with a lack of effect of phenylephrine at α 1A, α 1B or α 1D adrenoceptors. Indoramin produced inhibition of phenylephrine with a pKB of 8.42, consistent with the published values of indoramin for α 1A and α 1B.

These results support the conclusion that α 1L-adrenoceptors mediate relaxation to phenylephrine in rat gastric fundus.

Vane, J.R. (1957) Br. J. Pharmacol. Chemother., 12, 344-349.

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PC481

RACK1 regulates the trafficking of the G protein-coupled receptor for thromboxane A₂

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The thromboxane A₂ receptor (TP) is a G protein-coupled receptor implicated in the regulation of pharmacological events such as platelet aggregation as well as constriction and proliferation of vascular and bronchiolar smooth muscle cells. This receptor is expressed as two alternatively spliced isoforms (TP α and TP β) which shares the first 328 amino acids. Previous studies have demonstrated that alternative splicing of the C-tail of TP generates isoforms of the receptor showing distinct internalization characteristics. In order to identify putative proteins which are implicated in this specific regulation, we used the yeast two-hybrid system to screen for proteins that interact with the C terminus of the β isoform of the receptor (TP β). This screen identified Receptor for Activated C-Kinase 1 (RACK1) as a new TP β -interacting protein. Here, we show that RACK1 directly binds to the C-terminus and the first intracellular loop of TP β . We demonstrate with co-immunoprecipitation studies in HEK293 cells that there is a constitutive RACK1-TP β association, which is not modulated by stimulation of the receptor. We observed that cell surface expression of TP β was promoted when RACK1 was overexpressed, while it was inhibited when endogenous RACK1 expression was knocked down by siRNA. Our data suggest that the effect of RACK1 on the export of receptors is specific to some GPCRs since inhibition of RACK1 expression also affected cell surface expression of the angiotensin II type 1 receptor and CXCR4 but not of β ₂-adrenergic and prostanoid DP receptors. We also report that overexpression of RACK1 promoted, while knockdown of RACK1 by siRNA reduced, the agonist-induced internalization of TP β . Confocal microscopy revealed that RACK1 predominantly co-localizes with TP β at the plasma membrane in non-stimulated HEK293 cells and that agonist stimulation promoted the RACK1-TP β co-localization in intracellular compartments. Impaired cell surface expression of TP β in RACK1-depleted cells was also confirmed by confocal microscopy. Our data demonstrate for the first time a direct interaction between RACK1 and a GPCR and identify a novel role for RACK1 in the regulation of GPCR cell surface expression and internalization.

Parent *et al.* (1999). *J. Biol.Chem.*, **274**, 8941-8948

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PC482

Development of a non-imaging, homogenous assay format for analysis of arrestin recruitment as a detection method for generic GPCR screeningA. Fowler¹, T. Wehrman², K. Peng², D. Bassoni² and K. Olsen²¹Discoverx, Birmingham, UK and ²Discoverx, Fremont, CA, USA

Most approaches for GPCR screening involve detection of second messengers in cell-based assays, which differ depending on the receptor coupling mechanism. A generic approach is highly desirable for primary screening, and much interest has focused on β -arrestin for this purpose, particularly with the availability of imaging methods to follow arrestin-GFP fusion proteins. We have developed a generic cell-based screening approach using a modified form of enzyme fragment complementation (EFC) that allows researchers to detect GPCR interactions with β -arrestin. In this approach, the GPCR of interest is expressed with a short C-terminal peptide tag in a clonal cell background expressing a β -arrestin-EA fusion protein. The peptide tag has a weak affinity for the EA fragment, and complementation is only possible when β -arrestin binds to an activated GPCR and brings the two EFC components together. Data will be presented for Gi, Gs and Gq-coupled GPCRs analyzed with this novel screening approach.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC483

A genetic approach to study the function of latrophilin in *C. elegans*

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Black widow spider venom (BWSV) is known to cause neurotransmitter release from nerve terminals due to its content of high molecular weight proteins, the latrotoxins (LTX), that are known to bind with high affinity to three neural proteins in mammals. We have established *C. elegans* as a model organism to study the function of the binding protein, latrophilin (a member of the class B family of G-protein coupled receptors), and its role in regulating neurotransmitter release by latrotoxins, by showing that latrophilin is required for lethality of BWSV by RNAi experiments. However, a latrophilin-knockout worm is required for determining the function of the latrophilin gene. The lat-1(ok1465) allele has a deletion of the lat-1 gene, and we know that ~97% of lat-1(ok1465) homozygous worms arrest or die before adulthood, with only 3 adult offspring per animal; moreover, there is strong interaction between the lat-1 allele and various G-protein mutants. However, it is unclear if this lethality is due to the deletion in the lat-1 gene, or to adventitious mutations introduced during the process of mutagenesis to create this allele. When transgenic worms were created with the B0457 cosmid (containing the full sequence of the lat-1 gene), they rescued the lethality of lat-1(ok1465) worms, whilst concurrent injections with the unc-54::C marker plasmid yielded six surviving adults from >80 transgenic embryos, showing that the

rescue was not due to the unc-54::C. Our results show that the lat-1(ok1465) allele causes developmental lethality and also an increase in defecation cycle timing, and that the B0457 cosmid can rescue both these defects. This is strong evidence that the lat-1 gene is responsible for these defects. Current work aims to rescue the lat-1(ok1465) defects with a lat-1 cDNA construct, and to determine the role of this gene in mediating the toxicity of Black Widow Spider venom.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC484

Localisation of the chemokine MIP-1 β to human arteries and veinsK.L. Jones¹, R.E. Kuc¹, S. Butler¹, C. Holt¹, E.J. Mead¹, S.D. Katugampola², J.J. Maguire¹ and A.P. Davenport¹¹Clinical Pharmacology Unit, University of Cambridge, Cambridge, UK and ²Pfizer Global Research and Development, Sandwich, Kent, UK

Chemokines, or chemotactic cytokines, are a family of cytokines that act as attractant molecules for leukocytes and which have an emerging role in atherosclerosis (Reape and Groot 1999). The chemokine receptor CCR5 has been identified in vascular smooth muscle cells (Schechter *et al.*, 2000), perhaps suggesting ligands of this receptor, such as MIP-1 β (macrophage inflammatory protein-1 β , or CCL4), may also have a vascular localisation. MIP-1 β is chemoattractant for monocytes and T-cells and has a potential role in atherosclerotic lesion development. This is supported by localisation studies in animal models (Lutgens *et al.*, 2005) however localisation in human vasculature has yet to be characterised. The aim of this study was therefore to investigate the distribution of MIP-1 β in a panel of human vessels.

Messenger RNA (mRNA) was extracted from the smooth muscle layer of human vessels including saphenous vein and aorta and reverse transcribed. PCR was performed with MIP-1 β specific primers as used by Krzysiek *et al.*, 1999. For immunocytochemistry, 30 μ m sections of human vessels including saphenous vein, normal and diseased coronary artery, left internal mammary artery, radial artery and aorta, were incubated with rabbit anti-MIP-1 β antisera (Abcam plc, UK) and detection was via an avidin-biotin method. PCR products corresponding to the expected size for MIP-1 β cDNA were detected in arterial and venous smooth muscle, indicating the cellular expression of the MIP-1 β transcript. Expression at the protein level was shown by immunocytochemical staining in all tissues tested.

These data demonstrate the expression of MIP-1 β at both the mRNA and the protein level in all vessels investigated, suggesting widespread vascular distribution. This builds upon previous work in animal tissues to indicate a potential role for MIP-1 β in human atherosclerosis.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC485

Desensitisation of the nicotinic acid receptors

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The lipid-lowering qualities of nicotinic acid, a B3 vitamin, have long been exploited to prevent cardiovascular disease and its associated mortality. Fifty years on from its arrival on the market, the receptors on which this drug acts upon have recently been identified as the 'Nicotinic Acid Receptors'.

A family of three highly homologous G-Protein Coupled Receptors (GPCRs), HM74, HM74A and GPR81 couple to the Gi class of G-proteins and are characterised by their differing affinities for nicotinic acid. HM74A has been identified as the high affinity receptor activated by μM concentrations; HM74A > HM74 > GPR81. HM74 and HM74A differ by only 15 base changes at the DNA sequence level, resulting in a shorter C-terminal tail in the HM74A receptor.

Despite the widespread availability and clinical use of nicotinic acid, the clinical target and precise molecular mechanism by which nicotinic acid acts remains elusive.

In this project, we have chosen to examine potential differences in the regulation of the high and low affinity receptors. C-terminal tail swaps, C-terminal truncations and site directed mutagenesis studies have been adopted to characterise desensitisation mechanisms. Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, Eilert M, Ignar DM, Murdock PR, Stepkowski K, Green A, Brown AJ, Dowell SJ, Szekeres PG, Hassall DG, Marshall FH, Wilson S, Pike NB.

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I would like thank BBSRC and GSK for funding my project and Prof. Graeme Milligan for his supervision.

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PC486

Atheroma: Evaluation by Positron Emission Tomography

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Atheroma is ubiquitous in man and rupture of the plaque is the principal cause of most heart attacks and stroke. Initiating early in life, development of the plaque is protracted and progress is dependent upon a variety of genetic and extrinsic factors. The pathogenesis of atheroma is poorly elucidated, although it is becoming increasingly apparent that inflammation, driven by

the accumulation of macrophages at the lesion site, is the principal cause of instability and rupture.

Previously, we have demonstrated the potential of positron emission tomography (PET) to image inflammatory activity in both carotid atheroma in humans (1) and aortic atheroma in a rabbit model using [¹⁸F]-fluorodeoxyglucose (FDG), a marker of elevated metabolic activity (2). However, FDG accumulates in all living cells including vascular smooth muscle cells, reducing the specificity of PET signal for the inflammatory component of the plaque. A PET-labelled marker specific to macrophages would enable more accurate quantification of macrophage presence. The peripheral benzodiazepine receptor (PBR) is a unique class of diazepam receptor which is functionally distinct from the central diazepam receptor and performs a number of context-dependent functions including cholesterol transport. It is highly expressed in a number of tissues and cell types, including immune cells such as the macrophage. In vivo imaging of the PBR is possible by PET using recently developed radioligands including [¹⁸F]-FEDAA1106; N-(5-fluoro-2-phenoxyphenyl)-N-(2-[¹⁸F]-fluoroethoxy-5-methoxybenzyl)acetamide (3). Confirmation of the specificity of [¹⁸F]-FEDAA1106 for PBR expressed in activated macrophages in the atherosclerotic plaque is a necessary prequel to evaluating the efficacy of this PET radioligand in a rabbit model of atheroma. We have evaluated the binding of [¹⁸F]-FEDAA1106 to human and rabbit tissues known to express PBR and have shown specific binding ($K_d \approx 1.0\text{nM}$) in both species. Analysis of [¹⁸F]-FEDAA binding in human carotid and rabbit aortic atheroma *ex vivo* indicates that this radioligand preferentially binds to sites within the plaque (Fig. 1), correlating with macrophage presence (CD68) and PBR expression (determined by immunohistochemistry). The successful conclusion of these experiments will generate an efficacious clinical tool for determining atheroma instability.

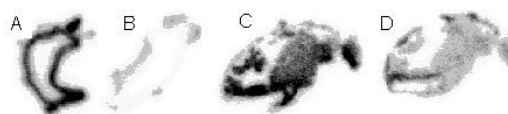


Fig. 1. [¹⁸F]-FEDAA1106 (0.5nM) binding to rabbit (A, B) and human (C, D) atheroma in the absence (A, C) and presence (B, D) of non-radioactive FEDAA (12 μM).

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PC487

Effect of A_{2A} adenosine receptor (A_{2A}AR) gene deletion on pro-inflammatory signalling pathway activation in aorta

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The A_{2A}AR is a critical non-redundant suppressor of inflammatory responses *in vivo*. While the receptor is known to elevate

cyclic AMP, the molecular mechanisms underlying its anti-inflammatory effects remain obscure. To address this, we have assessed activation of the NF- κ B and JAK-STAT pathways in aortas isolated from control and A_{2A}AR^{-/-} mice following treatment with or without LPS in vivo. While circulating cytokine levels were undetectable in vehicle-treated WT and A_{2A}AR^{-/-} animals, LPS-treated A_{2A}AR^{-/-} animals displayed significantly elevated levels of IL-1, IL-6, TNF α and GM-CSF compared with LPS-treated controls, consistent with a suppressive effect of A_{2A}AR expression on inflammatory responses. Interestingly, levels of total and IKK-phosphorylated I κ B α in isolated aortic extracts were significantly elevated in vehicle- and LPS-treated mice compared with WT animals, while total levels of RelA were unaffected. In addition, levels of tyrosine phosphorylated STAT1 and STAT3 were significantly elevated in aortic extracts from LPS-treated A_{2A}AR^{-/-} versus LPS-treated WT animals while total levels of each transcription factor remained unchanged. Together, these data suggest that major roles of the A_{2A}AR in vivo are to A) suppress NF- κ B pathway activation at the level of IKK-mediated phosphorylation of I κ B and B) limit activation of the JAK-STAT pathway by restricting cytokine-triggered tyrosine phosphorylation of STATs.

This work was supported by project grants from the BHF and Heart Research UK, and a PhD studentship from the BBSRC.

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PC488

Modulation of inflammation-dependent transferrin receptor expression by A_{2A} receptor agonists in macrophages: role of HIF1 and NF- κ B

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Inflammation causes several changes in body iron homeostasis, including iron sequestration in the reticuloendothelial system with ensuing hypoferraemia and anemia of chronic disease. A number of inflammatory stimuli have been shown to modulate macrophage iron homeostasis but little is known about the regulation of iron metabolism by anti-inflammatory signals. Adenosine accumulates extracellularly in inflamed areas where it acts as a reporter of tissue damage and activator of anti-inflammatory pathways. We have found that adenosine activates hypoxia inducible factor (HIF1) under normoxic conditions in macrophages (1). HIF1 is a transcription factor that in response to hypoxia turns on the expression of genes important for angiogenesis, glycolysis, cell survival, etc. On the other hand, HIF1 is activated also by typical inflammatory stimuli, such as LPS (2). As we and others have shown that transferrin receptor (TfR) expression is upregulated by HIF1 (3,4), the aim of this study was to understand the interplay between inflammatory and anti-inflammatory modulators on HIF1-mediated TfR expression. Therefore, we investigated the effect of adenosine and LPS/IFN γ on HIF-1-mediated activation of TfR gene expression in macrophages.

The expression of TfR was evaluated in mouse macrophage J774A cells exposed to adenosine or specific adenosine receptor agonists/antagonists. Adenosine, through the A_{2A} receptor-dependent pathway, dose-dependently increased TfR protein and mRNA levels and resulted in higher iron uptake. Transfection experiments with a reporter gene under the control of the TfR promoter, DNA and RNA bandshift analysis of HIF1 and iron regulatory proteins binding activity, respectively, indicated a role for transcriptional mechanisms.

We also found that exposure of J774A cells to LPS/IFN γ transiently and markedly induced TfR expression at the transcriptional level by means of HIF1 activation. Interestingly, pre-treatment of cells with adenosine prevented the LPS/IFN γ -mediated effect on HIF1 and the consequent increase of TfR expression. Ongoing experiments aimed at understanding the molecular basis of this inhibitory effect of adenosine seem to indicate a role for NF- κ B, which transcriptionally controls HIF1 (5) and whose LPS/IFN γ -mediated activation is blunted by adenosine.

Post-transcriptional down-modulation of TfR levels has been previously reported in macrophages exposed to LPS/IFN γ , the present data reveal novel mechanisms of transcriptional control of TfR expression regulated by an interplay of inflammatory and anti-inflammatory mediators. The consequent changes of TfR expression could be functionally involved in modulating iron retention in macrophages under inflammatory conditions.

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PC489

Regulation of extracellular adenosine level by equilibrative nucleoside transporters and ecto'5'-nucleotidase in inflammation

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Inflammation of endothelium is closely associated with the development of atherosclerosis. Extracellular adenosine levels are increased at the sites of inflammation. Such adenosine increase may serve to protect tissues from injury and potentially slow down the progression of atherosclerosis because adenosine is known to possess anti-inflammatory property. However, until now, the mechanism leading to the increased extracellular adenosine level during inflammation has not been understood. Two proteins are suspected to play roles in this mechanism: 1) equilibrative nucleoside transporters (ENTs), which control the rate of adenosine transport across cells; 2) ecto-5'-nucleotidase (ecto-5'Nu), which metabolizes extracellular adenosine monophosphate into adenosine. In our study, lipopolysaccharide (LPS) was used to induce inflammation in human umbilical vein endothelial cells (HUVEC). The results from RT-PCR and [3H]adenosine transport assay revealed that the expressions and activities

of ENTs in HUVEC were not altered by LPS stimulation. In contrast, biochemical assay demonstrated that the ecto-5'Nu activity in HUVEC was increased after incubation with LPS for 24 hours. This effect was dose-dependent, with an IC50 value of 1 ng/ml. In addition, western blot analysis showed that the protein expression of ecto-5'Nu in HUVEC was higher after stimulation by LPS. LY294-002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, was found to reduce the LPS-stimulated ecto-5'Nu activity. Therefore, it is suggested that the increase in extracellular adenosine level during inflammation may be, at least in part, related to the up-regulation of ecto-5'Nu through a PI3K signaling pathway.

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PC491

Xanthine oxidase-mediated calcium responses in human endothelial cells occur independently of enzyme activity

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Xanthine oxidase (XO) catabolises the conversion of purines to uric acid and hypoxanthine (HX)-XO has been used to investigate endothelial cell (EC) cytoplasmic calcium ($[Ca^{2+}]_c$) responses generated to both superoxide and hydrogen peroxide (H_2O_2)^{1,2}. The aim of this study was to investigate $[Ca^{2+}]_c$ responses to HX-XO in human vascular ECs isolated from the saphenous vein (SV) and compare responses to other studies. ECs were isolated from SV obtained surplus to bypass surgery. Cells grown to confluency on glass coverslips were loaded with fura-2. $[Ca^{2+}]_c$ responses were recorded to 3 or 30mU/ml bovine buttermilk XO (Sigma, UK) in the presence of 2mM HX. Histamine (10μM) was used as a comparator. Data are presented as mean±s.e.m.

SVECs responded to XO with $[Ca^{2+}]_c$ responses of $244±38$ nM at 3mU/ml and $345±55$ nM at 30mU/ml (n=5). XO-HX gave an initial peak $[Ca^{2+}]_c$ response followed by either calcium oscillations or a sustained $[Ca^{2+}]_c$ elevation. Histamine gave a $[Ca^{2+}]_c$ response of $683±68$ nM (n=5). EGTA abolished the sustained phase of the response to both XO and histamine but the peak $[Ca^{2+}]_c$ response and oscillations were maintained. Inhibition in the presence of 2-aminoethoxydiphenyl borate (2-APB), U73122 and phorbol 12-myristate 13-acetate (PMA) demonstrated that the XO-induced $[Ca^{2+}]_c$ response in SVEC was mediated by the generation of inositol 1,4,5-trisphosphate and was sensitive to protein kinase C activation. The presence of high levels of catalase but not superoxide dismutase (SOD) or 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) reduced the XO mediated $[Ca^{2+}]_c$ response. However XO-mediated $[Ca^{2+}]_c$ responses were maintained even in the absence of HX or the presence of allopurinol, which both abolished XO activity.

In conclusion, the HX-XO $[Ca^{2+}]_c$ response in SVEC occurs independently of enzyme activity. As our results are similar to those

of other investigators using XO to measure superoxide/ H_2O_2 -mediated EC $[Ca^{2+}]_c$ responses^{1,2} previous results may need to re-interpreted.

Table 1

Treatment	3mU/ml XO (% control)	30mU/ml XO (% control)	Histamine (% control)
EGTA (1mM)	129±22	98±8	93±11
2-APB (100μM)	17±10*	37±14*	16±5*
U73122 (3μM)	29±14*	40±16*	6±2*
PMA (1μM)	2±2*	16±4*	28±6
Catalase (1000U/ml)	15±5*	118±14	114±6
SOD (1000U/ml)	120±30	87±10	107±8
TEMPOL (2.5mM)	114±27	94±11	104±8
without HX	95±18	99±16	ND
Allopurinol (1mM)	98±6	106±18	ND

Effect of treatments on average peak $[Ca^{2+}]_c$ responses (n=3) in the presence of XO-HX or histamine. *Significantly (p<0.05) less than control by t-test.

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PC492

Resveratrol protects against irradiation-induced oxidative organ damage in rats

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The generation of the reactive oxygen metabolites plays an important role in the pathogenesis of irradiation-induced tissue injury. Resveratrol, a polyphenolic phytoalexin, is one of the most extensively studied natural products, with wide ranging biological activity and tremendous clinical potential (1). First identified from fruits and plants, in particular grapes and wines, its most noticeable effects are its anti-thrombogenic, anti-inflammatory, cardio-protective, neuro-protective, anti-aging, and cancer preventive and therapeutic activities (2, 3). The objective of this study was to examine the potential radioprotective properties of resveratrol (RVT) against irradiation-induced oxidative damage in the ileum and liver. Sprague-Dawley rats were exposed to irradiation (LINAC producing 6 MV photons at a focus) after a 10-days pretreatment with either saline or RVT (10 mg/kg/day) given orally, and treatments were repeated for 10 more days after the irradiation. Under ketamine anaesthesia (100 mg/kg intraperitoneally), each rat received a single whole-body X-ray irradiation of 800 cGy. Animals were returned to their home cages following irradiation. After decapitation, ileum and liver samples were obtained for the determination of tissue malondialdehyde (MDA; an index of lipid peroxidation) and glutathione (GSH, an antioxidant) levels, myeloperoxidase activity

(MPO; an index of tissue neutrophil accumulation) and collagen contents. Tissues were also examined microscopically. Lactate dehydrogenase (LDH), an indicator of tissue damage, and tumour necrosis factor- α were assayed in serum samples. Irradiation caused a significant decrease in GSH, which was accompanied by significant increases in MDA levels, MPO activity, and collagen content of the tissues studied ($P < 0.05$ - 0.001). Similarly, serum TNF- α and LDH were elevated in the irradiated rats as compared with the control group. On the other hand, RVT treatment reversed all these biochemical indices, as well as histopathological alterations induced by irradiation. Our results suggested that RVT supplementation reduces oxidative damage in the ileal and hepatic tissues probably by a mechanism that is associated with the decreased production of reactive oxygen metabolites and enhancement of antioxidant mechanisms. Thus, it may be suggested that supplementing cancer patients with adjuvant therapy of RVT may have some benefit for a more successful radiotherapy.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC493

The protective effect of resveratrol against chronic nicotine-induced oxidative damage of the urogenital system of the rats

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Resveratrol (3,5,4'-trans-trihydroxystilbene), a natural phytoalexin present in grapes, peanuts, mulberries and red wine, was shown to induce a number of endogenous antioxidants (1). Resveratrol (RVT) has additional pharmacological effects including anti-inflammatory properties, modulation of lipid metabolism and prevention of cancer (2, 3). The anti-inflammatory effect of RVT is related with its inhibitory action on oxidation, leukocyte priming and expression of inflammatory mediators. In our previous studies, we have also demonstrated that resveratrol improves renal damage in ischemia/reperfusion and sepsis-induced oxidative renal injury (4, 5). The present study was aimed to investigate the possible protective effects of RVT against nicotine induced oxidative damage in urinary bladder, corpus cavernosum and kidney tissues. Wistar Albino rats were injected intraperitoneally for 21 days with either nicotine hydrogen bitartrate (0.6 mg/kg/day) or saline along with the oral administration of RVT (10 mg/kg/day) or saline. Then the rats were decapitated and trunk blood was collected. In the serum samples

malondialdehyde (MDA), glutathione (GSH), BUN, creatinine, TNF- α levels and LDH activity were analyzed. Urinary bladder and kidney samples were taken for the measurement of tissue MDA and GSH levels, and myeloperoxidase (MPO) activity. Tissue samples were also examined histologically. Chronic nicotine administration caused a significant decrease in GSH levels and increases in MDA levels and MPO activity in both tissues, suggesting oxidative organ damage, which was also histologically verified. Furthermore, RVT restored the reduced GSH levels, while it significantly decreased MDA levels and MPO activity. Impaired renal function tests and elevated levels of LDH and TNF- α due to nicotine administration were reversed with RVT treatment. The present data suggest that RVT supplementation, possibly by its antioxidant effects, effectively counteracts the chronic nicotine toxicity and attenuates oxidative damage of bladder and kidney tissues, nominating resveratrol as a highly promising agent in avoiding the development of nicotine-induced toxicity in the urinary system.

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PC494

Ghrelin treatment protects against burn-induced pulmonary and gastric oxidative injury in rats

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Title only.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC495

Oxidative stress induces tyrosine phosphorylation of SHIP-1 in primary human T lymphocytes

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The second messenger phosphatidylinositol(3,4,5)tris phosphate is metabolised by both PTEN (a ubiquitously expressed 3' phosphatase) and SHIP-1 (a 5' phosphatase restricted to haematopoietic cells). However reactive oxygen species (ROS) inactivate

PTEN by causing its catalytic cysteine to form a disulphide bond with a neighbouring cysteine residue. ROS are generated in many signalling pathways and are also produced in some antileukaemic chemotherapies and disease states such as rheumatoid arthritis, where they may influence the survival of T lymphocytes.

It has previously been shown that in leukaemic T cell lines that expression of SHIP-1 protects against H₂O₂-induced cell death and promotes activation of the classical NF κ B pro-survival pathway (Gloire *et al.* 2006). Here we demonstrate that in primary human T cells and the leukaemic T cell line CCRF-CEM, SHIP-1 is phosphorylated and recruited to the cell membrane in response to H₂O₂. We further show that transfection of the SHIP-1 negative J6 Jurkat leukaemic T cell line with a constitutively active CD2:SHIP chimera protects against cell death in response to ROS. Finally we examine the role of SHIP-1 in apoptosis caused by cytokine deprivation and Fas ligand, both of which utilise ROS-dependent pathways.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC496

Inhibition of the TCR-mediated activation of naïve murine T cells by Cactus Pear Fruit Extracts

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Introduction

Cactus pear (*Opuntia Ficus Indica*) has recently been acknowledged as a source of bioactive compounds able to act as antioxidants in vitro and to decrease oxidative stress in vivo (1). Here we investigated the effects of extracts from fruits of Red and Yellow cactus pear cultivars on naïve murine lymphocytes, activated by the simultaneous engagement of TCR/CD3 and CD28 receptors.

Material and Methods

Cactus pear fruit extracts were obtained as previously reported (1). Naïve lymphocytes purification and treatment, proliferation assays, IL-2 production and CD69 expression assays were conducted as described by D'Acquisto *et al.* (2).

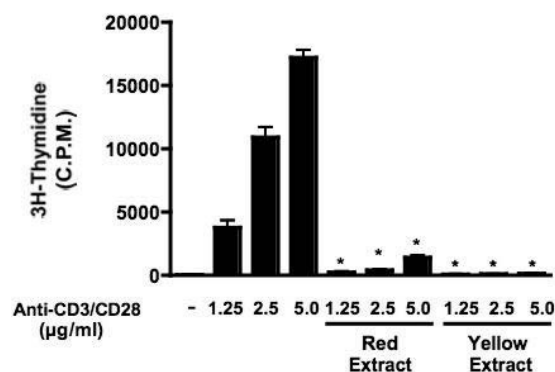
Results

Naïve T cells were stimulated with anti-CD3/CD28 either in the absence, or in the presence, of cactus pear fruit extracts. Anti-CD3/CD28 treatment (1.25 to 5.0 μ g/ml) induced a dose-dependent increase of cell proliferation (Fig.1) as well as IL-2 production (Fig.2). Co-incubation with either Red or Yellow cactus pear fruit extract (330 and 110 mg fresh fruit/ml, respectively) almost abolished cell proliferation, and significantly decreased IL-2 levels at all anti-CD3/CD28 concentrations (Figs. 1, 2). In addition, the expression of CD69, a marker of T cell activation, induced by anti-CD3/CD28 at 5.0 μ g/ml, was reduced by 41.7 $\pm$ 4.5 and 42.3 $\pm$ 4.3%, n=3, P<0.01, when lymphocytes were

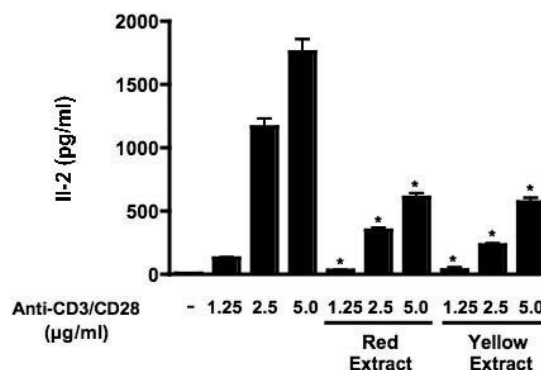
stimulated the presence of either the red or the yellow extracts, respectively.

Conclusions

Extracts from cactus pear fruits significantly counteract the TCR-mediated activation of naïve lymphocytes. A potential use of these natural, non toxic extracts in the treatment of autoimmune diseases, is therefore suggested. In addition, the anti-inflammatory effects on LPS-stimulated macrophages (manuscript in preparation), and the antioxidative protection on human LDL by the betalain components (3), suggest these extracts beneficial in diseases such as atherosclerosis where inhibition of LDL oxidation and modulation of macrophage and lymphocyte response has been proven to be crucial.



Effects of cactus pear fruit extracts, either red or yellow (330 and 110 mg fresh fruit/ml respectively), on anti-CD3/CD28-induced T cell proliferation. Results are the mean $\pm$ S.E. of three independent experiments. *Significantly different from anti-CD3/CD28 treated cells with a P < 0.01.



Effects of cactus pear fruit extracts, either red or yellow (330 and 110 mg fresh fruit/ml respectively), on anti-CD3/CD28-induced IL-2 production. Results are the mean $\pm$ S.E. of three independent experiments. *Significantly different from anti-CD3/CD28 treated cells with a P < 0.01.

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PC497

Altered sulphur amino acid profiles and increased glutathione degradation contribute to the oxidative stress associated with severe acute pancreatitis

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Introduction and Objectives: Glutathione (GSH) is a major intracellular amino thiol which participates in protein synthesis and the detoxification of free radicals (1). GSH metabolism is a complex compartmentalised inter-organ process, relying on the bioavailability of homocysteine (Hcy) and cysteine (Cys). GSH is degraded by membrane-bound gamma-glutamyltranspeptidase (gGT) to form cysteinylglycine conjugates (CysGly) that are involved in the de novo generation of Cys to maintain intracellular GSH levels during oxidative stress (2). GSH depletion is a consistent feature of the progression of a mild to severe attack of acute pancreatitis (AP), and in order to understand glutathione homeostasis during an attack we investigated the temporal relationship between plasma concentrations of the amino thiols Hcy, Cys and CysGly, total erythrocyte GSH (GSht) concentration, and the clinical course of AP.

Methods: Patients with AP were recruited within 24hrs of onset of pain. Peripheral blood (plasma) was analysed at 24hrs and 72hrs after the onset of abdominal pain for Hcy, Cys, and CysGly concentrations using HPLC with fluorescence detection (3). Serum gGT and erythrocyte GSht concentrations were determined using sandwich ELISA. The severity of disease was classified into mild and severe attacks based on the Atlanta criteria. **Results:** In total 41 patients with AP (mild 30, severe 11) were included. Overall Cys, Hcy and CysGly and gGT concentration were similar in the two groups at 24-hrs, although at 72hrs, CysGly levels were markedly higher ($p=0.002$), and erythrocyte GSht significantly lower ($p=0.033$) in severe attacks compared to a mild attack. During the course of the disease, significant increases were observed in amino thiol ($p < 0.01$) and GSht ($p=0.004$) concentrations for mild attacks, in whom plasma Cys correlated strongly with Hcy ($r = 0.77, p < 0.001$), and gGT activity [$r = 0.75, p < 0.001$]. No such relationship existed in severe AP in which increases in Hcy ($p=0.011$) were not paralleled by an increase in Cys. Furthermore significant decreases in erythrocyte GSht ($p=0.004$) and gGT activity ($p=0.012$) were observed. Levels of the GSH metabolite CysGly markedly increased ($p=0.018$), and this strongly correlated with erythrocyte GSht depletion ($r = 0.99, p=0.01$).

Conclusion: Glutathione depletion associated with severe acute pancreatitis is characterised by an increased rate of GSH degradation, as shown by increased CysGly levels. Our results also indicate that impaired GSH synthesis occurs despite adequate Cys supply.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC498

Measurement of 3-nitrotyrosine in plasma and synovial fluid of patients with inflammatory diseases

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Autoimmune diseases such as rheumatoid arthritis (RA) and Behcet's disease (BD) feature chronic inflammation, in which excessive levels of reactive oxygen (ROS) and nitrogen (RNS) species are produced by activated inflammatory cells. These may cause tissue damage and contribute to the development or progression of disease, and may also modify cellular proteins. Such oxidative/nitrosative modifications could potentially be utilised as biomarkers of the stage or severity of disease, should such correlations be reliably observed and measurable in patient samples. Previous studies have suggested that increased 3-nitrotyrosine (3-NT), is present in plasma from BD patients [1], and both plasma and synovial fluid in RA patients [2]. 3-NT is formed when peroxynitrite (ONOO-) reacts with accessible tyrosine residues in proteins and is considered to be a marker of excessive inflammation [3]. However, the specificity of the HPLC method used to measure 3-NT in RA has been called into question [4], and the increases in 3-NT in BD were minimal, and their biological significance unproven. We therefore sought to measure 3-NT levels simultaneously in plasma from healthy volunteers and patients with active BD, alongside plasma and matched synovial fluid from patients with RA, by sandwich ELISA. Our aim was to further clarify the levels of 3-NT that can be measured in such samples, and to determine whether the nitration of tyrosine residues acts as a biomarker of inflammatory status. In 3 of 4 tested samples from healthy subjects, 3-NT levels were below the limit of detection of the assay (2.1 nM). The fourth sample was at the lower limit of detection. Thus, as expected, nitrated tyrosine was present at low or undetectable levels in subjects with no apparent sign of inflammatory disease.

Surprisingly, 3-NT was not elevated in patients with active BD. None of the samples tested contained detectable levels of 3-NT. In contrast, however, 3-NT levels were markedly elevated in both the plasma and synovial fluid of some RA patients. Two samples showed 3-NT to be undetectable, while another patient had low levels. However, in a further 3 patients, 3-NT was markedly elevated. These samples contained an average of 503.0 ± 226.7 nM 3-NT in synovial fluid, and 784.0 ± 381.0 nM in plasma. In conclusion, elevated 3-NT may be useful as a potential biomarker of some, but not all, inflammatory conditions. Surprisingly, Behcet's disease is not associated with elevated 3-NT generation, despite being a chronic inflammatory condition.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC499

Diet and gastric nitrosative stress

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The incidence of cancer of the proximal stomach has increased markedly over the past 20 years. Unlike cancer of the more distal stomach, it is not related to *Helicobacter pylori* infection and occurs in healthy non-atrophic acid secreting stomachs. While the responsible agent remains unidentified, it is likely that environmental factors, such as the diet, play a role in the rising incidence of these cancers.

Saliva contains high concentrations of nitrite derived from the enterosalivary recirculation of dietary nitrate and its reduction by buccal bacterial. For many years, there has been interest in nitrite as a potential pre-carcinogen for gastric cancer. Acidification of nitrite in the stomach produces nitrosative species, which can form potentially carcinogenic N-nitroso compounds. Antioxidants such as ascorbic acid protect against this nitrosative chemistry by converting the nitrosative species to nitric oxide. However, nitric oxide diffuses rapidly to lipids, where it reacts with oxygen to form further nitrosative species (N₂O₃).

Using gas chromatography – ion trap tandem mass spectrometry (GC-MS/MS), we have investigated the effect of lipid on the formation of N-nitroso compounds from nitrite in a model of the human stomach. While we successfully demonstrated the inhibitory effect of ascorbic acid on N-nitrosation in a single-phase system, N-nitrosation was not inhibited by ascorbic acid in presence of 10% lipid. These results indicate that the presence of lipid can markedly alter the protective effects of antioxidants with respect to potentially carcinogenic nitrosative chemistry occurring in the human stomach, and illustrate how diet can influence gastric biochemistry.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC500

Signalling pathways underlying interferon-gamma-regulated expression of monocyte chemoattractant protein-1 and other key genes in macrophages implicated in atherosclerosis

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IFN-gamma regulates the expression of key genes in macrophages implicated in the control of cholesterol homeostasis and

the inflammatory response, two critical events in the pathogenesis of atherosclerosis. We are investigating the molecular mechanisms underlying the IFN-gamma-regulated expression of such genes in detail, with emphasis on monocyte chemoattractant protein-1 (MCP-1), a key mediator of monocyte trafficking during atherogenesis. The IFN-gamma-mediated induction of MCP-1 expression was attenuated by inhibitors of Janus kinase (JAK)-2, casein kinase 2 (CK2) and phosphoinositide-3-kinase (PI3K). The action of PI3K was mediated through its downstream target AKT. A combination of chromatin immunoprecipitation assays and electrophoretic mobility shift assays showed that signal transducer and activator of transcription (STAT)-1 interacted with IFN-gamma response elements in the promoter region of the MCP-1 gene. Dominant negative forms of JAK-1 and -2, STAT1, AKT and CK2 attenuated the IFN-gamma-induced activity of the MCP-1 gene promoter and an artificial promoter containing three copies of STAT1 response elements. Gene expression profiling and reverse transcription polymerase chain reaction showed that CK2 and PI3K were also required for the IFN-gamma-mediated regulation of a large number of genes implicated in atherosclerosis. The action of CK2 and AKT was mediated, at least in part, through the control of STAT1 phosphorylation at serine 727, which is required for maximal transcriptional activation by this factor. More recently, we have found that extracellular signal regulated kinase (Erk) also plays a key role in the regulation of STAT1 serine 727 phosphorylation by IFN-gamma. The interactions between these different pathways and their roles in the regulation of macrophage gene expression and cholesterol homeostasis are currently being investigated. These studies provide novel insights into signalling pathways underlying the IFN-gamma-mediated changes in macrophage gene expression during atherosclerosis.

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PC501

Concipient regulation of PKC δ in human platelets by phosphorylation of Tyrosines 311 and 565 and phospholipase C signalling

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PKC δ plays a complex role in platelets, having both positive and negative signaling functions. It is phosphorylated on tyrosine in response to thrombin and collagen, and it has recently been shown that residue Y311 is phosphorylated in response to PAR1 and PAR4 receptor activation. Here we show Y311 and Y565 to be phosphorylated in response to thrombin, and have addressed the interplay between phosphorylation and the classical lipid-mediated activation of PKC δ . Phosphorylation of both Y311 and Y565 is dependent upon Src kinase and phospholipase C activity in response to thrombin. Importantly however, direct allosteric activation of PKC δ with phorbol myristate (PMA) also induced phosphorylation of Y311 and Y565, dependent upon Src kinases, but not phospholipase C. Membrane recruitment of

PKC δ is essential for phosphorylation of the kinase on tyrosine, but tyrosine phosphorylation is not required for membrane recruitment of PKC δ . Both thrombin and PMA induce recruitment of PKC δ to the membrane, which for thrombin is by a PLC-dependent process. In order to address the functional role of tyrosine phosphorylation of PKC δ , we show phosphorylation to potentiate the activity of the kinase, although it does not play a role in membrane recruitment of the kinase. PKC δ is therefore regulated in a coincident fashion by PLC-dependent signals, recruiting it to the plasma membrane, and phosphorylation on tyrosine potentiating its activity.

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PC502

Purinergic regulation of epithelial sodium channels

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The epithelial sodium channel (ENaC) mediates amiloride-sensitive Na⁺ transport in epithelial cells. It is responsible for salt and fluid reabsorption in the distal collecting duct of the kidney, distal colon, lung and the excretory ducts of the exocrine glands. ENaC's function contributes to Na⁺ and K⁺ homeostasis, regulating blood pressure and maintaining the fluid layer lining the lungs. Diseases associated with defective regulation of ENaC include cystic fibrosis and respiratory infections such as those caused by respiratory syncytial virus¹ and pseudomonas aeruginosa². It has been suggested that the purinergic receptor agonists, UTP and ATP, are released during infections and feedback onto purinergic receptors to cause ENaC inhibition^{1,2}. Consistent with these findings, extracellular application of purinergic receptor agonists also reduces the activity of ENaC. The aim of our studies has been to decipher the key components of the signalling pathway by which purinergic receptor stimulation regulates ENaC activity. Fisher Rat Thyroid (FRT) cells expressing ENaC were used as a model cell line for our studies. Suspected genes involved with the signalling pathway were manipulated using cell transient transfection with plasmid DNA or siRNA. Short circuit currents were monitored using Ussing Chamber electrophysiology. We have found that the purinergic signalling mechanism which regulates ENaC activity involves the activation of apical and basolateral P2Y₂ purinergic receptors, activation of the $\beta\gamma$ -subunits of a pertussis toxin sensitive G-protein, and activation of phospholipase C β -4. In addition we found an increase in intracellular calcium and basolateral transport of potassium and chloride play a role in this mechanism.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC503

Application of a homogenous, non-imaging cell-based assay for rapid and direct detection of NHR translocation

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The Discoverx PathHunter assay platform provides a screening compatible assay format that is able to directly measure nuclear translocation events without imaging or a reporter gene. This is accomplished using positional complementation in cells engineered to express an inactive β -Gal acceptor fragment (EA) in the cell nucleus and a protein of interest in the cytoplasm that has been tagged with a short complementing peptide for β -Gal called ProLabel. This approach has been applied successfully to a number of targets, but is particularly effective for Nuclear Receptors (NHRs). Using clonal HEK and CHO cell backgrounds with the EA expressed exclusively in the nucleus, we have been able to transiently express NHR-ProLabel fusion proteins and measure translocation events after as little as one hour of compound treatment, often with signal-to-background ratios in excess of 10-fold. Data will be highlighted for a series of target, including GR, AR, PXR, LXR and VDR.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC505

TRP channel expression and signalling in normal human urothelial cells

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The human bladder and associated urinary tract is lined by a specialised transitional epithelium referred to as the urothelium. The urothelium was initially thought to be exclusively an impermeable barrier, however it has recently been shown to have a sensory role. Transient receptor potential (TRP) channel signalling was implicated in normal bladder function by Birder et al. (1) who showed that TRPV1 knockout mice displayed increased bladder capacity and a greater frequency of non-voiding contractions. Dysregulation of urothelial cell sensory signalling may contribute to the pathogenesis of dysfunctional syndromes, such as interstitial cystitis and overactive bladder syndrome. Although animal models provide a useful research tool, cross-species variation may limit their relevance to man. The relatively recent development of in vitro culture models of proliferative (2) and differentiated (biomimetic) (3) normal human urothelial (NHU) cells has permitted investigation of TRP signalling pathways in human urothelium for the first time. The aims of this study are:

1. Determine the expression of TRP channels in human urothelium and cultured NHU cells to validate the use of NHU cells as a model for investigating TRP channel signalling.

2. Identify and assess the modulatory effect of other sensory signalling pathways on TRP signalling in NHU cells.

Normal human urothelium was obtained at surgery with local research ethics committee approval and patient informed consent. In culture, NHU cells adopted a proliferative phenotype, however addition of 5% adult bovine serum and 2mM calcium to the cell medium induced a stratified epithelium that displayed characteristics of native urothelium with high trans-epithelial resistance and markers of terminal differentiation. TRPV1, TRPV4 and TRPM8, but not TRPA1, transcripts were identified by RT-PCR in cultured NHU cells. Ratiometric laser confocal microscopic techniques were used to detect calcium fluxes in cultured NHU cells in response to the perfusion of specific agonists to reveal the presence of functional receptors. Capsaicin (300µM), indicated the presence of functional TRPV1, while UTP (5µM) demonstrated the presence of purinergic P2Y receptors. Pre-treatment of UTP augmented the calcium flux following capsaicin perfusion and immunohistochemical experiments revealed increased immunolabelling with anti-TRPV1 antibodies following UTP treatment of NHU cells suggesting TRPV1 signalling is modulated by P2Y signalling.

In conclusion, proliferative and stratified NHU cells provide a useful tool for the investigation of normal human urothelial cell signalling. TRP channels are functionally expressed and the TRPV1 signalling pathway is modulated by P2Y receptor signalling, the significance of the in normal and deranged human bladder function has yet to be elucidated.

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PC506

Activation of extracellularly regulated kinases 1 and 2 in support cells during hair cell damage is a common signalling mechanism that may contribute to hair cell death

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Cellular stress results in the activation of members of the Mitogen Activated Protein Kinase (MAPK) family including c-Jun N-terminal kinases (JNK), p38 kinases and Extracellularly Regulated Kinases 1 and 2 (ERK1/2). JNK signaling is known to play a role during sensory hair cell death in the inner ear (e.g. Wang et al, 2003). Here, we investigated ERK1/2 activation in the inner ear using two different damage paradigms.

Cochlear cultures were prepared from postnatal day 1 rat cochleae and used after 1 day in vitro. The sensory hair cell epithelium was lesioned using a N2-laser which allowed precise timing of the damage onset. ERK1/2 activation was assessed using an antibody directed to the dually-phosphorylated form of ERK1/2. Laser lesioning induced the transient and specific activation of ERK1/2 in support cells but not in hair cells. The signal spread non-uniformly from the lesion site with maximum spread occurring 10 minutes after damage.

Aminoglycoside antibiotics are known to induce hair cell death both in vitro and in vivo (Wang et al, 2003). We treated cochlear cultures with neomycin (1mM) for 8 or 24 hrs to test whether ERK1/2 was also activated when hair cells were specifically targeted with aminoglycosides. Cultures were triple labeled with (i) a DNA stain; (ii) the activated ERK1/2 antibody and (iii) fluorescent phalloidin to label actin rich stereocilia at the apical surface of hair cells. Hair cell death was quantified by counting pyknotic nuclei. Treatment with neomycin for 8 hrs caused a significant increase in pyknotic inner hair cell nuclei compared to untreated controls. At this time point, ERK1/2 activation was observed in clusters of support cells surrounding dying hair cells. By 24 hrs, pyknotic outer hair cell nuclei were observed and again ERK1/2 were activated in the support cells. U0126, an inhibitor of MEK1/2, the MAPK kinase was used to test whether the activation of ERK1/2 plays a role in neomycin-induced hair cell death. At 8 hrs, neomycin induced inner hair cell death was significantly decreased in the presence of 10 µM U0126. However after 24 hrs, this protective effect was overcome and there was no significant difference between U0126-treated and untreated cultures. In outer hair cells a reduction in cell death was observed at both 8 and 24 hrs in the presence of U0126; however the effect was not statistically significant. These data suggest that ERK1/2 activation in support cells is a common signalling mechanism during hair cell damage and this activation can contribute to the subsequent death of hair cells.

Wang J et al (2003). *J Neurosci.* 23(24):8596-607.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC507

Oxidative stress mediates chlorpyrifos induced apoptosis in avian lymphocytes *in-vitro*

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Chlorpyrifos is commonly used pesticide for crop protection against pests and also in various public health operations. In the present study, the ability of chlorpyrifos to induce apoptosis and the influence of oxidative stress in induction of apoptosis was investigated; using avian lymphocytes model (white leghorn) in-vitro. Lymphocytes were treated with 10 –5 M for 24 and 48 h. MTT assay revealed decreased viability in time dependent way. Fragmentation assay showed DNA ladder upon agarose gel electrophoresis. Annexin V binding assay detected the phosphatidylserine exposure to outer leaflet of the plasma membrane. Electron microscopy showed margination of chromatin, membrane blebbing, which are the features of apoptosis. Western blot analysis revealed the expression of HSP 60, and other small molecular weight proteins, indicative of oxidative stress. The above results suggest, chlorpyrifos have the potential

to induce apoptosis and the oxidative stress might mediate the apoptotic death in chlorpyrifos-treated lymphocytes. Thus the study advocates the systematic use of chlorpyrifos when used for various public health operations.

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PC508

Insights into the mechanisms controlling Ca^{2+} signalling in myocytes of ureter and ureteric microvessels

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Mechanisms controlling Ca signalling in visceral and vascular smooth muscle cells can differ but interpretation is complicated because of differences in the type of tissue or vessel studied as well as experimental conditions. In the present study we have overcome these problems by using *in situ* imaging of Fluo-4 loaded ureters, isolated from humanely killed guinea pigs, to investigate and compare mechanisms controlling Ca signalling in visceral (ureteric) and vascular (ureteric arteriolar) smooth muscle cells in the same preparation under identical experimental conditions. The ureteric myocytes were electrically coupled and when stimulated by electrical field (EFS) via focal electrode with rectangular pulses (5–7V, 100msec in duration) with 40 s interval generated intercellular/inter-bundular fast propagating (6–7cm/sec), regenerative Ca waves. The wave could be initiated in any part of the ureter and propagated in all directions from the point of stimulation. Simultaneous recording of electrical activity and Ca imaging revealed that the intercellular Ca waves evoked by EFS were associated with action potentials, which were abolished in Ca-free solution or nifedipine. EFS did not induce any propagating intercellular Ca wave in terminal arterioles. High-K (60 mM) stimulation produced a sustained Ca rise in both types of cells which was blocked by Ca-free solution or nifedipine. Phenylephrine (PhE, 10 μM) stimulated both ureteric and arteriolar myocytes. In the ureteric bundles it produced 1.7 ± 0.1 times increase in the duration of the Ca transients but was ineffective in Ca-free solution. In contrast, vascular myocytes responded with asynchronous Ca oscillations in the form of intracellular Ca waves, travelling at a speed of 30–60 $\mu\text{m}/\text{sec}$. These Ca waves were highly resistant to nifedipine or removal of extracellular Ca (Ca free, 2 mM EGTA for 60–90min) ($n=17$). Both types of cells produced spontaneous Ca sparks which were potentiated by low concentrations of caffeine and abolished by ryanodine. Ryanodine increased the excitability in ureteric cells but had no effects on PhE induced Ca oscillations in the vascular myocytes. Inhibition of the SR Ca-ATPase by cyclopiazonic acid (20 μM) increased excitability of the ureteric cells but abolished PhE-induced Ca oscillations in vascular myocytes. 2-APB (50 μM) (an inhibitor of IP_3 signalling) had no effects on Ca signalling in ureteric cells but abolished PhE-induced Ca oscillations in vascular myocytes. These data suggest that Ca influx via

L-type Ca channels, mediated by the action potential, is the main mechanism controlling Ca signalling in ureteric myocytes. In contrast Ca release from the SR mediated by IP_3 Rs channels is the main mechanism controlling Ca signalling in vascular myocytes in the ureteric microvessels.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC509

Differential PDE4 expression contributes to altered cAMP levels in behaviourally diverse mouse strains: primary or secondary response to naturalistic stereotypy?

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Our lab recently validated the deer mouse (*Peromyscus maniculatus bairdii*) as model of OCD (Korff et al., 2007). Deer mice are useful models in that they engage in spontaneous stereotypic behaviour. This study compared cAMP levels, PDE4 activity, and PDE4 isoform expression of pharmacologically naïve deer mice to that of a non-stereotypic strain; C57Bl. Uniquely, deer mice display different degrees stereotypy, e.g. low versus high rates (Powell et al., 1999). Moreover, for the purpose of this study a distinction was made between low stereotypic (LSB), high stereotypic (HSB) and non-stereotypic deer mice.

The degree of stereotypy in *Peromyscus* was linked to the amount of cAMP under basal conditions in the prefrontal cortex and striatum, and correlated with PDE4 activity and expression in matching brain regions. HSB mice (8.51 pmol/mg prot) presented with the highest prefrontal cortex and striatal cAMP levels compared to LSB (7.05), non-stereotypic (6.84), and C57Bl mice (6.73). PDE4 activity was significantly higher in deer mice (3.81 pmol/min/mg prot) compared to C57Bl mice (1.86 pmol/min/mg prot).

Immunoblotting of PDE4 revealed that deer mice express significantly more prefrontal cortex PDE 4B4 and 4D4 and significantly less PDE 4A5, 4B1, and 4D1 isoforms compared to C57Bl. Striatal expression of PDE 4A5, 4B4, and 4D1 was significantly lower, and that of PDE 4D4 was significantly higher in deer mice compared to C57Bl.

In summary, neurochemical and molecular characterization of deer mice versus C57Bl mice indicate that raised stereotypic behaviour is characteristic of the deer mouse, and that raised stereotypic behaviour in these animals are associated with differential expression of select isoforms of the PDE4 family, which drive cellular release of striatal and prefrontal cAMP.

Korff S, Stein DJ, Harvey BH. 2007. Stereotypic behaviour in *Peromyscus maniculatus bairdii* (deer mouse): Validation and relevance for obsessive compulsive disorder. Submitted for publication.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC510

Detection of epoxyeicosatrienoic (EET) acid and hydroxyeicosatetraenoic (HETE) acid generating cytochrome P450s in pregnant human myometriumT. Pearson¹, D. Barrett² and R. Khan¹¹*Academic Division of Obstetrics & Gynaecology, University of Nottingham, Derby, UK and* ²*School of Pharmacy, University of Nottingham, Nottingham, UK*

Cytochrome P450 enzymes generate epoxyeicosatrienoic (EETs) and hydroxyeicosatetraenoic acids (HETEs) from arachidonic acid and act to decrease or increase smooth muscle contractility, respectively. As such, they may be important factors in the control and/or initiation of labour and differential expression of EETs or HETEs generating enzymes may therefore be predicted at key stages of pregnancy.

Myometrial biopsies, gathered from pregnant women at elective or emergency caesarean section (term, 38+ weeks) after full informed written consent were utilised in these studies. Samples (1.1 ± 0.5g) of uterine smooth muscle were washed and snap frozen in liquid nitrogen for Western blotting or enzymatically dispersed and cultured for immunocytochemistry within 24 hours of collection. For Western blotting, samples were incubated with antibodies to either CYP450 2C9/19 (EETs generating) or CYP450 4A (HETEs generating) enzymes following SDS-PAGE. The same antibodies were used for immunocytochemistry. Data is shown as mean ± S.E mean, where used a t-test was done to determine significance at the level P<0.05.

Immunoblotting showed clear signals for CYP450 2C9/19 and CYP450 4A enzyme expression. The measured molecular weight for CYP 4A was not different (t-test, P>0.05) between labouring (n=6) (46.4 ± 0.1 kDa vs 45.5 ± 0.2 kDa) and non-labouring myometrial samples (n=7), nor was there any significant difference in band density (26.4 ± 1 units vs 29.4 ± 1.8 units respectively, t-test P=0.19). Comparison of labouring (n=6) and non-labouring (n=7) samples for CYP 2C9/19 showed similar molecular weights at 44.4 ± 0.3 vs 45 ± 0.7 kDa (t-test P>0.05) respectively and no significant differences in band density 22 ± 8.2 units vs 33.7 ± 15 units respectively, (t-test P>0.09). Beta-actin loading controls for all groups had similar molecular weights (42 kDa) and no significant differences in band density (t-test, P>0.1). Strong immunoreactivity to CYP450 4A and faint reactivity to 2C9/19 was also found in cultured non-labouring smooth muscle cells. In conclusion, both the HETE (4A) and EET generating (2C9/19) P450 enzymes were detected in pregnant myometrium. Although no significant differences in expression of CYP 2C9/19 and CYP 4A were identified using Western blotting in non-labouring and labouring myometria, it may be argued that labouring myometrium is shifted more towards a HETE generating phenotype than that of EETs, with resultant HETE activity contributing towards successful labour. Additional studies are underway to validate these observations.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC511

PI 3-kinase-dependent membrane recruitment of centaurin-α2 is essential for its effect on ADP-ribosylation factor 6-mediated actin cytoskeleton reorganisation

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GTPase activating proteins (GAPs) of the centaurin family regulate the actin cytoskeleton and vesicle trafficking through inactivation of the ADP-ribosylation factor (ARF) family of small GTP-binding proteins (1-5). We report the functional characterisation of centaurin-α2, which is structurally related to centaurin-α1 ARF6 GAP (2). Centaurin-α2 contains an N-terminal GAP domain followed by two pleckstrin homology (PH) domains (N-PH and C-PH). In vitro, GFP-centaurin-α2 specifically binds phosphatidylinositol (PI) 3-kinase lipid products, PI 3,4-P2 and PI 3,4,5-P3 (PIP3), through the C-terminal PH domain. In agreement with this observation, GFP-centaurin-α2 was recruited to the plasma membrane from the cytosol in EGF-stimulated cells in a PI 3-kinase-dependent manner (2). Moreover, the C-PH domain is sufficient and necessary for membrane recruitment of centaurin-α2. Centaurin-α2 shows sustained kinetics of PI 3-kinase-mediated membrane recruitment in EGF-stimulated cells, due to its binding to PI 3,4-P2 (2). Centaurin-α2 prevents ARF6 translocation to and cortical actin formation at the plasma membrane, which are phenotypic indications for ARF6 activation, in EGF-stimulated cells. Moreover, the constitutively active mutant of ARF6 reverses the effect of centaurin-α2 on cortical actin formation. The membrane targeted centaurin-α2 is constitutively active. Together, these studies indicate that centaurin-α2 recruits in a sustained manner to the plasma membrane through binding to PI 3,4-P2 and thereby regulates actin reorganisation via ARF6 (2).

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC512

Investigation of the mechanisms of PtdIns5P production in HeLa cells

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Phosphatidylinositol 5-Phosphate (PtdIns5P) is a member of the Phosphoinositide (PI) family of phospholipids, which are intimately involved in many aspects of cellular function. PtdIns5P constitutes approximately 1-5% of total cellular PIs, though its levels have been found to fluctuate in response to certain stimuli and during the cell cycle. Levels of PtdIns5P have been shown to increase after thrombin stimulation in human platelets (Morris et al., 2000), during osmotic stress in mammalian and in plant cells (Meijer et al., 2001), or after insulin stimulation of 3T3-L1 adipocytes. A pool of PtdIns5P in the nucleus of murine erythroleukemia cells is increased 20-fold during the G1 cell cycle phase, and also increased 4-fold following UV irradiation (Jones et al., 2006). Additionally PtdIns5P levels have been shown to decrease in HeLa cells after histamine stimulation (Roberts et al., 2005). PtdIns5P is the least well understood member of the PI family, and its physiological function is presently obscure. Recent evidence suggests that it may act as a transducer of stress signalling, via its interaction with Inhibitor of growth protein-2 (ING2) and p53 regulation (Jones et al., 2006).

Although other members of the PI family are well characterised, little is currently known about the mechanisms of synthesis or breakdown of PtdIns5P. It can be produced in vitro via D5 phosphorylation of PtdIns by Type I PIPKins (Phosphatidylinositol Phosphate Kinases) and the Type III PIPKin, PIKfyve. An alternative route is via D3 dephosphorylation of PtdIns(3,5)P2 by members of the myotubularin family, or by D4 dephosphorylation of PtdIns(4,5)P2 by the *S. flexneri* effector IpgD and by two recently identified human enzymes, PtdIns(4,5)P2 4-Phosphatase Type I and Type II (Ungewickell et al., 2005). PtdIns5P is the preferred substrate of the Type II PIPKins which catalyse its phosphorylation at the D4 position, producing PtdIns(4,5)P2, and recent evidence suggests that the physiological role of these enzymes may be to remove PtdIns5P (Jones et al., 2006).

In this study, we demonstrate that PtdIns5P levels are increased in HeLa cells following short-term treatment with Pervanadate. To elucidate the cellular mechanisms involved in the PtdIns5P synthesis we have used siRNA knockdown and overexpression of enzymes suspected to be involved in PtdIns5P regulation. The effect of these on resting PtdIns5P and pervanadate elevated PtdIns5P levels, determined by specific PtdIns5P mass assay, are reported.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC513

Transcriptional repression of equilibrative nucleoside transporter 1 in human fetal endothelium from gestational diabetes is associated with activation of C/EBP homologous proteinM. Farias¹, C. Puebla¹, A. Vecchiola¹, M. Pastor-Anglada², P. Casanella¹ and L. Sobrevia¹

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Gestational diabetes reduces the equilibrative nucleoside transporter 1 (hENT1) expression and activity in human umbilical vein endothelial cells (HUVEC), an effect associated with nitric oxide (NO)-dependent reduction of *SLC29A1* (for hENT1) promoter activity (Farias et al., 2006). The human C/EBP homologous protein (hCHOP) is a transcription factor whose repressor effect on *BCL2* depends on NO in murine fibroblasts (McCullough et al., 2001). We performed a serial deletion analysis of a *SLC29A1* promoter region to study the involvement of hCHOP as transcriptional regulator of this gene in HUVEC.

HUVEC from normal (n=20) or gestational diabetic (n=20) pregnancies were cultured under standard conditions (Farias et al., 2006). hENT1 mRNA and protein levels, and hCHOP nuclear abundance were determined. Six fragments of *SLC29A1* upstream region (up to -3198 bp from ATG) were cloned into pGL3 vector (pGL3-hENT1⁻³¹⁹⁸, pGL3-hENT1⁻²¹⁵⁴, pGL3-hENT1⁻¹⁸¹⁰, pGL3-hENT1⁻¹⁶⁷⁰, pGL3-hENT1⁻¹¹¹⁴, pGL3-hENT1⁻⁷⁹⁵). These constructs were transfected into HUVEC by electroporation (320 V, 30 ms) and luciferase activity was compared with pRL-TK control vector. hCHOP specific binding to the consensus site for hCHOP-C/EBPα protein complex in the *SLC29A1* promoter (-1853 to -1841 bp) was evaluated by chromatin immunoprecipitation.

Reduced hENT1 protein (60 ± 5%) ($P < 0.05$, unpaired Student t test, mean ± S.E.M.) and mRNA (25 ± 3%, $P < 0.05$) levels, but increased hCHOP nuclear protein abundance (1.32-fold, $P < 0.05$) were detected in HUVEC from gestational diabetic compared with normal pregnancies. Transcriptional activity of pGL3-hENT1⁻³¹⁹⁸ or pGL3-hENT1⁻²¹⁵⁴ constructs was lower (27 ± 3% or 31 ± 2%, respectively, $P < 0.05$) in HUVEC from gestational diabetes compared with normal pregnancies. However, transcriptional activity was higher ($P < 0.05$) when HUVEC from gestational diabetes were transfected with pGL3-hENT1⁻¹⁸¹⁰ (1.3-fold), pGL3-hENT1⁻¹⁶⁷⁰ (1.4-fold) or pGL3-hENT1⁻¹¹¹⁴ (1.42-fold), compared with cells from normal pregnancies. hCHOP binding to *SLC29A1* promoter in HUVEC from gestational diabetes was higher (1.4-fold, $P < 0.05$) than in cells from normal pregnancies.

The reduced *SLC29A1* promoter activity exhibited by HUVEC from gestational diabetes could be due to increased binding of the transcription factor hCHOP to its consensus sequence in this genomic region. Since NO synthesis is higher in HUVEC from gestational diabetes and reduces hENT1 expression, this vasodilator could be crucial for hCHOP repression of *SLC29A1* promoter activity in this cell type.

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FONDECYT 1030781, 1030607, 1070865 (Chile). Agencia Española de Cooperación Internacional (AECI A/5484/06)(Spain). M. Fariás holds a CONICYT- and Faculty of Medicine-PhD fellowships (Chile). C. Puebla holds a VRAID-PhD fellowship (Chile).

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC514

AMP-Activated Protein Kinase (AMPK) contributes to glucose sensing in CRI-G1 Cells

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AMPK is a metabolic master switch activated by multiple stimuli (e.g. hypoglycaemia, muscle contraction and hypoxia) that increase the cellular AMP: ATP ratio (Rutter *et al.* 2003). Once activated AMPK inhibits ATP consuming processes and enhances ATP production partly, at least, by changes in fuel selection. Pancreatic β -cells however, display unique properties with regard to fuel sensing and energy maintenance. The β -cell isoform of hexokinase (hexokinase IV; also known as glucokinase) is only basally active at normal glucose levels, and is not negatively regulated by glucose-6-phosphate, in contrast to the liver isoform. Since β -cells are essentially nutrient deprived at normal glucose levels, insulin is secreted at a basal level. Upon raised extracellular glucose, glucokinase becomes more active, increasing ATP levels leading to closure of the ATP-sensitive K^+ -channel. This leads to membrane depolarisation, activation of voltage dependent Ca^{2+} -channels, influx of Ca^{2+} and induction of insulin secretion. To examine the influence of AMPK activity on glucose sensing, the glucose-insensitive insulin-secreting cell line, CRI-G1, was used.

Using patch-clamp electrophysiology, AMPK activation by acute exposure to AICAR (1 mM; 30-40 minutes) did not affect the membrane potential of CRI-G1 cells. However, acute removal of glucose induced a hyperpolarisation ($n = 9$), significantly greater in magnitude than in cells not exposed to AICAR ($n = 14$). To determine if this was due to an effect on glucose uptake, the uptake of the fluorescent glucose analogue, 2-NBDG was measured using confocal microscopy. AICAR did not significantly alter glucose uptake for exposure times up to 40 minutes. An *in vitro* enzyme assay for glucokinase revealed that AICAR (40 minutes) reduced glucokinase activity, as measured by the production of NADPH. Confocal immunofluorescence demonstrated that neither the total glucokinase expression nor the nuclear/cytoplasmic ratio of glucokinase changed during the time course of the experiment.

Thus, in CRI-G1 cells, AMPK activation sensitises cells to changes in glucose by limiting glucokinase activity when glucose levels are low. This contrasts with other tissues, where activation of AMPK leads to maintenance of ATP levels. This is surprising given that β -cells exhibit differential glucose sensing and that

large changes in ATP are required as the primary regulatory mechanism for insulin secretion. Additional studies are underway to define further the role of AMPK in regulating glucokinase activity and glucose sensing in pancreatic β -cells.

Rutter, G.A. *et al.* (2003) *Biochem. J.* 357, 1-16

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PC515

Sweet taste receptor interacting protein CIB1 is an inhibitor of $InsP_3$ -mediated Ca^{2+} -release

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In previous works of our group the calcium and integrin binding protein CIB1 has been identified and characterised as a specific interaction partner of the sweet taste receptor subunit TAS1R2 carboxyterminal domain.

Sweet and umami taste is mediated by three G-protein-coupled subfamily C receptors of the TAS1R-family, namely TAS1R1, TAS1R2, and TAS1R3. These receptors feature the typical large N-terminal "venus-fly-trap module" as well as a short intracellular C-terminus and function only as (hetero-)dimers recognising different classes of taste stimuli. While in humans the TAS1R1/TAS1R3 complex triggers mainly the perception of umami taste (taste of sodium glutamate), the TAS1R2/TAS1R3 exhibits the actual sweet receptor recognising natural ligands like sugars, D-amino acids, sweet tasting proteins and artificial sweeteners. The intracellular signalling cascade seems to involve a yet unidentified heterotrimeric G protein, phospholipase C- β 2 generating $InsP_3$, $InsP_3$ -receptor subtype 3 and the cation-conducting TRPM5 ion channel [1].

In the heterologous HEK293 sweet taste expression system the G protein chimera $G\alpha_{16gust44}$ links the sweet taste receptor dimer to an $InsP_3$ -dependent Ca^{2+} -release pathway. To further characterise the *in vivo* role of CIB1, which is endogenously expressed in HEK293 cells, we generated cell lines stably and transiently expressing shRNA against CIB1.

Investigating the general aspects of CIB1 influence on intracellular calcium signalling, various imaging systems were used, e.g. FLIPR and FURA-2 cuvette assays.

Applications of ATP, UTP and carbachol - ligands which increase intracellular calcium in an $InsP_3$ -dependent manner via endogenous receptors, revealed a more pronounced Ca^{2+} -elevation in HEK293 cells lacking CIB1 than in control cells. Overexpression of CIB1 lowered the Ca^{2+} response to UTP in comparison to mock transfected cells. Preincubating these cells with the SERCA inhibitor thapsigargin showed that the assayed changes of cytosolic calcium were due to its release from the endoplasmic reticulum.

In addition to that, HEK293 cells transiently overexpressing CIB1 also showed an increased release of calcium from intracellular stores after application of thapsigargin.

Taken together, this most likely corresponds with a general inhibitory function of CIB1 on $InsP_3$ receptors *in vivo*.

Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS. *Nature* 2006;444(7117):288-294.

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PC516

The neurotrophic actions of PACAP on human neuroblastoma cells: A role for EPAC

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The 38 amino acid neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP-38) induced differentiation of human neuroblastoma SH-SY5Y cells. Four-day treatment with PACAP-38 caused a concentration-dependent increase in neurite-bearing cells (5.6 ± 1.0 -fold of basal control at 100 nM; $n=3$) and a marked increase in the expression of the neuronal differentiation marker proteins, Bcl-2, GAP-43 and ChAT, as assessed by Western blotting ($n=3$). These effects were blocked by the PAC₁ receptor antagonist, PACAP6-38. The PAC₁ receptor couples to the robust activation of adenylyl cyclase [Lutz *et al.*, 2006], and the effects of PACAP-38 on SH-SY5Y cells could be mimicked by forskolin treatment, suggesting the involvement of a cAMP-mediated pathway. PACAP-38-induced differentiation was completely prevented by the adenylyl cyclase inhibitor, DDA, but not by the PKA inhibitor H89, or siRNA-mediated knock-down of the PKA catalytic subunit. Both PACAP-38 and forskolin were observed to stimulate the activation of ERK, p38 MAP kinase and JNK. PACAP-38-induced neuritogenesis was completely prevented by the MEK-1 inhibitor PD98059, and partially prevented by the p38 MAP kinase inhibitor SB203580. Activation of the guanine nucleotide exchange factor, EPAC (exchange protein directly activated by cAMP), by the specific activator 8-CPT-Me-cAMP, also partially mimicked the effects of PACAP-38, and led to the phosphorylation of ERK but not p38 MAP kinase. These data therefore indicate that PACAP-38, acting at the PAC₁ receptor, is a neurotrophic factor and that the mechanism involved is cAMP-dependent but PKA-independent, and involves EPAC-dependent activation of ERK as well as activation of p38 MAP kinase signalling pathways.

Lutz EM *et al.* (2006). *Mol Cell Neurosci* 31, 193-209.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC517

Effects of Eicosapentaenoic Acid (EPA) on murine C2C12 cell death during myoblast to myotube differentiation

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Skeletal muscle maintenance, repair and growth are dependent on satellite cell activation. These quiescent cells are activated to proliferate to allow ultimately formation of new myotubes, when for example muscle injury occurs. Murine C2C12 myoblasts can be induced to differentiate into myotubes and provide a convenient model for evaluating the effects of EPA, an omega-3 fatty acid credited with considerable health benefits, on this process. Previous studies have indicated that EPA can inhibit proteolysis of fully differentiated skeletal muscle myotubes (1,2).

For this investigation, C2C12 proliferating myoblasts were induced to differentiate by culture in growth medium containing 2% horse serum. EPA (0.01mM to 0.6mM), complexed with bovine serum albumin, was added to the differentiation medium at the start and myotube formation allowed to progress for 5 days. Indirect immunofluorescence microscopy with anti-myosin primary antibody (MF-20, DSHB University of Iowa) and Alexa-Fluor® 546 goat anti-mouse IgG secondary antibody (Invitrogen), followed by DAPI nuclear counterstaining were performed to evaluate myotube formation. A commercial cytotoxicity assay (CellTiter Blue Assay, Promega) was carried out to quantify cell death.

The cytotoxicity results, obtained after 5 days differentiation, show two distinct dose-dependent effects of EPA on cell death. At EPA concentrations >0.4mM, cell death increases significantly over that of untreated cells ($P < 0.05$). In contrast, at EPA doses between 0.01mM and 0.3mM, cell death is significantly reduced compared to untreated cells ($P < 0.05$). During myotube differentiation in the presence of low concentrations of EPA it was observed that there was an approximately 50% reduction in cell death. Data from immunofluorescence studies of cell death confirmed these findings. In conclusion, our data suggest that low doses of EPA exert a protective effect against cell death during myotube formation. Possible mechanisms could include an effect of EPA on the PI3K/AKT signalling pathway as this pathway has previously been shown to be involved with mediation of differentiation (4). Although the nature of this effect is currently being investigated, a previous study has demonstrated that EPA, administered at a similar dosage, can inhibit apoptosis of fully differentiated myotubes (3). Our findings support a potential therapeutic application for EPA during skeletal muscle regeneration following injury.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC518

Vasopressin increases basolateral membrane localization of the mUT-A3 urea transporter

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Facilitative urea transporters play a vital role in the production of concentrated urine in the mammalian kidney (1). The renal urea transporter UT-A3 is expressed on the basolateral membrane of mouse inner medullary collecting duct cells (2). Previous experiments using an MDCK cell line stably expressing mUT-A3 (MDCK-mUT-A3) have shown that the hormone vasopressin acutely regulates UT-A3-mediated basolateral urea transport (3). In this study, we have investigated the effect of vasopressin on the localization of UT-A3 transporters.

Utilising serial centrifugation to separate protein samples from various sub-cellular fractions, we probed immunoblots of MDCK-mUT-A3 protein with the UT-A3 antibody ML446 (2). The addition of 10^{-6} M vasopressin for 60 minutes shifted the predominant 40-45 kDa UT-A3 signal from 200,000g samples (intracellular membranes) to 17,000g samples (plasma membranes). Vasopressin therefore significantly increased the UT-A3 plasma membrane signal ($P < 0.05$, ANOVA, $n = 3$). In contrast, it had no such effect on a 100 kDa NaKATPase signal in the same samples (NS, ANOVA, $n = 3$), suggesting that it was a specific response. In addition, the presence of $10\mu\text{M}$ H89, a protein kinase A inhibitor, did not prevent the vasopressin-stimulated increase in UT-A3 plasma membrane signal ($P < 0.05$, ANOVA, $n = 3$). In conclusion, this study suggests that vasopressin increases basolateral urea transport in MDCK-mUT-A3 cells at least partly by increasing localization of UT-A3 to the basolateral membrane.

Fenton, R.A. et al., (2004) *Proc Natl Acad Sci* 101, 7469-7474.

Stewart, G.S. et al., (2004) *Am J Physiol Renal* 286, F979-F987.

Stewart, G.S. et al., (2007) *Am J Physiol Renal* - In Press, doi:10.1152/ajprenal.00183.2006

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC519

Chronic hyperosmolality increases mUT-A3 urea transporter expression in mIMCD-3 cells

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Renal facilitative urea transporters play a vital role in the urinary concentrating mechanism of the mammalian kidney (1). mUT-A1 and mUT-A3 are facilitative urea transporters that are expressed in mouse inner medullary collecting duct cells (2). The mIMCD-3 cell line is an osmotically-tolerant mouse cell line derived from the terminal part of the mouse inner medullary collecting duct (3).

Using a previously characterised antibody (ML446) (2), immunoblotting experiments detected ~45, ~60 and ~120 kDa mUT-A3 signals in protein samples from mIMCD-3 cells grown under 'normal' osmotic conditions (~300mOsm). Growing cells for up to two weeks in hyperosmotic media containing additional NaCl and urea (either ~500, ~700 or ~900mOsm) significantly reduced mIMCD-3 growth rates ($P < 0.05$, ANOVA, $n = 3$). However, there was a significant increase in expression levels of mUT-A3 ($P < 0.05$, ANOVA, $n = 3$). In contrast, no signals were detected in any samples using a mUT-A1-specific antibody (ML194) (2), showing that mIMCD-3 cells do not express mUT-A1. This study shows that mIMCD-3 cells express mUT-A3 but not mUT-A1 urea transporters. The mIMCD-3 cell line is therefore a suitable model for studying the effects of hyperosmolality on expression of mUT-A3 urea transporters.

Fenton, R.A. et al., (2004) *Proc Natl Acad Sci* 101, 7469-7474.

Stewart, G.S., et al., (2004) *Am J Physiol Renal* 286, F979-F987.

Rauchman, M.I., et al., (1993) *Am J Physiol* 265, F416-F424

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC520

Stochastic intracellular calcium dynamics of ventricular myocytes: A simulation study

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To investigate the mechanisms underlying the initiation and propagation of intracellular calcium wave, we developed a three-dimensional ventricular e-cell (3DVe-cell), where cell membrane, nucleus, ryanodine receptor clusters, Z-disk, cytoplasm are modeled as spatially distributed structures. The geometry of the 3DVe-cell is taken as a cylinder with diameter of $16\mu\text{m}$ and length of $100\mu\text{m}$ with the space step of $0.2\mu\text{m}$, which gives 80 by 80 by 500 3D matrix with 3.2 million grid points. RyR clusters are spatially distributed based on [1]. The detailed mathematical description of calcium sparks and calcium waves is a set of reaction-diffusion equations based on [2]. A normal excitation of the cardiac cell produces the membrane depolarization of the action potential. Ca^{2+} influxes (Ca^{2+} current through channels) predominantly in the T tubules activate most of the CRUs and incur SR releases, and then free cytosolic calcium concentration arises uniformly throughout the cell. In Figure (a), pseudo multi linescans of transverse section of the cell are obtained under different conditions, such as normal excitation following membrane depolarization, spontaneous traveling calcium wave, resting cell with sparse, and localized calcium sparks, see Figure (b). Spontaneous local Ca^{2+} release occurs in the 3DVe-cell when the Ca^{2+} sensitivity K_{poss} is high, i.e. RyR clusters have a high probability of opening, even when the Ca^{2+} concentration is low. To evoke calcium waves in the 3DVe-cell we use a low calcium sensitivity to avoid spontaneous initiation, and a high (8pA)

iCa. The evoked calcium waves propagate without decrement, and annihilate on collision, see Figure (c)(d).

Since nucleus does not actively participate in the CICR and has a lower diffusion coefficient compared to cytoplasm, such an obstacle allows the same wave to return on other side of the nucleus, can give rise to an intracellular micro re-entry [3].

The 3DVe-cell is used to illustrate how stochastic properties of Ca^{2+} sparks can lead to complex spatio-temporal intracellular wave processes and allows the incorporation of spatial data sets (protein distribution) into the geometry.

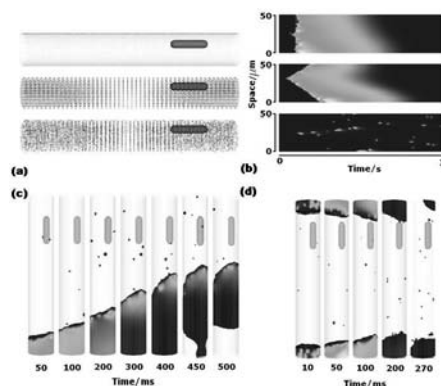


Figure (a) the 3D geometry of the virtual cardiac myocyte. (b) Pseudo line scans of Ca^{2+} concentration on 50 μm space (with transverse spacing of RyRs) for 1 second under different conditions. (c) 3D simulation of solitary wave initiated in one end. (d) Stimulus applied at both ends of the cell with $i_{\text{Ca}} = 5.0$, $K_{\text{poss}} = 800 \mu\text{M}$, waves fail to propagate due to relatively small release current i_{Ca} .

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PC521

Calcium signalling in vascular stem cells

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Cellular components of the vascular system are developmentally diverse and display considerable phenotypic plasticity. Circulating myeloid cells can differentiate into endothelial progenitor

cells (EPCs), or smooth muscle progenitor cells (SPCs) depending on the presence of specific extracellular cues: platelet-derived growth factor-BB (PDGF-BB) treatment of EPCs triggers differentiation into SPCs [1]. Such cells are potentially involved in repair of the vascular system, but could participate in disease processes, such as atherosclerosis [2]. Despite possible involvement of circulating stem cells in vascular health and disease, there are no published data characterising calcium signalling mechanisms that they utilise. Given the role of Ca^{2+} as a key cytoplasmic second messenger, this represents a deficit in understanding vascular stem cell biology. To address this issue, our laboratories characterised calcium signalling mechanisms present in EPCs, SPCs and differentiated human aortic smooth muscle cells (hASM). EPCs, SPCs and hASM express multiple subtypes of ryanodine receptor calcium release channel (RyRs) and L-type voltage-gated calcium channels (VGCCs). Levels of these proteins differ between cell types. Unstimulated cytoplasmic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_c$) were significantly lower in EPCs compared with the other cell types. This implies that differentiation of vascular progenitor cells toward a smooth muscle phenotype is associated with long-term increases in $[\text{Ca}^{2+}]_c$ and with alterations in calcium signalling machinery present. Vascular progenitors were stimulated with chlorocresol (CmC) to open RyRs, or by depolarisation with KCl to activate VGCCs. Both CmC and depolarisation caused rapid elevations in $[\text{Ca}^{2+}]_c$ in EPCs, SPCs and hASM, but magnitudes of these increases differ between the three cell types. Depolarisation-induced increases in $[\text{Ca}^{2+}]_c$ in EPCs indicate that these stem cells are functionally distinct from mature vascular endothelial cells, which do not express VGCCs [3]. Treatment of EPCs with PDGF-BB also triggers complex changes $[\text{Ca}^{2+}]_c$ within seconds of addition. These changes in $[\text{Ca}^{2+}]_c$ varied between individual cells and included decreases as well as increases in cytoplasmic levels of this ion. Overall, these studies indicate association between calcium signalling and differentiation toward a smooth muscle phenotype in vascular progenitor cells. Mechanisms by which calcium signals could modify vascular stem cell phenotype are currently under investigation within our laboratories.

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The authors thank Prof. Vincenzo Sorrentino, University of Siena, for anti-RyR antisera.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC522

Up-regulation of PKC- α and δ in tamoxifen-resistant breast cancer - role in insulin-like growth factor-stimulated cell proliferation

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Anti-hormone therapies such as Tamoxifen, are widely used to treat breast cancer patients. A small but significant number of

patients receiving tamoxifen will not respond or will develop resistance. Although many mechanisms have been suggested which may play a role in tamoxifen resistance, the mechanisms have not yet been fully elucidated. Tamoxifen resistant breast cancer (TamR) cell lines have been shown to have increased expression of a number of cell signalling mediators including EGF receptor [1], ERK-1/2 [2], Src [3] and PKC α and δ isoforms [4]. These cells are stimulated to proliferate by a number of growth factors including members of the Insulin like growth factor (IGF) family. We have previously shown that IGF-1 increases PKC- δ activity in the parent MCF-7 cell lines [5]. Now using adenoviral delivery of dominant negative forms of PKC- α and δ we have investigated the role of these PKC isoforms in tamoxifen resistant breast cancer cell proliferation.

IGF-I and II stimulate TamR cell proliferation 162% and 274% respectively v. control (n= 6). Expression of the PKC- δ dominant negative mutant inhibited control cell proliferation by 15% and significantly reduced growth stimulation by IGF-I (by 48%) and by IGF-II (by 70%). Similarly, the PKC- α dominant negative mutant inhibited control cell proliferation by 42%. Expression of dominant negative PKC- α inhibited IGF-I stimulated growth by 37% and IGF-II stimulated growth by 63%.

Thus increased expression of PKC- α and δ in tamoxifen resistant cell lines appear to contribute to the cells' proliferative response to both IGF-I and II, with PKC- δ appearing to have a particularly important role in IGF-II signalling.

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We would like to thank Prof R Nicholson for supporting this work, Prof David Murphy, Dr Carol Rickards and Dr Gavin Wilkinson for assistance with producing the dominant negative mutants and the Wales Office of Research and Development for Health and Social Care for providing Ian Lewis's PhD stipend.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC523

Pi3K constimulation in reproductive failure

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INTRODUCTION

The implantation and maintenance of Human pregnancy involves the immune system and the endocrine system; They regulate, communicate and interact closely by signal communication. Metabolic changes that result from physiological alterations at the boundary between mother and fetus, known as the FETOPLACENTAL UNIT (FPU). This interface is a major site of protein and steroid hormone production and secretion. Many

of the endocrine and metabolic changes that occur during pregnancy can be directly attributed to hormonal signals originating from FPU. Proper timing of these neuro-endocrine events within and between the placental, fetal and maternal compartments is critical in directing fetal growth and development as well as the coordinating the timing of parturition. The phosphatidyl inositol enzymes and other second messenger help in the communication and close interaction between the endocrine and the immune system.

PRINCIPLE

Signal communication between Hormonal receptors on the plasma membrane and the intracellular Ca²⁺ reservoir is accomplished by product of phosphatidylinositol metabolism thereby phosphatidylinositides are involved in all growth and signal transduction and are precursor to many other secondary messenger molecules involved in regulation of cellular activities.

LABORATORY ANALYSIS

120 women used for this research were grouped into six of (20) viz spontaneous aborters, primary infertile, secondary infertile, (Test group). Multiparous, pregnant and non pregnant (control group). Antithyroid antibody test, Pi3k assay and Ca 2+ Assay were done.

STATISTICAL ANALYSIS

Data of the parameters were measured as mean $\pm$ standard error of mean values. Differences between mean values were analysed using the t test. P < 0.05 was considered significance while > 0.05 were insignificant.

CONCLUSION

The unregulated increase in pi3k resulted into autoimmune disorder seen in the test sample, when mean of 72.3% failed Antithyroid antibody test. While means of 84% of the control sample passed the Antithyroid antibody tests. Therefore unregulated changes in Pi3k plays a critical role in modulating the metabolic and immunologic changes required for successful outcome in pregnancy.

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PC524

Optimisation of 2-D gel electrophoretic separation of dystrophic cardiac muscle

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Duchenne muscular dystrophy is characterized by a lack of the protein product of the dystrophin gene. Loss of dystrophin

interrupts the connection between extracellular matrix and intracellular cytoskeleton. The disease effects skeletal muscle with muscle weakness and degeneration. Cardiac muscle is also affected by the replacement of fibres by fatty tissue. Of the 90% of patients with the disease about 20% develop serious cardiomyopathies resulting in death. Mdx mice lacking dystrophin compare clinically to the human form. Crude sample preparation was carried out on total cardiac mouse muscle. Reagents for lysis buffers were chosen based on their effects, such as solubility, ability to act as reducing agent or reaction with other buffer components. Variations in these buffers were used to determine the electrophoretic effect on cardiac proteins. Separation was carried out by 2D-gel electrophoresis. Staining was carried out with colloidal comassie and proteins of interest were identified by MALDI-ToF mass spectrometry.

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PC526

Computational methods for the study of enzyme reaction mechanisms

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Enzymes catalyze chemical reactions. The protein lowers the overall activation energy needed for the reaction compared to the same reaction in solution. There are various 'strategies' of enzymes to achieve this, ranging from providing a suitable scaffolding for the reaction, to the active (covalent) participation of amino acid residues in the mechanism. A detailed understanding of the reaction mechanism in the enzyme environment is often critical for the design of efficient inhibitors or the engineering of proteins to perform different tasks.

The mechanisms can be studied computationally by determining the possible intermediates and how they are connected. Since the mechanisms involve bond breaking and forming, the computational study requires Quantum Mechanical (QM) methods. However, the computational cost of these methods scales unfavorably with the size of the system, and even state-of-the-art techniques cannot be applied to an entire enzyme.

To reduce the computational cost of conventional QM methods, one can employ hybrid QM/MM schemes. The molecular system is partitioned into a QM region and an (inexpensive) Molecular Mechanics (MM) region. The bond breaking and formation must be localized in the QM region, while the effect of the (protein) environment is treated at the MM level of theory.

Insights in the enzymatic mechanisms can be obtained from mapping the QM/MM potential energy surface.

We present methods for the investigation of QM/MM potential surfaces. Besides the location of equilibrium structures, our methods also allow the location of the connecting transition states that determine the activation energies. Normal mode analysis can then be used to characterize the resulting critical points, while reaction paths can be calculated to confirm the relationships between the critical points. The strengths of our methods are in the very high level QM methods that can be incorporated in the QM/MM scheme, the rigorous description of the QM/MM potential surface, and the ability to fully optimize the critical points.

We demonstrate our methods using various examples, such as the hydrogen peroxide reduction by Selenoprotein Glutathione Peroxidase and thermal isomerization of retinal in rhodopsins.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC527

2-D profiling of changes in N-linked glycosylation in aged rat skeletal muscle

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Sarcopenia is the rapid loss of muscle mass and strength during ageing. It can have a severe effect on the quality of life of the elderly. Exercise can protect and to a certain degree replace some of the lost muscle mass, but at a certain point the muscles' population of stem cells are depleted and muscle can no longer be rebuilt. Several factors are thought to cause sarcopenia such as reduced hormone levels, altered protein metabolism, degeneration of the nervous system and lifestyle.

Some other diseases that can cause muscle wasting such as Duchenne muscular dystrophy and type-II diabetes are known to have altered protein glycosylation. With all this in mind we are studying the changes in the aged muscle glycoproteome.

To do this a combination of 2-D gel electrophoresis and blotting were used to separate and immobilise proteins to a nitrocellulose membrane while peroxide-conjugated lectins were used to identify particular types of carbohydrate moieties on the muscle proteins.

N-linked glycosylation can play many vital roles in the way a protein functions; it can affect protein folding, protein degradation, targeting of proteins to intracellular organelles, as well as more widespread functions such as cell-cell communication. So through this work we are hoping to identify changes in the glycosylation and understand its effects on muscle ageing.

This work was supported by a principal investigator grant from Science Foundation Ireland (SFI-04/IN3/B614), as well as equipment grants from the Irish Health Research Board (HRB-EQ/2003/3, HRB-EQ/2004/2).

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PC528

A new method for studying single muscle fibre mechanics under 'natural' loading conditions

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The mechanical properties of muscle are traditionally studied under isotonic or isovelocit conditions. In contrast, during real-life movements isotonic or isovelocit conditions are unlikely, at least partly because loads to the muscle may comprise a combination of inertial, elastic, viscous, frictional, gravitational and/or other components. The responses (e.g. force output) to such loading may be complex and are not well understood. To understand them, we are attempting to examine simple systems but with more 'realistic' loading conditions before we add more complexity. In particular, we have developed a method to study the mechanical output of single, skinned fibre segments as they actively shorten against loads with "virtual" elastic and inertial components. Using single fibres enables direct measurements of fibre length changes and force.

Single skinned fibre segments are prepared from needle biopsy material from the human vastus lateralis muscle and mounted between a force transducer and a motor (He et al. 2000). Once fully activated (pCa 4.5) under fixed-end conditions, a rapid length change slackens the fibre. The resulting drop in tension triggers a newly designed feedback control circuit which, upon tension redevelopment, drives the motor such that the fibre shortens as though against a mass-spring system. The input to the circuit is the force applied by the fibre to the force transducer. Its output at any instant is the addition of two signals: one that is proportional to the double time integral of the force signal (inertial component) and one that is proportional to the force signal (elastic component). Figure 1 illustrates results from a trial.

This system allows the study of the mechanical responses of active single muscle fibres shortening against added series elasticity and inertia. More complexity may be added to the experiments by, for example, increasing load complexity, or by using intact muscle preparations. This provides a systematic approach to the study of how the complex response patterns observed during real-life movements are synthesized from simpler components.

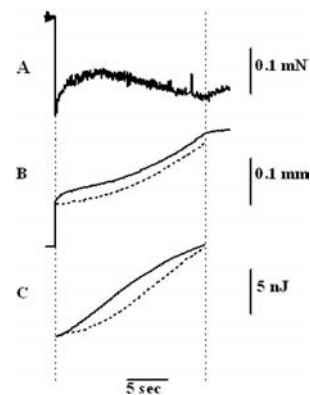


Figure 1. Time-course of some mechanical variables. A: Force; B: Fibre shortening (solid) and distance moved by virtual mass (dash); C: Work done by fibre (solid) and kinetic energy of mass (dash). Vertical cursors indicate the feedback control period. Initial fibre length 0.47 mm; average diameter 73 μm ; virtual compliance 0.36 m N^{-1} ; virtual mass 58 kg; temperature 11.0 $^{\circ}\text{C}$.

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PC529

An investigation into KCNQ channel expression in rat dorsal root ganglion (DRG) and axonal neuroma following a neuropathic pain model

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Neuropathic pain (NP) is a severe health problem which is further complicated by the lack of effective therapy. The search for new treatments have recently focussed on ion channel candidates; expression and activity of many ion channels relevant to pain signalling have been found to be altered following nerve injury (Markman & Dworkin, 2006). There is compelling evidence for the role of the M-type K^{+} channels in pain transmission as the membrane excitability of small nociceptive DRG neurons is tightly controlled by M channel activity (Passmore et al. 2003). To examine the importance of the M current in the development of ectopic firing produced by the neuroma, we are examining KCNQ2, KCNQ3 and KCNQ5 (M channel subunits) expression within the sciatic nerve neuroma and DRG of partial sciatic nerve ligation (PSNL) operated rats. Adult male Wistar rats were subjected to PSNL or sham surgery.

Under general anesthesia with 2% isoflurane, the nerve was exposed, partially ligated and sectioned. Quantitative RT-PCR and immunocytochemistry were used to reveal changes in KCNQ

expression levels in DRG and sciatic nerve neuroma 14 and 28 days following PNL. Preliminary data suggest an increase in ipsilateral DRG KCNQ2 expression following PNL in comparison to contralateral KCNQ2 expression. These studies seek to reveal the importance of M channel expression in the initial stages following peripheral nerve injury, both at the primary sensory neuron soma and at the site of nerve injury. This builds on investigations suggesting that KCNQ channels may be a novel analgesic target for neuropathic pain.

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PC530

Over-expression and characterization of soluble form of synthetic human bone morphogenetic protein-2 in *E. coli*

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Biologically active bone morphogenetic protein-2 (BMP-2) has been produced from *Escherichia coli* through in vitro refolding of inclusion bodies, but the refolding procedure is complicated and the overall yield is low or the refolding buffer contains additional reagents. Our goal was to develop a new, simple and inexpensive method to improve the production of biologically active hBMP-2. The design of a 345-bp synthetic hBMP-2 gene was based on the amino-acid sequence of hBMP-2. A DNA sequence containing 11 restriction sites located approximately every 30 bp throughout the entire length of the coding region was selected from the large number of possibilities. The codon usage of the resulting BMP-2 gene was modified to include those triplets that are utilized in highly expressed *E. coli* genes. A ribosome binding site was added 10 bases upstream of the coding region to direct the initiation of translation in *E. coli*. The spacer region (-1 to -9) was made A/T rich to reduce potential mRNA secondary structure in the vicinity of the translation start site. The hBMP-2 gene was assembled into pUC18, subcloned into pCold I vector, and over-expressed as a soluble protein in Origami B(DE3). hBMP-2 was homogeneously purified using heparin-Sepharose and FPLC Resource-Q column. hBMP-2 dimer (24 kDa) possessed similar level of activity as hBMP-2 from CHO cells, whereas hBMP-2 monomer (12 kDa) did not show any biological activity.

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PC531

Developmental changes in the electrophysiological properties of intracardiac ganglion neurones of the mouse *in situ*

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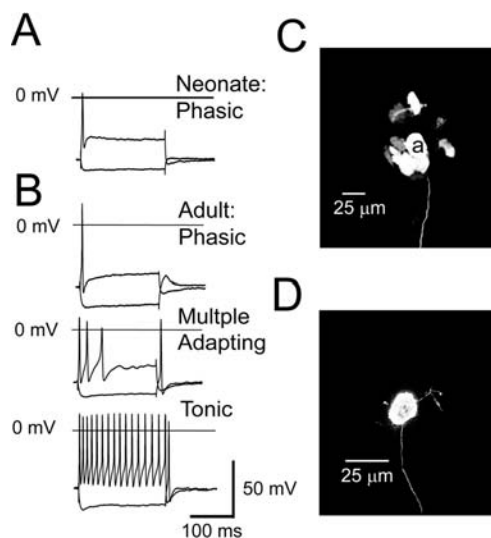
Within intracardiac ganglia (ICG) of adult animals different neurone types have been identified and classified according to their electrophysiological properties, the nature of the synaptic input they receive, and their morphological characteristics. These differences have been proposed to underpin differences in function.

The intrinsic properties of a neurone are not static, but can alter during development by the up- or down-regulation of expression of ion channels and their subunits. Recent reports have documented developmental changes in expression of GABA_A receptor channels and SK channels in rat ICG neurones (Fischer et al., 2005; Harper & Rimmer, 2006).

Diversity in the electrophysiological and morphological characteristics have been observed in ICG neurones from different mammals.

We have carried out studies to determine the neuronal types in the neonatal (5-9 days) and adult (> 6 weeks) BALB/c mouse. This information will be used to organize the characterization of specific genetically modified animals.

The electrophysiological properties of mouse ICG neurones undergo postnatal differentiation. Comparison of the discharge activity evoked by depolarizing currents in neonates and adults reveals marked differences. Neurones of neonates (6/6) fire only a single action potential over a large range of steps, but neurones in adult ganglia exhibit a wide range of activities from phasic (7/17), multiple adapting (8/17) to tonic discharge (2/17). The input resistance of neonates was in several instances unexpectedly similar to that of adults. Cell capacitance (C_i) was calculated from C_i = τ_m / R_i to provide an index of the size of the neurone. The reduction in R_i and increased C_i measured for neurones in the neonatal ganglion is consistent with gap junction coupling, increasing their apparent electrical size. Dye coupling (Neurobiotin) was found in 2/6 neurones in neonates but not in adults. These studies indicate changing patterns of expression of ion channels with maturation of the cardiac nervous system



Voltage responses obtained in response to long (200 ms) de- and hyperpolarizing current pulses, (2x threshold and -0.1 nA) recorded from neonatal (A) and adult ICG (B) neurones. Confocal images of a neonatal (P5) mouse intracardiac ganglion (C) showing presumptive evidence for dye-coupling between neurones (neurone 'a' was injected with the gap junction permeable tracer Neurobiotin, which spread to neighbouring neurones) and an adult mouse intracardiac ganglion neurone (D).

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PC532

Induction of 5-HT_{1A} receptor and NUDR mRNA in differentiated SHSY-5Y neuroblastoma cells

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Serotonin (5-HT) is an important neurotransmitter involved in depression and other affective disorders (Lucki, 1998). The release of 5-HT in neurons is mediated by somatodendritic 5-HT_{1A} autoreceptors (Ou, 2000). The presence of 5-HT_{1A} receptor is thought to be increased in depressed patients, producing a reduction in the synthesis of 5-HT (Lemondé, 2003). The nuclear DEAF-1 related (NUDR) protein represses the 5-HT_{1A} promoter region regulating both 5-HT_{1A} transcription and receptor expression (Lemondé, 2003). The SHSY-5Y neuroblastoma cell line is a well characterised system used in neurotransmitter studies. The aim of this study was to determine the presence of 5-HT_{1A} receptor and NUDR mRNA in this cell line at different time-points by real-time PCR, when differentiated with both nerve growth factor (NGF) and aphidicolin or retinoic acid (RA).

The SHSY-5Y cells were grown for different lengths of time (0, 3, 5, 7, 10 and 14 days) in the presence of either RA (10⁻⁵M) or NGF (100ng/ml) and aphidicolin (0.3μM) (LoPresti, 1992). RNA was extracted using SV total RNA isolation kit (Promega). cDNA was synthesised using Iscript cDNA synthesis kit (Biorad). For Real-time PCR relative expression ratios were normalised against housekeeping genes ubiquitin C (UBC) and tryptophan 5-monooxygenase activation protein zeta polypeptide (YHWAZ). Data are presented as means (±SEM). Values represent the relative fold change in 5-HT_{1A} mRNA compared to undifferentiated cells (time point 0).

At day 5 following stimulation with RA a significant 17.2±6.4 fold increase (n=3, p<0.01) in 5-HT_{1A} receptor mRNA was determined relative to undifferentiated cells (n=3). However, after day 5, 5-HT_{1A} receptor mRNA levels declined and the change in expression observed was not significantly different relative to undifferentiated cells (7-days 1.5±0.2, 10-days 0.6±0.2, 14-days 4.1±1.5, n=3). Differentiation with NGF and aphidicolin significantly increased 5-HT_{1A} mRNA levels at 10 days producing an 426±205 fold increase relative to undifferentiated cells (n=6, p<0.01). However, by 14 days of stimulation with NGF and aphidicolin, 5-HT_{1A} expression had significantly declined to a 4.2±1.8 fold (n=6) change in expression relative to control. A 2.6±1.7 fold increase in NUDR expression was observed at day 5 after treatment with RA (n=3). At day 10 cells differentiated with NGF and aphidicolin a 44.8±27.7 fold increase in NUDR expression was observed (n=6).

In conclusion, the results demonstrate that 5-HT_{1A} receptor and the 5-HT_{1A} promoter repressor NUDR mRNA are expressed in the SHSY-5Y cell line when differentiated with either NGF and aphidicolin or RA. The differentiated SHSY-5Y cell line may provide a model that will be useful to investigate 5-HT_{1A} receptor function.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC533

Transdifferentiated hepatocytes: an *in vitro* model for liver function

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Transdifferentiation is the conversion of one differentiated cell type to another with or without intervening cell division. We have previously demonstrated that treatment of the pancreatic cell line, AR42J-B13 (B13) with the glucocorticoid Dexamethasone (Dex) induces a transdifferentiation to a hepatic phenotype. Previously, we have demonstrated that a range of pancreatic markers are down regulated and liver markers are

induced during transdifferentiation. Here we have described our analysis of the functional nature of the transdifferentiated hepatocytes. The normal liver plays a major role in metabolic homeostasis, synthesis of plasma proteins, enzymes and cofactors and is crucial for detoxification. We determined the ability of the transdifferentiated hepatocytes to synthesise urea, glycogen, receptor mediated uptake of low density lipoproteins and metabolise xenobiotics via Phase I metabolising enzymes. Additionally, we have shown that the transdifferentiated hepatocytes are delineated directly from the pancreatic cells with no intervening cell division. For the first time, we describe here that transdifferentiated hepatocytes are able to integrate into the liver when transplanted into NOD/SCID mice. Our results give every indication that hepatocytes transdifferentiated from pancreatic cells are an effective model for studying liver function and have the potential to rescue liver disease.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC534

Calcium signalling in interstitial cells and smooth muscle cells in the guinea-pig bladder

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Interstitial cells (IC) have been shown to exist within the wall of the guinea-pig bladder (1). Previous work has shown that guinea-pig bladder IC display spontaneous Ca^{2+} signals (1, 2) and isolated IC respond to cholinergic stimulation by firing Ca^{2+} -transients. The aim of the present investigation was to study Ca^{2+} -signalling in IC and smooth muscle cells in vitro preparations. Bladders were removed from guinea-pigs, killed by cervical dislocation in accordance with Schedule 1 UK Home Office regulations. Small preparations, containing a few smooth muscle bundles were loaded with fluo-4AM and imaged using a confocal microscope.

After washing with Krebs to remove excess indicator, areas of the tissue in which spontaneous Ca^{2+} rises occurred were imaged with the confocal microscope. Two types of activity were typically present; Ca^{2+} -waves in smooth muscle cells (see below) and integrated, co-ordinated flashes in the whole smooth muscle bundle (amplitude F/F_0 0.61 ± 0.07 , duration 3.5 ± 0.5 sec, frequency 3.8 ± 0.9 per minute, $n=7$). In addition, spontaneous Ca^{2+} transients were observed in the IC found on the edge of the bundles or in the interstitium between two or more bundles. The Ca^{2+} -events in smooth muscle cells and IC were markedly different. The smooth muscle cells fired regular signals of 2.3 ± 0.2 s duration, amplitude 0.21 ± 0.03 at 9.2 ± 1.7 per minute ($n=8$) whereas the IC fired transients of duration 12.2 ± 2.4 s, amplitude 0.49 ± 12 , and less frequently at 1.3 ± 0.3 /min.

Application of $10 \mu\text{M}$ nifedipine abolished the co-ordinated whole bundle flashes but did not prevent Ca^{2+} -waves in individual smooth muscle cells (at 6.1 ± 1.6 /min control to 4.9 ± 1.0 /min in drug, $n=4$) nor the transients in IC (from 1.91 /min to 0.79 /min in nifedipine, $n=4$). Activity of the cells

was enhanced by depolarizing the tissue with 60mM K^+ external solution. Smooth muscle cells and IC both responded with an initial large increase in intracellular Ca^{2+} followed by oscillatory events. This was often accompanied by tissue contraction. In smooth muscle cells, carbachol ($30 \mu\text{M}$) increased frequency from 11 ± 1 /min to 21 ± 2.9 /min, $n=5$). Carbachol caused large Ca^{2+} transients (0.34 ± 0.02 amplitude) in IC followed by several oscillations.

In summary, spontaneous Ca^{2+} -signalling occurs in both smooth muscle cells and IC in the guinea-pig bladder. The signals differ significantly between the two cell types. Co-ordination of global flashes in bundles relies on influx through voltage-dependent channels. Both cell types responded to cholinergic stimulation.

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2. Hashitani H, Yanai Y, Suzuki H. (2004) J Physiol. 559:567-81

This work was funded by The Wellcome Trust and QUB School of Medicine

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC535

Spontaneous Ca^{2+} signalling in interstitial cells from the guinea-pig bladder

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Interstitial cells from the guinea-pig bladder have been reported to display spontaneous changes in intracellular Ca^{2+} (1, 2). This signalling has not yet been characterised and its functional significance is unknown. The aim of the present investigation was to study spontaneous Ca^{2+} -activity in isolated bladder interstitial cells. Bladders were removed from guinea-pigs which had been killed by cervical dislocation in accordance with UK Home Office regulations. Interstitial cells were enzymatically dispersed from small pieces of detrusor as previously described (1), loaded with fluo 4AM and imaged with a confocal microscope. Cells were washed with Hanks solution for 30 minutes to remove excess indicator before the commencement of recordings. The majority of IC did not display large, regular changes in intracellular Ca^{2+} , however, spontaneous activity was seen in an estimated 10% of cells. Ca^{2+} -signals had mean half-width durations of 8.8 ± 2.9 s (data from 7 cells), however the range was from 2.8 s to 23.9 s indicating the variability of the events. IC fired Ca^{2+} -transients at the rate of 10.5 ± 4.2 per minute and the mean amplitude, F/F_0 was 0.73 ± 0.2 ($n=7$ cells). The spontaneous activity persisted in the presence of xestospargin C, a blocker of IP_3 receptors. Application of $10 \mu\text{M}$ carbachol caused a large increase in intracellular Ca^{2+} and prevented generation of spontaneous signals ($n=6$). Normal activity returned on washout of the agonist. The findings of the present investigation suggest that bladder interstitial cells exhibit spontaneous Ca^{2+} signalling and that this activity is affected by cholinergic stimulation. McCloskey KD and Gurney AM. (2002) J. Urol. 168(2): 832-836.

Hashitani H, Yanai Y, Suzuki H. (2004) J Physiol. 559:567-81.

Funding from the Wellcome Trust and QUB School of Medicine is gratefully acknowledged.

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PC536

Reaction time and skin potential level of women under stressful conditions

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Background: It is known, that individual differences in the lateralized performance have important implications for individual differences in patterns of cognitive ability. (Banich M.T. et al, 1992). It was shown, that the right hemisphere emotional activation in the greater degree influences autonomic functions such as vasoconstriction responses and heart rate. (Spence S., 1996). It is considered, that Skin Potential Level (SPL) displays arousal level (Nishimura C., Nagumo J., 1995), and decreases in the state of weariness (Gorgo Yu., 1999).

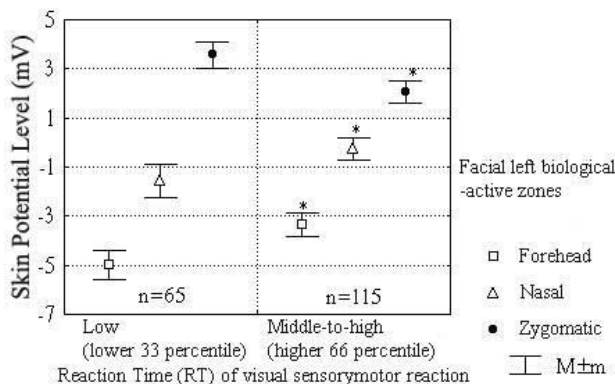
Objective: We have assumed that SPL displays an individual performance level.

Methods: For this purpose we carry out researches, in which performance level estimated on the Reaction Time (RT) of visual sensorymotor reaction. SPL was measured (Ag-AgCl electrodes with high (40 MΩ) entrance resistance DC amplifier) in five facial biological-active zones - forehead, superciliary, nasal, zygomatic and auricular (Gorgo Y., 1986) in healthy women (16-26 y, n=180) in the calm condition (CC) and in the stressed condition (SC) (during examinations).

Results: The SPL in three left facial zones significantly differed in groups of subjects with low (lower 33 percentile) and middle-to-high level RT (higher 66 percentile) in SC (fig.1). In the CC group such an interrelation wasn't revealed.

The higher performance level and sympathetic nervous system activation leads to SPL in forehead and nasal facial biological-active zones become more negative and in zygomatic zone it become more positive.

Conclusion: Facial SPL can display a performance level of women only with a high arousal level baseline (stress activation). The common increased emotional level is accompanied by increase of sympathetic nervous system activity and results to more expressed electrodermal reactions.



PC537

Cyclic AMP-mediated induction of suppressor of cytokine signalling-3 (SOCS3) through EPAC1 and C/EBP transcription factors

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Exchange protein directly activated by cAMP (EPAC) mediates protein kinase A (PKA)-independent cAMP signalling pathways. We recently showed that EPAC is responsible for cAMP-mediated induction of suppressor of cytokine signalling-3 (SOCS3) in human umbilical vein endothelial cells (HUVECs)(1). Using an EPAC-selective cAMP analogue (8-pCPT-2'-O-Me-cAMP) and siRNA knockdown of EPAC1, we have demonstrated that cAMP-mediated induction of SOCS3 in murine embryonic fibroblasts (MEFs) is also EPAC-dependent. MEFs null for CCAAT/enhancer binding proteins (C/EBPs) β or δ did not show increased expression of SOCS3 in response to EPAC activation; additionally, electrophoretic mobility shift assays and functional chromatin immunoprecipitation assays showed both an increase in C/EBP binding activity to consensus binding sites, and specific binding of C/EBPs to the SOCS3 promoter when HUVECs were treated with forskolin or 8-pCPT-2'-O-Me-cAMP. Transient overexpression of C/EBPs in HUVECs increased SOCS3 induction after EPAC activation, while antisense knock-down had the opposite effect. These data demonstrate that EPAC mediates cAMP-induced SOCS3 induction through the C/EBP family of transcription factors, and this represents a previously unrecognised mode of cAMP-controlled gene expression.

Sands W.A. et al. (2006) *Mol Cell Biol* 26, 6333-6346

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC538

Identification of functionally important residues in transmembrane domain 1 (TMD1) of rabbit PepT1, expressed in *Xenopus* oocytes

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PepT1 mediates the intestinal absorption and renal re-absorption of di- and tri-peptides and a great number of therapeutically active compounds (reviewed in Daniel et al., 2006). A 12 transmembrane domain (TMD) topology was proposed originally (Fei et al., 1994) and largely confirmed by epitope mapping, with the exception of TMDs 1&2 (Covitz et al., 1998). Recently, re-analysis of the sequence and homology modelling

of PepT1 have suggested that TMD1 may have been mis-assigned (Meredith & Price, 2007). Rather than being amino acid residues 7-25, TMD1 is predicted by MEMSAT-3 to be formed from residues 24-42. Homology modelling suggests TMD1 will be tilted relative to the bilayer and therefore to be membrane spanning will need to be longer, and would be formed by residues 13-42. This re-assignment would put the conserved charged residues E23, E36 and R34 into TMD1; interestingly, E23 and E26 are conserved in one of the signature motifs for the dipeptide/tripeptide permease superfamily PTR2 (Daniel *et al.*, 2006), while R34 is strongly conserved in higher organisms. Here we test whether these residues play a functional role in rabbit PepT1.

The mutants E23R, E26R, R34E and R34Q were generated using a PCR based protocol on rabbit PepT1-FLAG epitope tagged template (wt-PepT1), and expressed in *Xenopus* oocytes. Both membrane expression and the transport properties were determined by luminometry and uptake of $0.4\mu\text{M}$ [^3H]-D-Phe-L-Gln respectively using techniques previously described (Panitsas *et al.*, 2006). Data are means $\pm$ SEM.

As can be seen in Figure 1A, the uptake of D-Phe-L-Gln into oocytes expressing E23R-, E26R-, R34E- or R34Q-PepT1 at pH_{out} 5.5 was not significantly greater than that of non-injected oocytes, in contrast to those expressing wt-PepT1 ($p > 0.05$, Student's *t*-test, $n = 5$ oocytes per data point, representative of up to 4 preparations). When the level of protein surface expression was quantified by luminometry (Figure 1B), it is clear that E23R- and E26R-PepT1 are not expressed at the membrane, whereas wt-PepT1, R34E- and R34Q-PepT1 were significantly expressed ($p < 0.001$, $n = 12$ -18 oocytes per data point).

These findings suggest that the residues mutated are crucial for functional rabbit PepT1 expression. E23 and E26 are implicated in proper protein folding/trafficking, as when mutated neither was expressed, whereas R34 is important for PepT1 function, as there was no dipeptide uptake despite membrane expression.

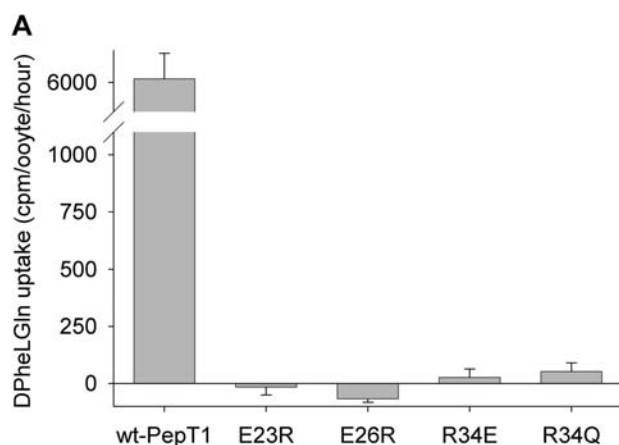
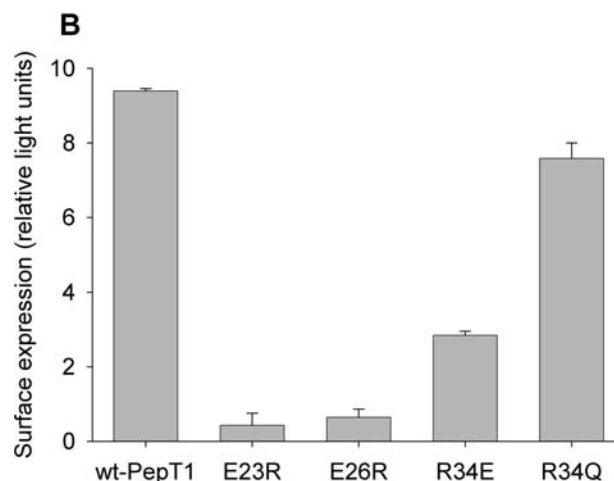


Figure 1: (A) PepT1-dependent uptake of [^3H]-D-Phe-L-Gln and (B) transporter surface expression in *Xenopus* oocytes expressing wt-PepT1, E23R-, E26R-, R34E- or R34Q-PepT1.



Daniel *et al.* (2006) *Physiology* **21**, 93-102

Fei *et al.* (1994) *Nature* **368**, 563-566

Covitz *et al.* (1998) *Biochem* **37**, 15214-15221

Meredith & Price (2007) *J Memb Biol*, in press

Panitsas *et al.* (2006) *Pflugers Arch* **452**, 53-63

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC539

Synovial hyaluronan secretion shows graded coupling with joint movement, not dependent on protein synthesis *de novo*

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Introduction. Hyaluronan (HA) regulates trans-synovial fluid drainage and aids joint lubrication. HA secretion (q_{HA}) is stimulated by joint movement (Wann *et al.*, this meeting), in keeping with its arthro-protective role. Here we tested the hypothesis that movement-stimulated q_{HA} is a graded response affected by movement duration and/or cycle frequency. We also tested whether movement-stimulated HA secretion (MSHA) requires transcription-translation.

Methods. Endogenous HA was washed out from cannulated knee joints of anaesthetised rabbits. The joint was left for 5h, either static or moved, then the secreted HA was recovered by further washouts. HA was analysed by HPLC. Moved joints were cycled passively at 0.5Hz for 0, 7%, 20% or 60% of the secretion period in the pattern 0, 1, 3 or 9 min in every 15min for 5h. In a second series joints were cycled for 20% duration (3/15 min) at 0, 0.17Hz, 0.5Hz or 1.5Hz. To investigate whether MSHA requires *de novo* protein synthesis, moved joints injected with cycloheximide (CX, 0.36mM) were compared with paired, Ringer-injected moved joints. As positive controls, q_{HA} was measured in static joints stimulated by phorbol-12-myristate-13-acetate (PMA, 0.35mM) in the presence or absence of CX. In a further study

moved joints were injected with actinomycin-D (AD, 11mM) and puromycin (PM, 0.3mM) to block both transcription and translation.

Results. Since joints vary in size, q_{HA} was normalised per 100 mg endogenous HA ($\%q_{HA}$). The $\%q_{HA}$ varied with the duration of movement, falling significantly from $7.82 \pm 0.52 \text{ mg h}^{-1}$ (100 mg^{-1}) ($\pm$ sem) at 60% duration ($n=5$) and $7.93 \pm 0.50 \text{ mg h}^{-1}$ (100 mg^{-1}) at 20% duration ($n=19$) to $5.29 \pm 0.25 \text{ mg h}^{-1}$ (100 mg^{-1}) at 7% duration ($n=5$) and $3.65 \pm 0.27 \text{ mg h}^{-1}$ (100 mg^{-1}) in static joints ($n = 24$) ($p=0.02$, 1-way ANOVA). Cycle frequency increased $\%q_{HA}$ from $4.87 \pm 0.49 \text{ mg h}^{-1}$ (100 mg^{-1}) in joints moved at 0.17 Hz to $7.27 \pm 0.63 \text{ mg h}^{-1}$ (100 mg^{-1}) in joints moved at 1.5 Hz. ($p=0.014$, $n=6$ pairs, paired t-test). Inhibition of translation by CX had no significant effect on MSHA (CX moved, $7.79 \pm 1.55 \text{ mg h}^{-1}$ (100 mg^{-1}); Ringer moved, $9.37 \pm 2.21 \text{ mg h}^{-1}$ (100 mg^{-1}) ($p=0.12$, $n=5$ pairs, paired t test). In positive controls CX reduced PMA stimulated $\%q_{HA}$ from $13.17 \pm 2.57 \text{ mg h}^{-1}$ (100 mg^{-1}) to $6.40 \pm 0.97 \text{ mg h}^{-1}$ (100 mg^{-1}) ($n = 3$ pairs). As with CX, AM+PM-treated moved joints showed no reduction in $\%q_{HA}$ ($p=0.29$, $n=5$ pairs, paired t test).

Discussion. HA secretion is stimulated by joint movement in a graded fashion, dependent on the duration and frequency of joint movement. The mechanosensitive transduction process was not dependent on transcription-translation.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC541

Role for phospholipase A_2 in tumour necrosis factor- α -mediated cytoprotection

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Introduction: The pleiotropic cytokine tumour necrosis factor- α (TNF- α) and the cytosolic phospholipase A_2 (cPLA $_2$) are involved in the cellular inflammatory response and ischaemic injury. During ischaemia TNF- α is endogenously generated by muscle (1,2) activating cPLA $_2$ (3). In addition to this, low dose TNF- α has been shown to confer cytoprotection in muscle (2). Furthermore, cPLA $_2$ activation is known to result in the generation of mediators of cellular injury, apoptosis and necrosis (4). We have therefore investigated the role of cPLA $_2$ in low dose TNF- α mediated cytoprotection in a muscle cell line.

Methods: Murine C2C12 myoblasts were grown to 50% confluency and differentiated into myotubes. Myotubes were then included in one of the following experimental groups: normoxic control; ischaemic control; ischaemic preconditioning (IPC); TNF- α preconditioning (TNF-PC); and TNF-PC with AACOCF3 (cPLA $_2$ inhibitor, TNF-I). Following a simulated ischaemic insult cell viability was determined using trypan blue exclusion. In addition cPLA $_2$ phosphorylation state and cellular localisation was assessed using western blotting and fluorescence immuno-histochemistry combined with 3-D imaging techniques and analysis.

Results: Exposure to simulated ischaemia reduced cell viability when compared to the normoxic control (from $81.6 \pm 2.0\%$ to

$24.8 \pm 3.9\%$, $n \geq 3$, $p < 0.05$). IPC, TNF-PC and TNF-I prevented the ischaemia induced decrease in cell viability ($57.6 \pm 3.7\%$, $65.8 \pm 3.4\%$ and $53.7 \pm 1.1\%$, $n \geq 3$, $p < 0.05$ vs. ischaemic control). cPLA $_2$ phosphorylation was reduced in the ischaemic control and TNF-PC groups ($41.1 \pm 0.5\%$ and $45.3 \pm 5.0\%$ respectively, $n \geq 3$, $p < 0.05$ vs. normoxic control). Phosphorylation was further reduced in the IPC group ($20.3 \pm 5.2\%$, $n \geq 3$, $p < 0.05$). In addition to these changes, differences were seen in the cellular localisation of the cPLA $_2$ in the various groups, with a predominantly cytosolic distribution seen in the normoxic control and ischaemic control groups, and a more nuclear localisation seen in the IPC and TNF-PC groups.

Conclusion: This data suggests an additional level of regulation in the TNF – cPLA $_2$ axis, with a possible role for cPLA $_2$ in deciding cell fate.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC542

High fat feeding of healthy mice alters metabolic and functional characteristics of hearts and myocytes

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We have recently shown that feeding apolipoprotein E knockout (apoE $^{-/-}$) mice a high-fat diet for 6 months accelerates progression of coronary disease and induces changes to cardiac metabolism and function. These changes could be due to coronary disease and/or direct effect of high-fat feeding. Wild-type mice are resistant to coronary disease (Breslow 1996) and therefore, when fed a high-fat diet, provide an excellent model to investigate the effect of high-fat diet on cardiac function, independent of coronary disease.

Male C57/129 wild-type mice were weaned onto a normal rodent diet, and then at approximately 8 weeks old, animals were either switched onto high fat, Western-type diet (21% fat; 0.15% cholesterol) or were maintained on normal rodent diet. Animals were fed their respective diets for 6 months. Histological examination of hearts confirmed absence of coronary disease in both groups. Isolated hearts from both groups were used to measure cardiac metabolites and function. Isolated myocytes were used to measure mitochondrial flux (NAD $^{+}$ /NADH), contractile function and calcium transients.

Mice fed high-fat diet had significantly increased levels of cardiac lactate (from 52 ± 4 to 76 ± 6 nmol/mg protein) and decreased glycogen content (from 0.09 ± 0.005 to 0.06 ± 0.008 mg/g wet weight) but had similar levels of energy rich phosphates. Evidence of metabolic stress in high-fat fed hearts was

confirmed in isolated perfused myocytes which showed increased NAD⁺/NADH ratio (0.34 ± 0.02 vs. 0.29 ± 0.01). These metabolic differences did not alter functional characteristics of isolated perfused myocytes and hearts. However the rate of rise and decline of calcium transients were slower in high-fat fed myocytes. Intact perfused hearts from high-fat fed animals were significantly more vulnerable to reperfusion injury than those from animals fed normal diet.

In conclusion, high-fat diet has profound metabolic effects on the myocardium that is independent of coronary disease. These changes appear to alter vulnerability to reperfusion injury.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC543

Microseconds protein-ligand molecular dynamics simulations by using a hybrid force field

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All-atoms molecular dynamics (MD) simulations are a very powerful tool to predict structural, dynamical, and thermodynamical properties of biological molecules and their complexes with ligands. Unfortunately, the current computational power constrains this analysis to time scales of ~100 ns, too short to follow several important biological processes, which evolve on a much longer time scale.

To bridge the gap between time scales of feasible simulations and those of biologically relevant processes, several simplified methods (coarse-grained models) have been proposed in which a small number of interaction sites are used to represent the systems [1-3].

Unfortunately, since the chemical details are often neglected, these models are not suitable for investigating processes involving molecular recognition. Nevertheless, such processes are usually highly localized and involve small protein portions.

A hybrid approach (MM/CG) has been developed in which a small portion of the protein (i.e. the binding pocket) is treated at the level of detail allowed by classical MD, while the remainder of the protein is treated at the CG level [4].

The method was tested on three proteins of great pharmacological relevance, belonging to the protease class (HIV-1 PR, human β -secretase and OmpT from *E. coli*) and was able to reproduce both the large-scale motions and the local features of the active sites of the considered proteins. Nevertheless, these simulations were performed in a few days, whereas the corresponding MD simulations would require months on the same computers. MM/CG approach emerges as a powerful tool to investigate long (in the order of μ s) dynamics of enzymes, which may impact on function, as well as an efficient and fast tool for computational structural biology. By allowing running more

numerous and longer simulations, MM/CG on one hand is expected to improve the confidence of the results and on the other, it may strengthen the interrelation between molecular biology experiments and simulations.

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PC544

Bicarbonate-dependent fluid secretion by the human airway cell line Calu-3

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The Calu-3 epithelial cell line expresses the cystic fibrosis transmembrane conductance regulator (CFTR) and is widely used as a model for the acinar cells of human airway submucosal glands. We have examined the ionic basis of cAMP-stimulated fluid secretion by control and CFTR knock-down Calu-3 monolayers cultured at the air-liquid interface. Exposure of control cell monolayers (4.8 cm²) to cpt-cAMP (200 μ M) + forskolin (10 μ M) evoked fluid secretion of 154 ± 11.8 μ l fluid/day, which was reduced to 20 ± 3.6 μ l/day in a Calu-3 variant stably expressing short hairpin RNAs complementary to CFTR mRNA. Control secretions contained ~25 mM bicarbonate and became strongly alkaline (pH 9.58 ± 0.14) when equilibrated with room air. Replacing basolateral HCO₃⁻ with Hepes and decreasing pCO₂ from 5% to 0.035% nearly abolished fluid transport and reduced the pH of secretions to 7.09 ± 0.01 . Acetazolamide inhibited fluid secretion by 37% under control conditions and abolished residual secretion under bicarbonate-free conditions, indicating a strict dependence on bicarbonate. Apical glybenclamide (100 μ M) and Inh-172 (10 μ M) inhibited fluid secretion by 30%, whereas apical DIDS (100 μ M) and basolateral bumetanide (10 μ M) had no effect. We conclude that sustained, cAMP-stimulated fluid secretion by Calu-3 monolayers involves CFTR but is strictly bicarbonate-dependent. The results are consistent with a model proposed previously for pancreatic ducts (Gray et al., 1988; Novak & Greger, 1988), where apical bicarbonate efflux is mediated by DIDS-insensitive anion exchangers, and CFTR channels mediate chloride recycling rather than transepithelial chloride secretion. Gray, M. A., Greenwell, J. R., & Argent, B. E. (1988). Secretin-regulated chloride channel on the apical plasma membrane of pancreatic duct cells. *Journal of Membrane Biology* **105**, 131-142.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC545

Plasma antioxidants, nitric oxide and trace metals in Plasmodium falciparum-infected pregnant Nigerian women

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Pregnancy and malaria are two aggravating conditions resulting in increase in the production of reactive oxygen species. Studies had been conducted to investigate the antioxidant status of pregnant females with or without malaria parasitemia. Twenty four Plasmodium falciparum infected patients attending Adeoyo Maternity Hospital, Ibadan, Oyo State were compared with their age matched non-malarial pregnant women who served as control group. Antioxidant enzymes (catalase and superoxide dismutase), total antioxidant activity, nitric oxide as an index of oxidative status, some haematological parameters and trace metals were measured. There were significant increases in catalase activities, selenium, iron and cadmium levels in the primigravidae malaria pregnant subjects as compared to their corresponding control group. Superoxide dismutase (SOD) activities and zinc levels are significantly decreased in malaria pregnant women in secundigravidae and multigravidae as compared to their corresponding control group. Zinc levels in the third trimester in the pregnant malaria subjects were lower as compared to their corresponding control group. There were increased oxidative stress and concomitant antioxidant levels in malaria in pregnancy as compared to their corresponding control group. Also, there was significant positive correlation between nitric oxide level and catalase activities in pregnant malaria group ($r = 0.918$, $P < 0.01$)

The incidence of malaria in pregnancy is highest in nulliparous as compared to primiparous and multiparous women. It may be concluded that defense mechanisms against free radical damage are enhanced as pregnancy progresses and that maternal anemia as indicated in the low level of haemoglobin in pregnant malaria subjects remains the most frequent consequence of malaria during pregnancy.

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We acknowledge the assistance of the staff of Adeoyo Maternity Hospital, especially Dr. A. Adekanmbi, the Chief Consultant of the hospital. Also, the ethical committee of the Oyo State Health Management Board, for the approval to carry out the studies and the permission to use the hospital facilities.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC546

N-methylated peptide inhibitors of β -amyloid aggregation and toxicity

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The key pathogenic event in the onset of Alzheimer's disease (AD) is believed to be the aggregation of the β -amyloid peptide ($A\beta$) into toxic oligomers. Molecules that interfere with this process may therefore act as therapeutic agents for the treatment of AD. N-methylated peptides (meptides) are a general class of peptide aggregation inhibitors that act by binding to one face of the aggregating peptide, but are unable to hydrogen bond on the other face, due to the N-methyl group replacing a backbone NH group. We optimised the structure of meptide inhibitors of $A\beta$ aggregation, starting with the KLVFF sequence that is known to bind to $A\beta$. We varied the meptide length, N-methylation sites, acetylation and amidation of the N- and C-termini, side chain identity and chirality, via five compound libraries. Inhibitor activity was tested by Thioflavin T binding, affinity chromatography, electron microscopy and an MTT toxicity assay. We found that inhibitors should have all-D chirality, have a free N-terminus, but an amidated C-terminus, and have large, branched hydrophobic side chains at positions 1–4, while the side chain at position 5 was less important. A single N-methyl group was sufficient. The most active compound, D-[(chGly)-(Tyr)-(chGly)-(chGly)-(mLeu)]-NH₂ (SEN304), was more active than a wide range of previously reported peptide inhibitors. Its related non-N-methylated analogues were insoluble and toxic.

A model for learning and memory is long term potentiation in a brain slice. While $A\beta$ inhibits long term potentiation, SEN304 is able to reverse this toxic effect at nanomolar concentrations. SEN304 is able to reduce the concentration of $A\beta$ oligomers by inducing their aggregation into a non-toxic morphology, as shown by light scattering, electron microscopy and gel electrophoresis.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC547

The arterial lactate to pyruvate ratio and cerebral blood flow velocity at the onset of exercise

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The mechanism(s) responsible for the increase in cerebral blood flow velocity (CBFV) at the onset of exercise is unknown but may relate to the arterial lactate-to-pyruvate ratio (L/P). In order to evaluate this hypothesis, blood was drawn from the radial artery and the internal jugular vein immediately before and during the first 120 s of ergometer cycling in 8 young trained males. The CBFV was appreciated with transcranial ultrasound Doppler. At rest, mean heart rate (HR) was 78 ± 15 (SD) bpm, mean arterial pressure (MAP) 108 ± 16 mmHg and CBFV 42 ± 9 cm s⁻¹. The arterial lactate concentration was 1.1 ± 0.3 mM, that of pyruvate 85 ± 45 μ M and the L/P ratio 13.1 ± 4.2 , and similar to the jugular vein (13.5 ± 2.3). Five seconds after the onset of exercise, HR was 100 ± 19 bpm, MAP 104 ± 17 mmHg, CBFV 43 ± 11 cm s⁻¹ and the arterial and venous L/P ratio 13.1 ± 3.8 and 12.9 ± 1.2 , respectively. The CBFV stabilised ~25% above baseline after 50 s of exercise, while the arterial and venous L/P ratio increased to 15.3 ± 2.7 and 14.7 ± 1.6 , respectively. Approximately 25% of the change in CBFV after 5 s and ~50% after 60 s of exercise could be attributed to changes in plasma L/P ratio. These results qualify the arterial L/P ratio as one possible signal for exercise induced elevation of cerebral blood flow.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

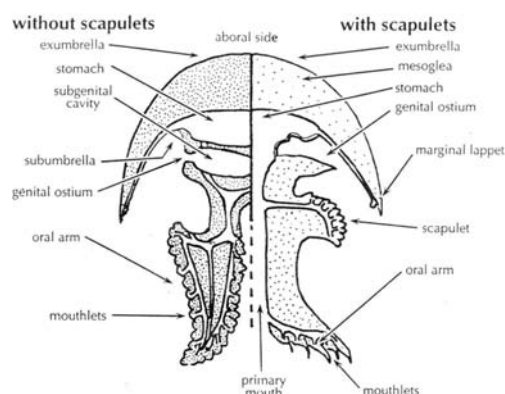
Eutima, Aequorea, Liriope, Diphyes, Lensia, Rhizostoma, Catostylus, Cassiopea). Includes: Phialucium carolinae, Eirene kambara, E. hexanemalis, Liriope tetraphylla, Diphyes disper, D. chamisonis, Obeliasp., Lensia subtiloides, Aequorea parva, Phiallelasp., Eutimalevuka, Octophialucium indicum, O. medium, Cassiopea andromeda, Catostylus perezii, Rhizostoma sp. The frequency and percentage of frequency for each species was measured. The most frequency was observed in Juvenile medusae (38/5%), E. kambara (22/18%), D. chamisonis (11/6%), Ph. carolinae (4/9%) respectively. In this study, the total abundance of medusae was 610/73 No/m³. the mean annual abundance of medusae was 43/7 No/m³. and the most mean abundance of medusae were observed in March ($23/71 \pm 8/35$ No/m³) but there was no medusae in September.

Among species Eirene kambara was the most dominant species in the year study.

Comparison frequencies in Doragh and Ghazaleh estuary showed no significant differences. The effect of temperature, salinity and pH as three main environmental parameter on medusae frequency investigated. Only significant relation was found between medusae frequency and temperature in two creek.

Indices of diversity showed the most diversity of month is March and the less diversity of month is January.

Diversity indices distinguished a median level diversity in medusae population in Western north of Persian Gulf.



PC548

Identification and determination of density of medusae in estuary Doragh and Ghazaleh in Iran

L.M. Dehmordi and A.S. Savari

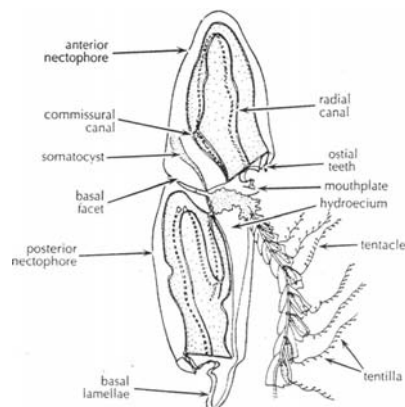
university, marine biology, Khoramshahr, khozestan, Iran

Abstract

This study was carried out on identification and density determination of Coelenterate medusae in Doragh and Ghazaleh creeks in Mahshahr area in Khozestan Province (Persian Gulf).

Coastal waters from July to June.

Monthly sampling were collected from 2 creek with 300 μ m mesh size plankton net in oblique tow from near bottom to surface. and sample of macro medusae were collected from local fisherman. In general: 16 species of medusae were identified belonging to 2 class (Hydromedusae, Scyphomedusae), 4 order (Leptomedusae, Trachymedusae, Siphonophora, Rhizostomeae), 11 family (Phialuciidae, Campanulariidae, Eirenidae, Aequoreidae, Geryoniidae, Diphyidae, Rhizostomatidae, Catostylidae, Cassiopeidae) and 13 genus (Phialucium, Octophialucium, obelia, Phialella, Eirene,



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PC549

Development of an *ex vivo* receptor occupancy assay for progesterone receptor antagonists

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Endometriosis is a widespread condition affecting approximately 10% of women of reproductive age worldwide (Eskenazi & Warner, 1997). Clinical studies have demonstrated the effectiveness of the progesterone receptor (PR) antagonist mifepristone (RU486), in the treatment of this disease (Kettel et al, 1996). The aim of this work was to establish a native tissue binding assay for determining the potency and *ex-vivo* receptor occupancy (RO) of PR antagonists in the rabbit.

With Pfizer ethical approval, sexually immature New Zealand White rabbits (700-900g) were dosed daily (q.d) for 2 weeks with 0.1mg/kg ethinyl estradiol. Animals were killed and snap frozen uterine tissues used for either homogenate binding (Issar et al., 2006) or autoradiography (ARG), adapted from Brown et al., 1995. Cryostat sections (20µM) or cytosolic tissue fractions (160µl) were incubated with 1.5nM [³H]R5020 (Perkin Elmer, UK) in the absence (total binding) or presence (non-specific binding) of RU486 (10µM). Dried tissue sections were imaged using a BetaImager for 8h. In homogenate binding experiments, bound and unbound fractions were separated via charcoal centrifugation. For IC₅₀ determination, sections were incubated with a range of concentrations (10pM–10µM). Specific binding (counts/mm²) was quantified using Betavision and IC₅₀ analysis performed using an in-house curve fitting programme. Following optimisation experiments to minimise incubation times, *ex-vivo* RO studies were performed comparing untreated animals with animals dosed q.d. with RU486 (3mg/kg) for 5 consecutive days. Animals were sacrificed 1h post-dose. Data are expressed as mean ± standard error of the mean.

Both homogenate binding and ARG produced specific binding >70% at PR. RU486, progesterone and dexamethasone inhibited [³H]R5020 binding with IC₅₀ values of 16.6±2.3nM, 57.2±11.7nM and >1µM respectively (n=4). In contrast to the reported 18h incubation for *in-vitro* studies (Brown et al., 1995), using our optimised conditions, we found no difference in % specific binding after 18h or 30min and therefore selected this incubation time for all subsequent *ex-vivo* RO studies. In tissue

sections from rabbits dosed with RU486 (3mg/kg), an effective dose in the rabbit McPhails test of endometrial arborisation (Elger et al, 2000), a mean RO of 74±3% (n=4) was calculated. In summary, this study has shown that ARG is a suitable method for determining the binding affinity of PR ligands in native rabbit endometrium. In addition, we have shown for the first time, optimised binding conditions for an *ex-vivo* RO assay for PR. The RO data obtained with RU486 in this pre-clinical model may be used as a benchmark to profile other PR ligands to build confidence in the prediction of clinically efficacious doses.

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PC550

Comparison of S-adenosyl-L-methionine and N-acetylcysteine protection of hepatotoxicity when given after acetaminophen exposure

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Acetaminophen (APAP) is a popular over the counter medication for treatment of pain and fever. APAP is relatively safe when taken at the recommended dose. However, in excessive doses, it is associated with hepatic toxicity. The purpose of the present study was to examine the protective effect of SAME when administered after APAP. The rationale for these studies was to evaluate the effectiveness of SAME when administered after APAP dosage in order to model what occurs in the clinical setting. A second goal of this study was to compare the potency of SAME to N-acetylcysteine (NAC), the current treatment for APAP toxicity. Male C57BL/6 (16-25 g) mice were used in all studies. Each treatment contained an n = 5 animals/group. The mice were randomly divided into 6 groups: vehicle (VEH), APAP, SAME, SAME plus APAP (SAME+APAP), NAC and NAC plus APAP (NAC+APAP). The animals were injected intraperitoneal (ip) with 250mg/kg APAP or water. One hr later, SAME and SAME+APAP groups were injected (ip) with 1.25 mmol/kg SAME. One hr after APAP or VEH injection, NAC and NAC+APAP groups were injected (ip) with 1.25 mmol/kg NAC. Hepatic toxicity was assessed 4 h after APAP injection by monitoring hepatic glutathione (GSH) levels, changes in alanine aminotransferase (ALT) levels, lipid peroxidation, liver to body weight ratios and examination of hepatic tissue under light microscopy. Normal liver function was noted in the VEH, SAME and NAC groups. Hepatic toxicity was apparent within 4 h in the APAP treated groups. SAME protected the liver from APAP-induced damage as the SAME+APAP group had lower ALT values when compared to the APAP group. SAME+APAP mice also

had less induction of lipid peroxidation and higher hepatic glutathione levels than the APAP group. Evaluation of hepatic tissue under light microscopy showed extensive centrilobular necrosis in the APAP group and only mild hepatic changes in the SAME+APAP group. NAC treatment 1 h after APAP (NAC+APAP) reduced APAP hepatic toxicity. Plasma ALT values and histology supported a finding of NAC attenuation of APAP toxicity. Further studies investigated the protective effects of SAME against APAP-induced hepatotoxicity on 4-hydroxynonenal (4HNE) adducted proteins and protein carbonyls. The results of this study have found that SAME protects the liver from production of protein carbonyls and 4HNE adducts. In summary, APAP hepatic toxicity occurs within 1 h after injection in mice. SAME administered 1 h after APAP was successful at reducing APAP hepatic toxicity. SAME administered 1 h after APAP also reduced the extent of oxidative stress associated with APAP.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC551

Melanin concentrating hormone receptor localisation and binding affinity of small molecular antagonists in sections of rat brain using [¹²⁵I]-S36057

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Melanin concentrating hormone (MCH) binding to central MCH1 receptors leads to an increase in food intake (Hervieu et al., 2000). Although molecular biology techniques have identified MCH containing neurons and mRNA for MCH1 in rat brain (Hervieu et al., 2000), a lack of suitable radioligands and optimised binding conditions have limited the ability to determine MCH1 receptor distribution and compound potencies in native tissues. The aim of this study was to optimise the conditions for [¹²⁵I]-S36057 binding in sections of rat brain.

With local ethical approval, Sprague-Dawley rats (250-350g) were killed by decapitation, brains rapidly removed and snap frozen in isopentane. Cryostat sections (20 µm) of rat brains were pre-incubated in assay buffer containing 25 mM HEPES, 5 mM MgCl₂, 0.5% BSA, 1 µM phosphoramidon, 10 µM captopril and 1 mM PMSF, pH 7.4 for 15 min. Subsequently, sections were incubated with 50 pM [¹²⁵I]-S36057 (Perkin Elmer, UK) for 90 min, in the absence (total binding) or presence (non-specific binding) of MCH (1 µM). Sections were rinsed in 50 mM Tris and apposed to phosphoscreen for 12 hr. For IC₅₀ determination, coronal sections capturing the caudate putamen were incubated with a range of concentrations (10 pM – 10 µM). Specific binding (counts/mm²) was determined using ImageQuant and IC₅₀ analysis was performed using an in-house curve fitting programme.

To obtain 65-70% specific binding, greater than the reported 30% in rat brain homogenates (Audinot et al., 2001), it was essential to have 15 min pre-incubation, protease inhibitors and a wash time of 2 x 2 min. There was no difference in specific binding between genders. The highest density of receptors were localised

to the caudate putamen, with moderate levels in pre-frontal and frontal cortex, anterior olfactory nucleus, substantia nigra, amygdaloid nuclei, hypothalamic nuclei, brain stem and cerebellar cortex. Low levels of receptors were observed in the thalamus. Receptors were not detected in the deep cerebellar nuclei and inferior colliculus, in contrast to the reported gene expression (Hervieu et al., 2000). Two small molecule MCH1 antagonist, SNAP-94847 and GW-803430 (Rokosz & Hobbs, 2006) inhibited [¹²⁵I]-S36057 binding with IC₅₀ values ± s.e.mean of 1.26 ± 0.11 nM and 15.1 ± 3.6 nM (all n=4) respectively.

In summary, we have reported for the first time, optimised binding conditions using [¹²⁵I]-S36057 in cryostat sections of rat brain to demonstrate reproducible high specific binding, discrete MCH1 receptor protein localisation and binding affinity of small molecule antagonists. This methodology may prove useful to determine native tissue invitro binding data for lead compounds in other species and tissues.

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Rokosz LL & Hobbs DW (2006). *Drug News Perspect* 19(5), 273-285

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC552

An improved method of determining ex vivo glucocorticoid receptor occupancy using [3H]Dexamethasone in rats

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Inhaled corticosteroids are the drugs of choice for asthma and other airway allergy diseases (Barnes et al, 2004). Despite direct delivery into the lungs inhaled corticosteroids are absorbed into the systemic circulation with the potential to cause side effects (Barnes et al, 2004). In this study we evaluated [3H]Dexamethasone (DM) as a suitable radioligand for assessing glucocorticoid receptor (GR) occupancy in lung and side effect tissues. With local ethical approval, Sprague-Dawley rats (250-350g) were sacrificed by decapitation. Lung, thymus, liver, kidney and brain were resected and frozen in isopentane. Saturation experiments were performed by incubating cytosolic fraction of tissue homogenate with 0.1-40nM [3H]DM (Perkin Elmer,UK) in the absence (total binding) or presence (non-specific binding) of DM (1µM) for 3h at 40C. Bound and unbound fractions were separated via charcoal centrifugation (Hochhaus, 1995) or rapid filtration through GF/B filters. Following ex vivo optimisation studies, fluticasone propionate (FP) receptor occupancy (RO) was measured by comparing control animals with animals dosed by the intratracheal route with 100µg of FP nanosuspension at 1,2,4 and 8h post dose. Data are expressed as mean±standard error of the mean.

In vitro, [3H]DM bound with high affinity with specific binding >80%. Saturation analysis showed [3H]DM had similar affinity for the GR expressed in tissues examined (5.4-12.4nM), consistent with previous studies using [3H]DM binding in rat liver

(Kenji et al, 1985.) and human lung (Philip et al, 1974). However, levels of GR expression varied between tissues with Bmax (mean fmol/mg protein $\pm$ s.e.mean) rank order of thymus (267 $\pm$ 10) > liver (228 $\pm$ 31) > lung (136 $\pm$ 26) > kidney (91 $\pm$ 10) > brain (44 $\pm$ 4). Time dependant decrease in ex vivo RO was seen in lung and liver following 100 μ g intratracheal dosing of FP with no difference in RO values comparing either 3h or 18h incubation or filtration versus charcoal separation. Based on this data we selected 3h incubation time and filtration for all subsequent ex vivo RO studies. In contrast, previous ex vivo assays have been performed using [3H]triamcinolone with 18h incubation time using charcoal centrifugation (Hochhaus et al, 1995). Further ex vivo time course studies with FP demonstrated consistently higher RO in lung compared to liver. Interestingly, similar RO values to the lung were also observed in the thymus. In summary we have reported an improved way of measuring ex vivo GR occupancy by reducing incubation time with [3H]DM and using filtration methods. This may allow us to relate pharmacological responses to RO in various tissues in pre clinical models of asthma and differentiate compounds based on their therapeutic index.

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Kenji H. et al., (1985) Biochimica et Biophysica Acta 843, 171-179

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

The maximal relaxation caused by NaHS was 70% or more of CCh-induced precontraction in most preparations, except for the guinea-pig bronchi in which less than 20% relaxation was induced by NaHS. In the gastrointestinal preparations, EC₅₀ values (mM) for NaHS were 0.11 in the ileum, 0.20 in the tanea coli, 0.17 and 0.09 in the terminal cavity and ampulla, respectively, of sphincter of Oddi from guinea-pigs, and 0.43 and 0.27 in the distal and proximal colon, respectively, from rats. Glibenclamide, an inhibitor of K⁺_{ATP} channels, at 10 μ M did not affect either EC₅₀ values or the maximal effect for NaHS in any preparations. In the bronchial preparations of mice, the EC₅₀ for the relaxant activity of NaHS was 0.83 mM, and was not affected by glibenclamide, antagonists of NK₁ and NK₂ receptors, and inhibitors of soluble guanylyl cyclase, COX-1 and COX-2. Na₂SO₃, a metabolite of H₂S in the mammalian body, did not induce relaxation in a range of 0.01-1 mM in the sphincter of Oddi of guinea-pigs and bronchus of mice.

Conclusions: In the gastrointestinal and bronchial smooth muscle preparations used in the present study, NaHS appears to cause relaxation, the activity being similar among distinct tissues and species, except for the guinea-pig bronchus that poorly responds to NaHS. The NaHS-induced relaxation in any preparations is considered independent of K⁺_{ATP} channels. Further, it is also clear that the relaxant effect of NaHS in mouse bronchus does not involve endogenous neurokinins, nitric oxide and prostaglandins.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC553

Effects of hydrogen sulfide on the tone of gastrointestinal and airway smooth muscle preparations from rodents

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Introduction: Hydrogen sulfide (H₂S) is now considered a gaso-transmitter in the mammalian body. In the arterial smooth muscle, H₂S activates ATP-sensitive K⁺ (K⁺_{ATP}) channels, leading to membrane hyperpolarization followed by relaxation. However, the H₂S-induced vasorelaxation also involves K⁺_{ATP} channel-independent mechanisms. In the present study, we examined and characterized relaxant activity of H₂S in the gastrointestinal and bronchial smooth muscle preparations from guinea-pig, rats and mice.

Methods: Male Hartley guinea-pigs, ddY mice and Wistar rats were sacrificed by abdominal exsanguinations under urethane (1.5 g/kg, i.p.) anesthesia. The ileum, sphincter of Oddi and tanea coli from guinea-pigs, the main bronchi from mice and guinea-pigs, and the colon from rats were used for isometric tension recordings. Effects of cumulatively applied NaHS, a donor of H₂S, were observed in the smooth muscle precontracted with carbachol (CCh) at 1 and 10 μ M for bronchial and gastrointestinal preparations, respectively.

Results: NaHS in a range of 0.01-3 mM induced concentration-dependent relaxation in all preparations precontracted with CCh.

PC554

Rat mesenteric system perfusion *in situ* and intraduodenal administration *in vivo* as a tool for dosage forms equivalence testing

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The rate of intestinal absorption was observed in the study in order to evaluate equivalence of two batches of oral suspension dosage forms containing an active ingredient widely used in certain hepatic disorders treatment. The following experimental methods were applied: a) rat mesenteric system perfusion *in situ* and b) intraduodenal administration in rat. Rats were anesthetized with urethane (1.5 g/kg, i.p.) during the whole experiment. Mesenteric system was single-pass perfused with an influx cannula entering arteria mesenterica superior and with an efflux cannula coming out of vena portae. The system was supplied with Krebs-Henseleit solution (pH 7.4) under a constant pressure of 15 kPa saturated with 95% O₂-5% CO₂. 6 ml of suspension was given into the intestinal lumen. The small intestine was tied under the bile duct entry and in the place of ileum-caecum junction. In another experimental *in vivo* set up was the suspension administered intraduodenally in rats with tied small intestine in the same way as in the previous experiment. Blood samples were taken from aorta abdominalis at 1h, 2h, 3h and 4h after suspension administration. Perfusion samples were collected during the following 90 min. Concentrations of absorbed

portion were measured. The results were compared each together and used for dosage form equivalence evaluation as well as for optimization of equable absorption in the terms of pharmaceutical dosage forms changes.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC555

Increasing lung volume in humans does not modulate respiratory-related sensory evoked potentials

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In some disease conditions, an increase in lung volume (hyperinflation) may contribute to the sensation of dyspnoea. Cortical processing of respiratory-related sensory information can be investigated using transient inspiratory occlusions (TIOs). This afferent stimulation results in respiratory-related sensory evoked potentials (RREPs) which exhibit early (Nf, P1: within 100 ms of stimulus onset) and late (N1, P2, P3: 100 – 350 ms after stimulus) latency components (Davenport et al., 2002). With this context, we hypothesised that the processing of sensory information from the respiratory system may be modulated by changing lung volume and that such changes might contribute to the sensation of dyspnoea. To test this we determined changes in the RREP response to TIO in healthy volunteers induced by increasing lung volume.

Surface EEG electrodes were placed to record RREPs at frontal, central and parietal cortical sites. RREPs were elicited using TIOs of 0.3 s in 15 healthy subjects with ethical approval. Subjects were instructed, in response to a tone, to voluntarily inspire from their functional residual capacity to their inspiratory capacity (IC) whilst maintaining a targeted constant flow rate. In each trial, TIOs were presented in a randomised order, either early in the targeted inspiration at a low volume ($10.3 \pm 1.9\%$ IC; group mean $\pm$ se) or late in the inspiration at a high volume ($70.5 \pm 1.2\%$ IC) of the subject's IC. No significant differences (ANOVA) in amplitude (Table 1; group mean $\pm$ se, μ V) or latency were observed in the early or late components of the RREP between low and high volumes.

This study demonstrates no modulation of respiratory-related sensory information by increasing lung volume and suggests that changes in the processing of sensory information, associated with hyperinflation, may not contribute to the sensation of dyspnoea in lung disease.

Table 1. Changes in RREP amplitudes induced by increases in lung volume

	Nf	P1	N1	P2	P3
Low volume	-2.9 ± 0.7	1.2 ± 0.3	-5.2 ± 0.8	6.2 ± 1.6	7.3 ± 1.9
High volume	-2.5 ± 0.5	1.3 ± 0.3	-3.9 ± 0.7	4.9 ± 1.2	7.0 ± 1.2
P value	0.35	0.89	0.16	0.16	0.79

Davenport PW et al. (2002). J App Physiol 93, 894-902

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC556

Thiazide diuretics directly stimulate a sodium chloride cotransporter (SLC12a3) in bone-forming cells, the osteoblasts

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Thiazide diuretics are commonly used for the treatment of hypertension and their use has been associated with an increased bone mineral density in patients undergoing such therapy. Their molecular target is the thiazide-sensitive sodium-chloride cotransporter (NCC), which is expressed in the kidney distal tubule. It has been proposed that, by inhibiting the kidney NCC, thiazides produce a positive calcium balance and drive the observed bone-sparing effect. However, a direct skeletal effect of thiazides and the expression of NCC in bone cannot be ruled out and, indeed, has been hypothesized.

We have previously reported the presence of NCC mRNA and protein in cells of the osteoblast lineage in freshly frozen undecalcified preparations of human and rat bone, as well as in tissue culture models of osteoblasts, the bone-forming cells [1].

Here we investigated the effect of metolazone, a thiazide-like diuretic, on the differentiation of human osteoblast-derived cells (MG63) and on fetal rat calvarial cells (FRC), a model for osteoblasts, and detected the consequence of an NCC overexpression on FRC differentiation. Our results indicate that chronic metolazone treatment (1-100 μ M) dose-dependently promoted the expression of osteoblast differentiation markers, Runx2 and osteocalcin, measured by western blotting, in the absence of a proliferative effect ($n=3$; $p<0.05$ by ANOVA, Tukey post hoc test). This effect was specific, as the expression of an early differentiation marker, type I collagen, was not affected. In FRC cells, metolazone dose-dependently increases the formation of mineralized nodules, quantified by von Kossa staining ($n=6$; $p<0.05$ by ANOVA, Tukey post hoc test). These findings are specific to thiazide diuretics and are not mimicked by the loop diuretic bumetanide. Finally, overexpression of NCC in FRC cells significantly increased metolazone-induced mineralization ($n=3$; $p<0.05$ by ANOVA, Tukey post hoc test).

Taken together, these results provide strong evidence for a direct action of thiazides on bone, through NCC, an effect which is enhanced by NCC overexpression. Our findings support the view that thiazides should be considered as diuretics of choice in elderly, osteoporotic patients.

[1] Dvorak MM, Carter DH, Riccardi D. (2002) Thiazide-Sensitive Sodium Chloride Co-Transporter (NCC) in Cryosections of Rat and Human Bone. J. Bone Miner. Res. 17:SA194 Suppl.1.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC557

Exploring the dynamics of the neuronal endoplasmic reticulum with a novel lentiviral vector

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The neuronal endoplasmic reticulum (ER) is an extensive, complex endomembrane system, containing channels and receptors that permit its lumen to act as a dynamic calcium store. In response to electrical or chemical stimulation, ER calcium can be released under strict spatiotemporal control to regulate processes ranging from synaptic plasticity to growth cone guidance (1). Currently, there is much controversy over the nature of the ER in neurons and how this intersects with calcium signalling. We hypothesise that in neurons the ER is a dynamic structure with a single continuous lumen. To test this notion and define the relationship between ER-dynamics and calcium signalling in healthy and diseased neurons, we engineered a novel ER-targeted GFP construct, termed LV-pIN-KDEL, (derived from our pIN-G trafficking reporter, 2), in a 3rd generation replication-defective, self-inactivating lentiviral vector system capable of mediating gene transduction in diverse dividing and non-dividing mammalian cells, including neurons.

Following its expression in HEK293 (or COS-7) cells, LV-pIN-KDEL (or its parent pIN-KDEL) yielded a pattern of fluorescence that co-localised exclusively with the ER marker sec61 β but no other major organelles. We found no evidence for cytotoxicity and only rarely inclusion body formation. To explore the utility of the probe in resolving the ER in live cells, HEK293 or COS-7 cells were transduced with LV-pIN-KDEL and, after 48h, imaged directly at intervals from 1min to several hours. LV-pIN-KDEL fluorescence reveals the endoplasmic reticulum as a tubular lattice structure whose morphology can change markedly within seconds. Although GFP can be phototoxic, the integrity of the cells and ER is retained even after light exposure for periods up to 24h.

Using LV-pIN-KDEL we have imaged the ER in diverse fixed neuronal cultures and, using real-time imaging, found evidence for extensive, dynamic, remodelling of the neuronal ER in live hippocampal cultures and brain slices. Supported by BBSRC (UK). Verkhratsky A (2005). *Physiol Rev* 85, 201-79.

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PC558

Chronically elevated levels of phosphorylated p38 MAPK and ERK 44 in response to psychological stress in rodents

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The Mitogen Activated Protein Kinases (MAPKs) are stimulated by various types of stress. Repeated exposure to psychological

stress leads to increased levels of phosphorylated p38 MAPKs, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) levels in the brain. However, effects of psychological stress have not been extensively studied in peripheral tissue, particularly skeletal muscle. Restraint stress has been shown to increase p44/p42 ERK in the rat heart and 1 day of hindlimb suspension has been shown to activate p38 MAPK in rat soleus muscle. Therefore, the purpose of this study was to determine levels and phosphorylation status of various MAPKs in gastrocnemius muscle after chronic psychological stress. Adult male Wistar rats were used. Animals in the Immobilisation stress group (I; n = 10) were subjected to immobilisation for 2 hours/day for 28 days. For these 2 hours the animals were placed in small cages preventing movement. Animals in the Control (C) group were not immobilized (n = 10). After 28 days of stress (or control), 24 hours after the last exposure to immobilisation, animals were decapitated, blood was collected and gastrocnemius muscle was removed. Levels of total and phosphorylated p38, ERK 44, ERK 42 and JNK were measured by western blot analysis, and corticosterone levels by radioimmunoassay (RIA). ANOVA was performed and specific differences were identified using Bonferroni correction. Statistical significance was accepted at $P < 0.05$; data are presented as mean $\pm$ SD. Chronic stress significantly increased basal serum corticosterone (I: 81 ± 11 vs C: 22 ± 7 ng/ml, $p < 0.001$) demonstrating the stress response. There were significantly increased levels of phosphorylated p38 MAPK (I: 137.5 ± 8.67 vs C: 70.93 ± 7.44 ; $p < 0.001$) and phosphorylated 44kD ERK (I: 118.70 ± 5.11 vs C: 72.61 ± 9.23 $p < 0.005$). There were no changes in phosphorylated ERK 42 or phosphorylated JNK levels ($p > 0.05$). The results demonstrate that upregulation of p38 MAPK and ERK 44 may play a role in the response to chronic psychological stress in skeletal muscle of rats.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC559

Temperature dependence of the deoxygenation-induced cation pathway in red cells from sickle cell patientsK.A. Gehl¹, J.C. Ellory² and J.S. Gibson¹

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Red cells from sickle cell disease (SCD) patients contain the abnormal HbS, (and are here termed HbS cells). Lack of a negatively charged residue allows neighbouring HbS molecules to aggregate when deoxygenated. The rigid polymers of Hb distort cell shape (hence the name SCD) and contribute to the microvascular occlusions, characteristic of the disease. They are also associated with abnormal membrane permeability. In particular, when deoxygenated, HbS cells exhibit a deoxygenation-induced cation permeability, termed P_{sickle} which elevates intracellular $[Ca^{2+}]$ and contributes to cell dehydration (Joiner, 1993; Lew & Bookchin, 2005), markedly encouraging polymer formation. It thus contributes to the vascular pathology of SCD. P_{sickle} , however, remains poorly understood and its molecular

identity is unknown. To improve our understanding of this pathway, we investigated the temperature dependence of P_{sickle} . Blood samples obtained from homozygous HbSS SCD patients with consent and ethical approval were washed in MOPS-buffered saline (in mM: 145 NaCl, 5 glucose, 10 MOPS, pH 7.4). Red cell aliquots (c.10% haematocrit) were deoxygenated for 10 min at room temperature before varying the temperature (37, 20, 10 and 0°C). K^+ influx (30 min) was measured using $^{86}\text{Rb}^+$ as a K^+ congener, added in isotonic KNO_3 , final $[\text{K}^+]$ 15 mM. Ouabain (100 μM), bumetanide (10 μM) and clotrimazole (10 μM) were present. Data are given as means $\pm$ SEM. In the first series of experiments, influxes were compared at 37°C and 0°C. In all cases, at the lower temperature influx was much reduced (mean inhibition $76 \pm 14\%$, $n=3$, $p<0.05$). In a further series of experiments, the temperature profile of influx was determined. Expressed as a percentage of the influx at 37°C, which was $1.71 \text{ mmol}/(1 \text{ cells.h})$, influxes at 37, 20, 10 and 0°C were $100 \pm 31\%$, $70 \pm 28\%$, $35 \pm 11\%$ and $23 \pm 7\%$, respectively ($n=4$).

These findings show that, as temperature was reduced, the activity of P_{sickle} was progressively inhibited, with a Q_{10} of about 1.5. This temperature dependence differs significantly from that shown by the "passive" cation leak in normal human red cells and in those from cases of hereditary stomatocytosis (Stewart *et al.*, 1980; Stewart, 2003), some of which involve band 3 mutations, consistent with an unique physical basis. These observations are relevant to understanding the nature of P_{sickle} .

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PC560

A possible role of nitric oxide in the relaxant effect of resveratrol on isolated rat corpus cavernosum

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Introduction: Epidemiological studies have shown a putative correlation between the moderate consumption of red wine and a lowered risk of coronary heart disease (1). Biological mechanisms proposed for red wine-derived phenolic compounds include estrogenic activity, antioxidant activity, inhibition of platelet aggregation, modulation of lipid metabolism, and proliferation of smooth muscle cells (2, 3). Resveratrol (RVT), a stilbene polyphenol found in grapes and red wine, exhibits vasorelaxing effects, mainly via a NO-dependent mechanism. Nitric oxide is an important mediator for the relaxation of corpus cavernosal muscle which is the fundamental event in erection. Thus, this study was designed to investigate the effect of RVT on

phenylephrine-induced contractile response of the rat corpus cavernosum strips. **Materials and methods:** Corpus cavernosum strips were suspended in oxygenated Krebs buffer at 37°C. Isometric contractions were recorded using a force-displacement transducer coupled to a polygraph. The strips were placed under a resting tension of 1 g and after a 60-min of equilibration, the contractile response to phenylephrine (10-5M) followed by relaxation responses to cumulative addition of RVT (10-10-10-4 M) was recorded. Then, the responses of the strips to RVT were repeated in the presence of a non-selective NOS inhibitor L-NAME (10-5M). **Results:** The phenylephrine-precontracted corpus cavernosum strips showed relaxation in a dose-dependent fashion by RVT added cumulatively. The responses of the strips to RVT showed a significant reduction at all concentrations of RVT in the presence of L-NAME ($p<0.05$ -0.001). **Conclusions:** The results of the study suggest that RVT causes relaxation of isolated rat corpus cavernosum strips by a mechanisms involving NO.

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PC561

Transcriptional regulation of human pulmonary artery smooth muscle cell responses to chronic hypoxia

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Pulmonary hypertension (PH) is a major cause of death and impairment of quality of life. Chronic exposure to hypoxia alone is sufficient to cause major structural changes to pulmonary arteries (Groves *et al.*, 1987), as well altering the biochemical and functional phenotypes of all of the vascular cell types. Furthermore, these hypoxia-induced changes are site specific and vary between the large and small vessels of the pulmonary vasculature. Although traditionally it has been stated that PH is a disease of distal lung circulation, more current research has shown that structural changes in larger vessels contribute directly to right ventricular work and failure thus changing blood flow within the smaller vessels (Stenmark & Mecham, 1997).

In order to determine which genes are involved in this remodelling process, we have utilized an affymetrix gene chip array to determine the gene expression profile of human pulmonary artery smooth muscle cells (HPASMC) cultured for 24 hours under nonhypoxic ($p\text{O}_2 \approx 150 \text{ mmHg}$) and hypoxic conditions ($p\text{O}_2 \approx 7 \text{ mmHg}$). Analysis for differential gene expression was done using the Welch t-test, the Bayesian t-test and the moderated t-test. Only probe sets which passed all three statistical tests and showed either a 1.5 fold increase or 1.5 fold decrease in expression were chosen for further investigation. Of the 188 individual genes which met these criteria, 133 showed an increase

in expression in response to chronic hypoxia and 47 genes were downregulated. The gene chip array data was validated by performing quantitative PCR on a random selection of genes which were shown to be altered by chronic hypoxia. As smooth muscle cells are known to differentiate quickly, HPASMC of the same passage were stained with α -smooth muscle actin to show that they had the correct phenotype when the experiments were performed. Furthermore, HPASMC were also shown to respond to the vasoactive peptide Endothelin-1 in calcium imaging experiments, which is another indicator of smooth muscle function (Sinha et al., 2006).

The largest category of genes which were regulated by hypoxia were proteins involved in metabolism, of which many have previously been reported to be regulated by hypoxia inducible factor 1 α (HIF-1 α). In addition, numerous proteins involved in collagen synthesis, cell proliferation / motility, oxidoreduction and solute transportation were differentially expressed. These results suggest that in addition to the effect that vasoactive substances released from the endothelium have on the contractile and proliferative state of smooth muscle cells (Aaronson et al., 2002), hypoxia can directly regulate genes within HPASMC which are important in responding to injury.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC562

Muscarinic receptor subtypes mediating the release of urinary bladder-derived relaxant factor

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The release of a previously unrecognized smooth muscle relaxant factor from rat urinary bladder has been demonstrated by a coaxial bioassay system (1-3). In the present study using the same bioassay consisting of rat urinary bladder as the donor organ and rat anococcygeus muscle as the assay tissue, we have investigated the muscarinic receptor subtype(s) mediating the release of bladder-derived relaxant factor. The anococcygeus muscle that was placed in the lumen of the bladder was mounted under a resting tension of 1 g in an organ bath filled with Krebs-Henseleit solution, and was precontracted with phenylephrine (1-3

μ M) before each experimental procedure. In this coaxial bioassay system, acetylcholine induced concentration-dependent relaxation response that was also repeated with lower potency by muscarinic M1 receptor agonist McN-A-343. The pD₂ values of acetylcholine and McN-A-343 calculated from their concentration response curves were 5.91 ± 0.18 and 3.45 ± 0.34 , respectively. The relaxation response to acetylcholine was inhibited by non-selective and subtype selective muscarinic receptor antagonists. The rank order of potency (pKB) was estimated as: M3 antagonist darifenacin (9.36 ± 0.11) > M3, M1 antagonist 4-DAMP (9.30 ± 0.11) > non-selective antagonist atropine (9.09 ± 0.15) > M1 antagonist telenzepine (8.56 ± 0.21) > M4 antagonist tropicamide (6.63 ± 0.17) > M2 antagonist AF-DX 116 (6.01 ± 0.21). The pKB values of subtype selective muscarinic antagonists have revealed that M1 and M3 muscarinic receptors are involved in the release of urinary bladder-derived relaxant factor.

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PC563

Divergent constrictive responses to agonists in permeabilised human arteries

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Background: Agonist-mediated sensitisation of myofilaments to calcium is known to play a role in the regulation of vascular tone in human myometrial arteries during pregnancy (Wareing et al. 2005). Pregnancy complications such as intrauterine growth restriction and pre-eclampsia are known to involve alterations in vasoreactivity of myometrial arteries to constrictive agents such as the thromboxane mimetic U46619 and arginine vasopressin (AVP). Understanding the mechanisms by which these molecules act to regulate tone of human arteries in normal pregnancy will therefore be of importance in elucidating changes accompanying the above complications that result in elevated vascular resistance. Aims: To assess the level of agonist-dependent sensitisation of tone to sub-maximal activating calcium in permeabilised myometrial arteries using a selection of vasoactive agonists known to be present in the circulation: U46619, AVP and sphingosine-1-phosphate (S1P). Methods: Arteries were dissected from biopsies obtained during Caesarean section, following written informed consent, and mounted in a wire myograph. Agonist-induced constriction (to 10 μ M S1P, 1 μ M U46619

and 10nM AVP) was assessed in vessels permeabilised with α -toxin and already sub-maximally constricted with pCa6.7 solution by ~30% (relative to a previous constriction to pCa4.5 solution). The involvement of rho-associated kinase (ROK) in any agonist-mediated sensitisation of tone to pCa6.7 was established using the inhibitor Y27632 (10 μ M). Results: U46619 (N=5) or AVP (N=4), added to pCa6.7 solution, produced constrictions over and above that of pCa6.7 alone; these were of similar magnitude to preceding constrictions to pCa4.5 alone (1.1 $\pm$ 0.2 and 0.9 $\pm$ 0.3 fold of that to pCa 4.5 respectively). S1P (N=5), however, produced a far smaller constriction (0.3 $\pm$ 0.2 fold change compared to pCa 4.5). The constriction to U46619 was reduced to 17% of maximal constriction, that of AVP to 40% and that of S1P to 12% in the presence of Y27632. Conclusion: Interestingly, receptor-mediated Ca²⁺-sensitisation of human arterial tone, at least at pCa6.7, is agonist-dependent. Irrespectively, ROK inhibition substantially reduced the sensitisation to U46619, AVP or S1P. These data indicate that vasoactive agents may induce constrictions by evoking different magnitudes of Ca²⁺-sensitisation, a finding of importance when considering potential mechanisms by which circulatory agents promote increased vascular resistance associated with hypertensive disorders e.g. for pregnancy, pre-eclampsia or IUGR.

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PC564

A novel nociceptive role for luminal hydrogen sulfide in mouse colon

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Background and Aims: Hydrogen sulfide, a gasotransmitter, can be formed from L-cysteine through enzymatic pathways in the mammalian tissues, and also by sulfate-reducing bacteria in the colonic lumen. It has been reported that intraperitoneal (i.p.) injection of sodium hydrosulfide (NaHS), a donor for hydrogen sulfide, suppresses responses to colorectal distention in rats (Distrutti *et al.* 2006). In contrast, we have shown that NaHS enhances T-type calcium channel (T-channel)-mediated membrane currents in neuronal model cells, and that intraplantar injection of NaHS produces mechanical hyperalgesia through activation of T-channels in rats (Kawabata *et al.* 2007). To clarify roles for luminal hydrogen sulfide in colonic nociceptive processing, we thus examined if intracolonic (i.col.) administration of NaHS could cause visceral pain and/or referred hyperalgesia in mice.

Methods: Male ddY mice received i.col. administration of NaHS or capsaicin, and visceral nociceptive behavior was observed for 30 min. The mechanical nociceptive sensitivity of the abdomen

was then determined by use of von Frey filaments. For immunostaining of phosphorylated ERK (p-ERK), the mice received i.col. NaHS or capsaicin under anesthesia with i.p. urethane at 1.5 g/kg, and, after 5 min, the spinal cord was fixed transcardially with paraformaldehyde solutions. The excised spinal cord was separated into three segments, T13-L1, L2-L4 and L5-S1, and serially sectioned for staining of p-ERK.

Results: Like capsaicin, NaHS, administered i.col. at 0.5-5 nmol/mouse, caused visceral pain-like nociceptive behavior followed by development of mechanical abdominal hypersensitivity to stimulation with von Frey filaments. The pro-nociceptive effect of i.col. NaHS as well as capsaicin was also accompanied by increase in the number of p-ERK-positive cells in the superficial layers of the T13-S1 spinal dorsal horn, particularly at T13-L1 levels. The visceral nociception and referred abdominal hyperalgesia caused by i.col. NaHS were blocked by mibefradil, a T-channel blocker, or 5,5'-dithio-bis-(2-nitrobenzoic acid), an oxidant, but not modified by glibenclamide and verapamil, inhibitors of ATP-sensitive potassium channels and L-type calcium channels, respectively.

Conclusions: Our study shows that colonic luminal NaHS/hydrogen sulfide causes visceral nociception and referred abdominal hyperalgesia accompanied by excitation of spinal nociceptive neurons in mice, and indicates that the underlying mechanisms involve redox modulation of T-channels.

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PC565

The relaxant effect of Aqueous Garlic Extract (AGE) on isolated rat corpus cavernosum tissue

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Aqueous Garlic Extract (AGE) has previously been shown to have relaxant effects on isolated rat aorta rings (1). Several studies have suggested the relaxant effect was mediated by endothelial nitric oxide and vasodilator prostaglandins (2). The aim of the present study was to investigate the effect of fresh AGE on phenylephrine-induced contractile responses.

1g fresh garlic was squeezed and extracted in 10ml distilled water for the stock solution. Further dilutions were also prepared by distilled water. Briefly, corpus cavernosum strips were suspended in oxygenated Krebs buffer at 37°C and isometric contractions to phenylephrine (10-5M) were recorded and AGE extract was added to the bath cumulatively to maintain increasing concentrations of 150 μ g/ml, 300 μ g/ml, 450 μ g/ml and 600 μ g/ml. Then the tissue was washed out twice and pretreated L-NAME (10-5M) or indometasine (10-3M) or both.

The increasing concentrations of AGE produced a relaxation on corpus cavernosum tissues which were contracted by phenylephrine. The relaxant responses were partially blocked by L-NAME preincubation, but not indometasine preincubation

demonstrating that the relaxant effect of AGE on corpus cavernosum tissue was partially mediated by NO.

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PC566

Evaluation of *Cuminum cyminum* for its potential against postmenopausal osteoporosis in ovariectomized rats

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Phytoestrogens, the estrogen-like substances found in many plants, are being explored as an alternative to steroids for the treatment of estrogen-related disorders including cardiovascular diseases, menopausal symptoms, postmenopausal osteoporosis, etc. *Cuminum cyminum* (CC) is a phytoestrogen-rich plant. Fruits of CC are reported to contain estrogenic compounds like β -sitosterol, stigmasterol and luteoline. The present study investigated anti-osteoporotic potential of methanolic extract of fruits of CC (MCC) against postmenopausal osteoporosis in rats. Ovariectomized (OVX) Sprague Dawley rats were used as an experimental model for postmenopausal osteoporosis. Ovariectomy was performed under ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. MCC (1000 mg/kg, in two divided doses) and vehicle (0.5% CMC) were administered orally to OVX animals and Sham control/OVX control animals, respectively, for 10 weeks. Estradiol (0.15 mg/kg) was used as the standard for comparison. At the end of 10 weeks blood (for estimation of calcium (Ca), phosphorous (P), alkaline phosphatase (ALP) and tartarate-resistant acid phosphatase (TRAP)); urine for estimation of Ca, bones (for bone density, ash density, Ca content and mechanical strength) and uteri were collected. Students' t test at 95% CI was used for statistical analysis.

Treatment with MCC caused decrease in bone turnover as indicated by the significant decrease in serum ALP with concurrent decrease in serum TRAP. MCC did not alter calcium and phosphorous homeostasis which was seen by no effect on serum Ca and P as compared to Sham control and OVX control. MCC caused significant decrease in urinary excretion of Ca as compared to OVX control. There was increase in bone and ash density, significant increase in Ca content and Ca:P ratio of bone and significant increase in mechanical strength of the bone as revealed by three point bending test in tibia and femoral neck strength test. Serum total cholesterol was measured to assess the effect of MCC on increased cholesterol levels due to ovariectomy. MCC treatment did not cause any increase in uterine weight, uterine peroxidase (UPO) activity and total protein content of uterus.

The results of the study underline the anti-osteoporotic potential of MCC in postmenopausal osteoporosis with additional benefit of cholesterol lowering capacity.

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PC567

Effect of oral MSG administration on regulation of appetite

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It is well known that parenteral neonatal monosodium glutamate (MSG) administration has toxic effects on arcuate nucleus neurons, leading to significant brain lesions, neuroendocrine disturbances and obesity (1-3). The present study was undertaken to investigate whether oral MSG administration was able to produce the same effect. Thirty pregnant Wistar rats were separated in three groups; the first two groups received specific diets containing 0, (control group, CT group) or 4 g MSG/Kg BW, (oral treatment group, OT group) from day 14th of pregnancy onwards. The third group of mothers received standard rat chow, but their offspring was neonatally injected with MSG (4mg/g BW) on alternate days for the first 10 postnatal days (day 2, 4, 6, 8, 10), (parenteral treatment group, PT group). The other groups received saline injections on the same days. After weaning, MSG feeding was continued in the first two groups at the same concentrations in the offspring until 6 months of life. Body weight of animals as well as food intake were measure periodically. Half of the pups were sacrificed at 1 month and the rest at the 6 month of life. Blood samples were collected for Leptin radioimmunoassay determination. Hypothalamus were collected as well for neuropeptide Y (NPY) and Leptin receptor (R-leptin) mRNA expression determinations using real time PCR.

Even though no changes were observed in body weight in PT group with respect to control group at one month life, at six months life, AP group showed lower body weight and body weight gain than the other two groups ($p < 0.001$). By contrast, the group of rats treated orally with MSG 4g/Kg BW presented lower body weight than the other two groups at one month of life ($p < 0.001$), but at six months there were no differences in body weight and body weight gain between this group and CT. In addition, food intake was significantly augmented in OT group respect to CT and PT groups ($p < 0.01$). Plasma Leptin levels were not significantly modified in orally treated group with respect to control at 1 and 6 month of life. Nevertheless, this hormone was markedly increased in PT group at 1 month ($p < 0.05$) and 6 month ($p < 0.01$) of life. At six month life, both groups, orally and parenterally MSG treated groups showed significantly reduced expression levels of NPY and R-leptin mRNA with respect to control group ($p < 0.05$).

A In conclusion, all these data support that MSG administered orally is able to produce disturbances in nutritional and endocrine parameters in the same way as parenteral administration.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC568

Pharmacological evaluation of *Cuminum cyminum* for estrogenic activity

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Cuminum cyminum (CC), a phytoestrogen-rich plant is used in many polyherbal formulations for its possible estrogenic activity. Fruits of CC are reported to contain estrogenic compounds like β -sitosterol, stigmasterol, apigenin and luteoline. The present investigation is aimed at evaluation of estrogenic potential of *Cuminum cyminum* using *in vitro* and *in vivo* models.

Estrogenic activity of methanolic extract of *Cuminum cyminum* (MCC) was evaluated *in vitro* using MCF-7 cell proliferation assay (E-screen). *In vivo* evaluation included uterotrophic assay in ovariectomized (OVX) rats. Ovariectomy was performed under ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. The dose and dosage (200 mg/kg p.o., for 21 days) was selected on the basis of preliminary evaluation performed for estrogenic activity.

MCC was fractionated with n-hexane (MCCF1), diethyl ether (MCCF2), chloroform (MCCF3) and water (MCCF4). All the fractions were evaluated by E-screen over the concentration range of 1 ng/ml to 10 μ g/ml. The chloroform fraction (most active fraction) was evaluated *in vivo* at 25, 50 and 100 mg/kg in OVX rats. MCC significantly increased (w.r.t. vehicle control) MCF-7 proliferation over the concentration range of 1 ng/ml to 100 μ g/ml with the maximum increase of 115.78% at 1 μ g/ml. *In vivo* evaluation of MCC at 200 mg/kg in OVX rats caused significant increase in absolute and relative weight of uterus along with the increase in uterine peroxidase (UPO) activity and significant increase in total protein content.

MCCF1, MCCF2, MCCF3 and MCCF4 increased cell proliferation maximum by 178.49% (at 100 ng/ml) with MCCF1; 177.98% (at 10 ng/ml) with MCCF2; 208.68% (at 10 μ g/ml) with MCCF3 and 108.74% (at 1 μ g/ml) with MCCF4. MCCF3 when evaluated *in vivo*, showed significant increase in absolute and relative weight of uterus and increase in glycogen content with no effect on UPO activity at 25 mg/kg. The higher doses 50 mg/kg and 100 mg/kg did not show any estrogenic activity.

Results of *in vitro* and *in vivo* estrogenic evaluation signify the estrogenic potential of methanolic extract of *Cuminum*

cyminum. All the fractions have also been found to be estrogenic *in vitro*. The estrogenic potential of the most active chloroform fraction was confirmed by *in vivo* uterotrophic assay. Thus *Cuminum cyminum* can be a good candidate for developing new estrogenic herbal drug which can be used as uterotrophic or anti-fertility agent.

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PC569

Expression of growth factor receptors at key pre- and postnatal stages of mouse gonadal development

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The key role of growth factors in regulating proliferation and triggering differentiation pathways in gonadal development is now well established (1). Having previously demonstrated the importance of Fgf9 in the mouse (2), we now report the expression of a number of other growth factor receptors implicated in gonadal sex differentiation. Glial cell-line derived neurotrophic factor (GDNF), epidermal growth factor (EGF), bone morphogenetic protein 4 (BMP4) and nerve growth factor (NGF) have been selected as candidates for study. Expression *in vivo* for their respective receptors GFR α 1, EGFr, BMPr1 and trkA, has been monitored at critical stages from embryonic day (E)13.5 to postnatal day (PN) 25 to address the question whether the receptors are uniformly expressed in both sexes at various timepoints. CBA strain mice were killed humanely according to Schedule 1, and the excised gonads processed for histology and immunocytochemistry (1). Expression of GFR α 1 was observed in the ovary only at PN25, limited to the oocyte cytoplasm; however, in the testis, immunostaining in Sertoli cells and spermatogonia at PN7, 15 and 25 was strongly positive. Leydig cells were negative throughout. EGFr showed strong staining in E13.5 and E16.5 testes, but by E18.5 onwards it was only weakly positive with immunostaining for EGFr found in spermatogonia, Sertoli cells, and spermatocytes and Leydig cells at PN7. In the female, weak staining only was seen in embryonic ovaries, although in postnatal stages, the receptor was strongly expressed in oocyte cytoplasm. Expression of BMPr1 was seen in both males and females throughout embryonic development, mostly in somatic cells, as was the expression of trkA. All growth factors may continue to play a proliferative role in the male where spermatogonia are still mitotic in the adult, so it likely that expression of receptors such as GFR α 1, observed in the developmental period in this study, will continue throughout life. Staining patterns in the ovary, where expression is mainly restricted to the oocyte, suggest that

growth factors may have a more specific functional role other than merely proliferation in the female. In view of this differential expression of growth factor receptors in the two sexes, further work will test these effects of growth factors in vitro where they may be investigated both independently and in potential synergistic combinations.

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PC570

Association of Omega-3 therapy with changes in tendon structural properties in healthy older adults

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Collagen protein is ubiquitous in connective tissue, including tendons and there is reason to believe that Eicosapentaenoic acid (EPA), the main acting agent in Omega-3 therapy (O3T), may alter collagen protein synthetic response and thus influence tendon mechanical properties. Indeed EPA has been associated with increased collagen and elastic fibres expression in aged human skin (Kim et al, 2006). O3T has also been used to address ageing associated decreases in range of motion and increased joint pain (Gruenwald et al, 2002). It is unclear however whether the effects of O3T on the joints are merely increased lubrication or whether the therapy has any direct effect on the intrinsic properties of the collagenous tissues around the affected joint. We therefore investigated whether a 12-week course of oral O3T (three 1g pills taken in the pre-prandial state daily, each containing 180mg EPA) would have an effect of the structural and mechanical properties of the patellar tendon. Subjects: Nine healthy, community-living participants (age 77±1years, height 1.65±0.02m, weight 74±4Kg), gave their consent to participate in this study which was approved by the local Institutional Ethics Committee. All procedures accorded with the principles of the World Medical Association's Declaration of Helsinki. A combination of isokinetic dynamometry, electromyography, B-mode ultrasound imaging and magnetic resonance imaging were used to assess tendon properties using a method previously described (Onambele et al, 2007). Pre – post O3T comparisons were performed using paired student t-test. Tendon dimensions of length (4.52±0.17cm pre vs. 4.48±0.24cm post, P>0.05), and cross-sectional area (76.8±8.9 mm² pre vs. 76.5±8.9 mm² post, P>0.05) did not change with O3T. Maximal voluntary isometric force capacity also did not show any changes (2390±245N pre vs. 2327±121N post, P>0.05). Interestingly however, there was a 28% (P<0.0001) increment in tendon elongation during voluntary contractions (see Figure 1), which translated into a 14% (P=0.015) decrement in tendon stiffness.

→ Figure 1 near here

The present study demonstrates that 12 weeks of Omega-3 supplementation in elderly subjects alters the viscoelastic properties of the human patella tendon. It is as yet unclear whether this is a positive physiological outcome in a context where ageing is normally associated with decreased tendon stiffness and the associated decreased ability to maintain adequate functional abilities such as stable standing posture (Onambele et al, 2006).

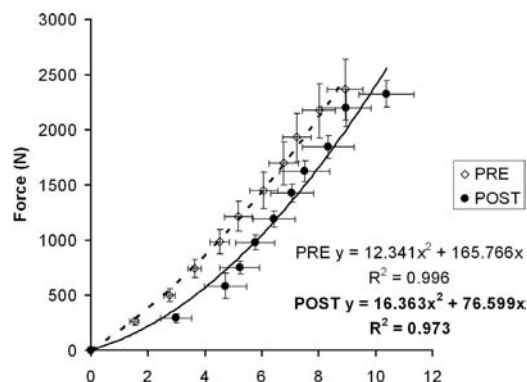


Figure 1. Tendon force-elongation relationship with omega-3 therapy. Data are mean±SEM.

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PC571

Reversed-phase high performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS) analysis of phenolics in selected Malaysian traditional vegetables and their total antioxidant activities

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Anacardium occidentale and *Centella asiatica*, among the most popular Malaysian traditional vegetables, locally known as 'gajus' and 'pegaga' were screened for phenolics and analysed for the quantification of individual compounds by reverse-phased high performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS2). Yellow variety of *Anacardium occidentale* showed the highest total phenolic content of 415 ± 20 mg GAE per kg fw. to that of red variety (361 ± 18 mg GAE per kg fresh weight, fw.) respectively measured using Folin-ciocalteu assay [1]. However, *Centella asiatica* had shown lower phenolic content of 100 ± 7.8 mg GAE per kg fw. compared to *Anacardium*

occidentale. 15 phenolic compounds in red and yellow varieties of *Anacardium occidentale* and 11 phenolic compounds in *Centella asiatica* mainly flavonol glycosides and chlorogenic acids were identified and quantified. The antioxidant activities were measured using two different assays, an on-line high-performance liquid chromatography (HPLC) method in conjunction with the analysis of the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS+) assay [2] and Ferric Reducing Antioxidant Potential (FRAP) assay [3]. The yellow variety of *Anacardium occidentale* showed the highest antioxidant activities (59 ± 1.9 mM Trolox equivalent (TE) in ABTS+ assay and 4.6 ± 0.3 mM Fe²⁺ in FRAP assay) to that of red variety (47 ± 2.1 mM TE and 3.3 ± 0.1 mM Fe²⁺) and *Centella asiatica* (8.5 ± 0.1 mM TE and 0.3 ± 0.0 mM Fe²⁺). The antioxidant activity are correlated with the total phenolic content and contributions from other compounds such as vitamin C, carotenoids and triterpenes to the total antioxidant activity should also be considered. However, the level of carotenoids in this study did not show any correlation to total antioxidant activities of these two plant species. Total phenolic content and total antioxidant activities in *Anacardium occidentale* and *Centella asiatica*

Traditional Vegetables	Total Phenolic Content (1)	Total Antioxidant	
		ABTS (2)	FRAP (3)
<i>Anacardium occidentale</i> (Red variety)	361 ± 18	47 ± 2.1	3.3 ± 0.1
<i>Anacardium occidentale</i> (Yellow variety)	415 ± 20	59 ± 1.9	4.6 ± 0.3
<i>Centella asiatica</i>	100 ± 7.8	8.5 ± 0.1	0.3 ± 0.0

1 - Result expressed as mg gallic acid equivalent (GAE) per kg fresh weight (fw). (n=3).

2 - Result expressed as mM Trolox equivalent (TE) (n=3).

3 - Result expressed as mM Fe²⁺ (n=3).

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spontaneous activity is achieved by a complex interaction of nerves, smooth muscle cells and interstitial cells. The aim of the present investigation was to assess the effects of several potassium channel blockers on spontaneous activity in the guinea-pig bladder. Bladders were removed from guinea-pigs, killed by cervical dislocation. Bladders were opened longitudinally; mucosa removed and detrusor strips mounted in organ baths for tension measurements at 35°C. Spontaneous activity typically developed during equilibration of the tissue to an initial tension of 1g. Regular activity was then recorded for at least 30 min and then blockers of various types of potassium channel were added. Application of TEA caused an increase in the amplitude of spontaneous contractions in a concentration-dependent fashion. 10mM TEA increased amplitude from 5.5 ± 1.4 mN to 43.8 ± 4.8 mN, n=14 tissue strips from 4 animals, $p < 0.0001$, EC₅₀ of 3.31 ± 0.17 mM). This was accompanied by a small increase in contraction frequency from 2.0 ± 0.4 /min to 3.4 ± 1.2 /min ($p = 0.055$). 4-Aminopiridine (0.1mM) increased amplitude from 3.21 ± 1.07 mN to 4.96 ± 1.39 mN, $p = 0.023$ and increased frequency from 1.6 ± 1.6 /min to 3.7 ± 2.7 /min, n=18 strips from 5 animals, $p = 0.0062$. The presence of KCNQ channels was investigated using XE991, a blocker of KCNQ channels. 10μM XE991 increased contraction amplitude from 5.1 ± 1.5 mN to 13.3 ± 2.8 mN, n=11 strips from 3 animals, $p = 0.0005$. The effects on frequency were less clear with 0.5μM increasing frequency from 2.5 ± 0.3 /min to 3.00 ± 0.2 /min, $p = 0.104$, however, higher concentrations did not further affect frequency. Linopirdine, a less potent analogue of XE991 had similar effects, increasing amplitude from 2.5 ± 0.4 mN to 5.3 ± 1.1 mN, n=5 strips from 2 animals, $p = 0.067$. There was little effect on frequency. Application of flupirtine, an opener of KCNQ channels, reversibly decreased contraction amplitude from 11.3 ± 1.4 mN to 3.4 ± 0.5 mN, n=16 strips from 4 animals, $p < 0.0001$. There was little effect on frequency. The findings of this study show that BK, delayed rectifier and KCNQ channels are all involved in spontaneous contractile activity of the guinea-pig bladder. It is likely that they contribute to repolarisation of the action potential and regulation of the resting membrane potential in smooth muscle cell and interstitial cells. To date, there is little evidence pointing to the presence of KCNQ channels in bladder cells; the present results provide evidence that such channels are present.

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PC572

Potassium channels and spontaneous activity in guinea-pig bladder

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The bladder has dual roles of storage and removal of urine. During the filling phase, low intravesical pressure is achieved through background spontaneous contraction of the smooth muscle. This

PC573

Anti-viral peptides against HCV

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Introduction: With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health.

A vaccine against HCV infection is not available, and current antiviral therapies are characterized by limited efficacy, high costs, and substantial side effects. Binding of the virus to the cell surface followed by viral entry is the first step required for the initiation of infection. Because this step represents a critical determinant of tissue tropism and pathogenesis, it is a major target for host cell responses and a promising target for new antiviral therapy. Envelope protein 2 (E2) of HCV initiates the association with the target cell surface [1] and it has been further shown that a 33 amino acid peptide derived from the C-terminal region of human lactoferrin (LF) possess binding activity to E2 and prevents HCV infection in HCV-infected human hepatocyte-derived PH5CH8 cells [2]. Small peptide molecules or more stable peptidomimetics may therefore present an interesting approach to chemotherapeutic prevention of HCV infectivity. Objective: The objective of this study is to determine the peptidic pharmacophore needed for E2 binding. In this regard, five peptides derived from the 33-residue LF fragment were synthesized and tested for E2 binding. Results: N-terminally biotin-tagged peptides (two 33-mers, two 27-mers, and a 17-mer) with sequence derived from LF were synthesized by Fmoc solid-phase peptide synthesis (SPPS). Of these, two of the peptides were with two-point mutations each. The synthesis involved manual addition of amino acids on Wang resin using the BOP/HOBt activation protocol. The in vitro binding affinity of these peptides with respect to the human lactoferrin (positive control) has been determined by an ELISA-based assay. E2 from two genotypes, 1a and 1b was used. Further, the solution conformation of these peptides has been studied by molecular dynamics simulations and CD spectroscopy. Conclusion: The results obtained from this study are used to design a non-peptidic small molecule that exhibits the same pharmacophore arrangement of functional groups in search for a potent anti-HCV agent.

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PC574

Mustard oil-induced alterations of hind limb withdrawal responses in the anaesthetized rabbit measured by movement analysis

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Application of the chemical irritant mustard oil to the hind limb of anaesthetized rabbits can produce long-lasting increases in withdrawal reflexes which are dependent on the reflex studied and the location of the noxious stimulus (Harris & Clarke, 2003). These previous studies recorded EMG responses, so alterations in hind limb movements were implied rather than actually observed. The aim of the present study was to use a movement analysis system to examine the effect of mustard oil on actual movements of the hind limb.

Experiments were performed on 13 rabbits lightly anaesthetized by a continuous i.v. infusion of Saffan (between 12 and 46 mg kg⁻¹ h⁻¹). Withdrawal responses in the left hind limb were evoked by noxious pinch of the skin using toothed forceps fitted with a pressure transducer (mean force 1.5 ± 0.2 kg for 1.4 ± 0.2s). Stimuli were randomly applied to eight locations but only data in response to pinching the ipsilateral heel or toes are included here. Movements of the limb were measured using a CODAmotion movement analysis system which allowed 3D joint angles at the hip, knee and ankle to be calculated via detection of strategically located infra-red emitters. Following recording of control responses a mustard oil conditioning stimulus (50µl; 20% in paraffin oil) was applied to one of six locations (maximum of three sites per animal) and pinch-evoked movements subsequently obtained. Only data after mustard oil application to the heel or the plantar MT joints are reported.

In animals receiving mustard oil to the heel, the control response to noxious pinch at the heel was extension of the ankle (by 4.8 ± 1.4 degrees, mean ± S.E.M.) and flexion at the knee and hip (by 6.4 ± 2.1 and 4.7 ± 1.6 degrees, respectively). After application of mustard oil to the heel, extension at the ankle joint in response to heel pinch was significantly increased (paired t-test, p < 0.05; n = 8) to a mean of 9.7 ± 2.0 degrees, but flexion at the knee or hip joints was not significantly altered reaching 6.8 ± 1.1 and 6.4 ± 1.3 degrees, respectively. In animals receiving mustard oil to the MT joints, the control response to noxious pinch at the toes was usually flexion at all three joints: mean angle changes at the ankle, knee and hip were 2.7 ± 0.7, 3.9 ± 1.6 and 1.6 ± 0.9 degrees, respectively. Application of mustard oil to the MT joints led to a significant increase in flexion at both the ankle and knee joints in response to toe pinch (paired t-tests, p < 0.05; n = 7): post-mustard oil angle changes were 7.6 ± 1.6, 9.0 ± 1.5 and 3.3 ± 0.9 degrees at the ankle, knee and hip respectively. These data show that a noxious conditioning stimulus can facilitate certain movements of the hind limb to pinch stimuli. In support of previous findings, the increases appear to enhance the protective function of the movement.

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PC575

Kolaviron attenuates ethanol-induced oxidative damage on male reproductive parameters

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Oxidative stress significantly impairs sperm functions (Emanuele et al., 2001) and the crude extract of *Garcinia kola* has been shown to reduce fertility in male rats (Akpantah et al., 2003). In this study, the effects of kolaviron, a biflavonoid from *Garcinia kola* seeds were examined on ethanol-induced oxidative damage in Sprague-dawley rats.

25 adult males were used. These were divided into 5 groups; group 1 served as control, which received corn oil (the vehicle for kolaviron), groups 2, 4, and 5 received kolaviron (100mg/Kg body weight), groups 3, 4, and 5 were administered ethanol (15%v/v body weight). All groups were treated for 4 weeks except group 5, which was treated for 8 weeks. Group 4 was given ethanol and kolaviron simultaneously while group 5 was treated initially with ethanol (4 weeks) and then kolaviron (4 weeks). Sperm morphology, histology and weights of organs, and MDA tests were assessed.

Kolaviron, has no significant effect on male reproductive functions, but significantly ($p < 0.05$) attenuated the damaging effects of ethanol on metabolic and reproductive organs as well as sperm functions. The antioxidative property of kolaviron was also confirmed in this study. Kolaviron a natural antioxidant (Farombi et al 2002), may therefore be used in the treatment of male fertility problems associated with oxidative stress damage.

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PC576

The development of a muscle-machine interface for use with an *in vitro* model of skeletal muscle

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The transition between muscle and bone *in vivo* is mediated by tendon, which acts to eliminate the impedance mismatch and stress concentrations present between the compliant muscle and the stiff bone. The force produced by muscle contraction is transmitted first through the myotendinous junction, then through parallel collagen fibres of the tendon and finally through the enthesis or osteotendinous junction. All three of these structures have been shown to differ in mechanical properties along their length, resulting in a smooth transfer of force between structures. Currently, the method used to engineer skeletal muscle involves the use of silk sutures to anchor the muscle construct, providing an attachment point to allow functional testing or stretching of the constructs. However, due to the impedance mismatch between the muscle and the suture, the muscles detach from their anchor points under stretch. The aim of this study is to produce a cell-embedded scaffold to function as a muscle-machine interface. Scaffolds consisting of polyethylene glycol diacrylate (PEGDA) hydrogels, incorporated with the cell adhesion peptide GRGDS to improve cell attachment and structurally reinforced with hydroxyapatite (HA) were produced for this purpose by UV crosslinking. The addition of HA alone or in combination with GRGDS did not affect the swelling properties of the hydrogel with the average length increasing by $41.1 \pm 10.6\%$ and width increasing by $142.1 \pm 17.9\%$ after 7 days in growth

media. To test the biocompatibility of the gels, PEGDA-HA, PEGDA-GRGDS and PEGDA-GRGDS-HA hydrogels were seeded with primary tendon fibroblasts and cell counts were performed at 6, 24 and 48hrs to quantify cell attachment and growth on the hydrogels. All gel types allowed cell attachment and growth with cell counts of 6333 ± 1100 (PEGDA-GRGDS), 4000 ± 943 (PEGDA-GRGDS-HA) and 3000 ± 157 (PEGDA-HA) after 48hrs. As PEGDA alone is non-adhesive to cells, this suggests that hydroxyapatite is promoting cell adhesion. The PEG-GRGDS-HA hydrogels were then cast in theta-shaped glass capillary tubes and the scaffolds were preloaded with primary tendon fibroblasts. After 4 days of incubation, sectioning and staining of the constructs showed DAPI and phalloidin staining primarily on the outside of the scaffolds, with isolated cells within the matrix. These studies suggest that the PEGDA-GRGDS-HA hydrogel may be a suitable scaffold for co-culture with an engineered muscle monolayer in the development of a muscle-machine interface to replicate the myotendinous junction in an *in vitro* model of skeletal muscle physiology.

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PC577

Teratogenic effect of lithium carbonate in early development of Balb/C mouse

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Lithium carbonate is used as a standard treatment for manic depression. While researchers have investigated the teratogenic effects of the lithium carbonate on embryos of various animals in later stages of development, very limited work has been done on the probability of effects at early stages of development. In this study, the teratogenic effect of lithium carbonate was investigated on the early development of Balb/C mouse embryos. A therapeutic dose (i.e. 300 mg/ kg b.w.) was injected into mice intra-peritoneally on days 3.5, 4.5, 5.5 and 6.5 of pregnancy. Then, on day 15.5 of gestation, embryos were collected from the pregnant animals. Among the embryos, 69.7% were healthy, 15.4% resorbed, 4% showed lordosis, 7.3% were undeveloped and 7.9% had eye malformations. A significant increase ($P < 0.05$) in the number of hepatic megakaryocytes and nucleated red cells were also observed among experimental embryos.

Analysis of maternal serum proteins prepared from dissected animals showed a significant increase or decrease ($P < 0.05$) in the levels of serum proteins albumin, α_2 globulin, β globulin and γ globulin.

This research on early developmental stages has shown that pregnant mothers need to be aware of possible teratogenic effects at early stages of pregnancy, though it has been thought that the egg envelope can prevent teratogens from entering. In this case, mothers may need to stop lithium carbonate treatment before they make a decision to be pregnant.

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PC578

Influence of neutering on collagen in the canine urinary bladder: evaluation by quantitative morphometric analysis

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Acquired urinary incontinence (AUI) in the bitch is a debilitating incurable condition hypothesised to be hormonally related as it affects 20% of neutered (Arnold et al., 1989) vs. <1% of intact bitches (Holt et al., 1993). AUI in the bitch is associated with a decreased maximal urethral closure pressure but this is not a defining attribute and its exact pathophysiology remains unknown. In post menopausal women UI is linked with changes in percentage collagen within the bladder and detrusor instability (Charlton et al., 1999). The aim of this study was to determine if neutering alters the collagen percentage in the canine urinary bladder wall.

Sections (2cm x 2cm) of bladder (lateral dome) were dissected from entire and neutered male and female dogs (ME, MN, FE and FN) euthanased by barbiturate overdose with informed owner consent. Dogs were of varying breed, weight (7-45kg) and age (1-14yrs). Tissue was fixed in 10% phosphate buffered formalin, wax embedded and sectioned (2µm) for Masson Orange G stain. Images (5 sections/bladder) were evaluated morphometrically by light microscopic stereologic analysis (40X) to determine collagen percentage. Data are expressed as mean ± s.e.mean (n= No. of dogs), with statistical differences determined by ANOVA (Bonferroni post-test).

Percentage collagen in the bladder was significantly increased ($P<0.001$) in FN 49.4 ± 3.3 (n=8), compared to FE 19.4 ± 2.2 (n=12), ME 19.5 ± 1.3 (n=21), and MN 20.3 ± 1.1 (n=9). There was no difference between percentage collagen in ME, MN or FE groups and there was no effect of weight or age on statistical analysis. The increase in collagen in the FN occurs due to greater collagen deposition between and within muscle bundles. This has the potential to affect bladder function by altering action potential conduction.

Strikingly this result contrasts with our previous work (Coit et al., 2006) which showed that neutering causes a marked decrease in the bladder responsiveness to muscarinic and neurogenic field stimulation equally in both sexes. The loss of gonadal oestrogen, following neutering, has been put forward as a cause for AUI. As oestrogen prevents proliferation of fibroblasts and the deposition of collagen in coronary vessels (rats) (Blacher et al., 2000) we postulate that a lack of oestrogen, following neutering in the bitch, may contribute to the observed increase of collagen within

the bladder wall. While this increase may predispose bitches to AUI, the sex difference in this study clearly suggests another mechanism underlies the change in bladder responsiveness. This will require further study but could lead to the development of new targets and therapies for treating AUI in both humans and dogs. ARNOLD, S., Arnold, P., HUBLER, M., CASAL, M. & RUSCH, P. (1989). *Schweiz Arch Tierheilkd*, 131, 259-63.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC579

Single cell-based assay for system biology studies

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A comprehensive understanding of elementary processes in individual single cells in development or adulthood is a challenging task, while essential in several molecular biology disciplines like in neural, stem cell and developmental biology. In this work we present an innovative transfection method with respect to already existing devices. The novelty comprises the advantage to address individual single adherent cells simultaneously, delivering one or more compounds or macromolecules to different single cells at the same time (multisite electroporation) or to the same cell at different times (serial electroporation) permitting a temporally controlled transfection. For our experiments we used several primary and immortalized cell lines (HDF, BAEC, NHEK, CHO, 3T3, COS). Cells were grown by standard culture procedures, directly on a 61 gold microelectrodes array integrated in a microfabricated biochip. Through a PC driven system, single cells were stimulated in the presence of different molecules of interest. We assessed the possibility to tune the delivery to individual (single) cells of different compounds such as 23bp fluorescein labeled DNA oligonucleotides, pcDNA3.1-EGFP, custom siRNA for EGFP silencing, fluorescein-labeled antibodies and other reporter molecules. This method will provide a cell-based assay as a powerful tool to screen various molecules giving the possibility to accurately restrict the transfected area and to follow the behaviour of the targeted cells. The technique can be used to get further insights not only into gene expression/silencing, but also into other areas of research such as single cell analysis, single cell biophysics, cell-cell signaling and microkinetics studies. For example, it will provide a useful tool for studying gene expression by simply applying the electric pulse in the presence of various stimuli

(structural proteins, genetic construct, transcription factors and cell-cycle regulators) and observing the cell reaction directly under microscope revealing cellular and molecular events of biology in real time.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC580

Functional analysis of the actin binding domain of Nesprin-1, giant protein at the nuclear envelope

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Nesprins, also known as Nuance and Enaptin, are extremely large actin-binding proteins associated with the inner and outer nuclear membranes. Nesprins contain N-terminal actin binding domain (ABD) followed by coiled coil rod domain and the C-terminal transmembrane domain.

Previous studies showed that the His-tagged ABD of Nesprin-1 binds and cross-links actin filaments into tight bundles. We have reexamined its actin-interaction properties using tag-free ABD and found that it binds to actin filaments without affecting their polymerization rate, but does not promote filament bundling. ABD also quenched the fluorescence of pyrene-labelled actin upon binding in a dose-dependent manner. This effect was used to determine the binding kinetics by stopped flow, and the Kd of the ABD.F-actin complex was estimated as 3.1 μ M. Next, we assayed the ABD/actin interaction in the presence of myosin and found that the ABD disrupts the actomyosin complex in a dose dependent manner. Competitive binding of the ABD and myosin to actin filaments was also measured by light scattering during actomyosin dissociation after flash photolysis of caged ATP. ABD efficiently displaced myosin from actin filaments without affecting kinetics of myosin dissociation. Furthermore, ABD efficiently blocked motility of actin filaments in the myosin-based assay, partially dislodging them from the myosin-coated surface.

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PC581

The influence of maternal diet on postnatal growth, development and physiology

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Alterations in maternal diet during pregnancy have been shown to change fetal and postnatal growth and physiology; here, the

sensitivity of early embryonic development to maternal diet was assessed.

Virgin female MF-1 mice were mated and randomly assigned to one of three diets, (a) normal protein diet of 18% casein (NPD), (b) isocaloric low protein diet of 9 % casein (LPD), or (c) LPD for the preimplantation period (3.5 days) then switched to NPD for the remainder of gestation (Emb-LPD). At birth, pups were weighed and litter size adjusted to a mean of 3 males, 3 females. Offspring were weighted weekly thereafter. Offspring behavioural patterns were assessed using an open field test. Blood pressure measurements were recorded using tail cuff. At 22 weeks, male mesenteric arteries were mounted on a wire myograph and the vasoreactivity of the vessels recorded. At 28 weeks, offspring were sacrificed, organs were weighed and stored. Female left kidneys were processed into glycol methacrylate and glomerular number determined using stereology.

At birth, Emb-LPD females were significantly heavier than controls ($p < 0.05$). Egg-LPD females were significantly heavier than controls for several weeks up 28 weeks of age ($p < 0.05$).

At 15 and 21 weeks of age, both male and female LPD and Emb-LPD offspring had a significantly elevated systolic blood pressure when compared to NPD offspring ($p < 0.05$). In myography, no significant differences were observed between male treatment groups. LPD females had significantly elevated serum angiotensin converting enzyme (ACE) activity, a known regulator of blood pressure through the generation of angiotensin II, a potent vasodilator. Emb-LPD and LPD females displayed significantly reduced heart:body weight ratios when compared to control females ($p < 0.05$).

Globally, Emb-LPD females showed significantly elevated open field activities (distance travelled and vertical counts), and significantly decreased time spent resting, when compared to controls ($p \leq 0.05$).

These data support the hypothesis that alterations in maternal diet during early embryonic development result in significantly altered patterns of postnatal growth, systolic blood pressure, and behaviour patterns.

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PC582

Lung, haematological and liver function parameters in workers occupationally exposed to cement dust in Sokoto, Nigeria

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Although the major pollution problem in cement factories is cement dust emission into the factory environment (ILO, 1999), there is paucity of data on the effect of cement dust exposure in cement factory workers in Nigeria. Evidence from experimental animals suggests that cement dust may be toxic to the liver (Kanta

et al., 1986) and bone (Pond et al., 1982). We hypothesized that such liver and bone lesions may also occur in man. Consequently we have measured haematological, liver and lung function parameters in the workers of the Cement Factory of Northern Nigeria, Sokoto.

Randomly chosen factory workers [$n=56$; years of exposure = 10.2 ± 5.6 (mean $\pm$ SEM) years] and an unexposed but matched group ($n=96$) were studied following informed consent. Data collection was effected by way of a structured questionnaire to determine use of protective gears e.t.c. Vital capacity (VC) and forced expiratory volume in 1 second percent ($FEV_1\%$) were measured at ATPS (ambient temperature and pressure saturated with water vapour). 10ml of blood was drawn. Some was anticoagulated and used to measure haematological parameters using the Quantitative buffy coat (QBC) II machine. Serum was obtained from the remaining and used to estimate the liver function parameters using the RAN-DOX test kits. Results are expressed as mean $\pm$ standard error of mean (SEM). Statistical analysis was carried out using the unpaired student t test. $P<0.05$ was taken as statistically significant.

Questionnaire responses indicated that protective gears were used by all workers. The VC [3.7 ± 0.5 ; versus $4.1 \pm 0.5L$; $P<0.001$], $FEV_1\%$ [78.4 ± 3.1 ; versus $89.0 \pm 1.5\%$; $P<0.005$], haemoglobin concentration [12.1 ± 0.4 ; versus $14.1 \pm 0.2g/dl$; $P<0.0005$] and packed cell volume [39.4 ± 6.9 versus $42.6 \pm 4.6\%$; $P<0.02$] were significantly lower in cement factory workers than in matched controls while the white cell [8.5 ± 0.6 versus 7.3 ± 0.2 ($\times 10^9/l$); $P<0.05$] and platelet counts [281.9 ± 20.3 versus 185.6 ± 5.6 ($\times 10^9/l$); $P<0.001$] were significantly higher. The serum liver function tests showed no significant difference except alkaline phosphatase [32.9 ± 0.8 versus $53.9 \pm 7.2(U/l)$; $P<0.01$] which was significantly lower in the cement factory workers than in the control subjects.

The results of the present study suggest that chronic exposure to cement dust may have deleterious effects on the lungs, the haemopoietic system and the liver.

International Labour Organization, (1999): Encyclopaedia of occupational health and safety vol. 3, 4th edition, pp 93.44-93.46 Geneva.

Kanta J et al. (1986) Br J Exp Pathol 67, 889-899

Pond WG wt al. (1982) J Anim Sci 54, 82-88

Thanks to the workers for volunteering and the management of the Cement factory of Northern Nigeria, Sokoto, Nigeria, for permission to carry out this study.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC583

Role of the C-terminal in the trafficking of the mouse urea transporter mUT-A3

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Urea is the largest solute component of urine and efficient urea handling is crucial in the generation of maximally concentrated urine. Urea movement across the nephron is facilitated by the UT-A urea transporters. Previously we have shown that UT-A3 is expressed on the basolateral membrane of the inner medullary

collecting duct (Stewart et al, 2004) and that 7 amino acids between residues 450–456 in the C-terminus are important for urea transport (Collins & Cooper, 2006). The current study further investigates the role of this 7aa region.

All clones were N-terminally tagged with eGFP. Mutations were introduced using quikchange and confirmed by sequencing. Oocytes were isolated from *Xenopus laevis*, injected with 1.5ng of the appropriate cRNA or 50nl of water. Urea transport was assessed by a 90s uptake of ^{14}C -labelled urea, 3–4 days post injection (Fenton et al, 2000). Each group contained at least 10 oocytes from 2 or more animals. Statistical analysis was by one way ANOVA coupled with Student-Newman-Keuls test and significance was assumed at the 5% level. MDCK II cells were grown on permeable filters and transiently transfected with 1 μ g of cDNA. Cells were fixed and the localisation of each construct determined by immunofluorescence and confocal microscopy. The apical membrane was labelled with TRITC-conjugated peanut agglutinin and the basolateral membrane was labelled using a primary antibody against β -catenin and a Cy-5 conjugated secondary antibody. Transfections were replicated 3–5 times for each construct. The C-terminus sequence of mUT-A3 is 449VRRSEEEKSP-NGD460. As found previously, urea uptake was observed for the K456X mutant but not the V449X mutant. A stop codon was inserted at 453, and the E453X mutant also retained the ability to transport urea. When transiently expressed in polarised MDCK II cells, mUT-A3, E453X and 456X co-localised with β -catenin in the basolateral membrane. In contrast, in cells transfected with V449X, fluorescence was exclusively intracellular. These results suggest that the C terminal of mUT-A3 is important in trafficking. The V449-K456 region reveals a high density of negatively charged residues and a putative PKA phosphorylation site (450RRS452). Starting with the E453X mutant we mimicked phosphorylated/dephosphorylated states at S452. With a charged residue at 452 (S452D-E453X) urea uptake was intact, but if the charge was removed (S452A-E453X), urea transport was lost. Returning to the full length clone, mutating the region of negative charge (452SEEE455) to 452AAAA455 or 453AAA455 did not interfere with urea uptake or trafficking. The last residue of mUT-A3 is negatively charged. Our results suggest that the presence of a negative charge at the end of the protein rather than the composition of the region V449-K456 is important for delivery to the plasma membrane.

Stewart et al. (2004) Am.J.Physiol. 286:F979-87

Collins and Cooper (2006) J.Physiol. Proc Physiol Soc 2 PC31

Fenton et al. (2000) 279:C1425-31.

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PC584

An *in vitro* investigation of the role of oxidative stress in photodynamic therapy (PDT)

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Aminolaevulinic acid induced photodynamic therapy (ALA-PDT) is a highly selective way to ablate tissue. A key application

of ALA-PDT is in the treatment of non-melanoma skin cancer, in particular basal cell carcinoma (BCC). BCC forms in basal cells deep in the epidermis and is the most common type of non-melanoma skin cancer accounting for approximately 75% of the total.

ALA-PDT is a ternary treatment for BCC involving three components: ALA, a pro-drug for the natural photosensitiser protoporphyrin IX (PPIX), light (636 nm) and tissue oxygen. The combination of these, causes cells to be destroyed via the production of reactive oxygen species (ROS) through a necrotic/apoptotic process depending on the PDT conditions employed. These ROS are generated from Type I and II photochemical reactions.

The current study investigated the molecular mechanisms of ALA-PDT induced apoptosis in cells (fibroblasts, keratinocytes and squamous carcinoma cells), assessing the amount/type of cell death occurring and also the reactive oxygen species being produced. This is with a view to enhancing treatment protocols. As positive controls in experiments when investigating the effect of ALA-PDT and cell death, fibroblasts were exposed to tumour necrosis factor α (25 ng/ml; TNF α) and hydrogen peroxide (1, 10, 100, 1000 μ M). Cells were assessed for type of death by using annexin V binding and fluorescence microscopy after 45 hours. The data showed that there was a statistically significant increase in % of apoptosis compared to the non-exposed control groups ($p < 0.05$; Student's t-test and two-way ANOVA, $p < 0.01$ respectively).

After setting up a reliable and consistent PDT system, fibroblasts were exposed to 0.5 mM of ALA for 6 hours and thereafter exposed to a red light (630 ± 15 nm; Paterson) for 5 minutes. The cells were assessed for cell death 16 hours after irradiation. The PDT exposed group demonstrated a statistically significant increase in % of apoptosis compared to the non-exposed control group ($p < 0.01$; Student's t-test).

This in vitro cell model will be further employed to explore the effects of pro- and anti-oxidant agents on cell death induced by ALA-PDT.

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PC585

Investigating chromatin regulation by the repressor element 1-silencing transcription factor (REST) and its effect in cardiac hypertrophy

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The most common disorder causing sudden cardiac death in young people is the cardiac disease, hypertrophic cardiomyopathy (HCM). Recent studies have suggested that HCM is more common than previously reported and it is now estimated that approximately 1 in every 500 people in the UK and USA suffer from the disease. HCM is characterised by cardiac hypertrophy, an abnormal thickening of the heart muscle and while most affected individuals show few or no symptoms, others suffer heart failure, arrhythmias and sudden death. The reason for the emergence of these symptoms in some people, but not others, remains

unknown but since the transcriptional levels of a number of genes are indicative of the disease phenotype, the control of transcriptional programs could provide one mechanism to alleviate HCM.

The genes encoding the brain and atrial natriuretic peptides (BNP and ANP) are normally highly expressed in the foetal heart with levels reducing during development. High levels of expression of these genes are observed in adult ventricular myocytes in cardiac hypertrophy. One transcription factor that is important in repressing ANP and BNP expression in the normal adult heart is the repressor element 1-silencing transcription factor (REST). The aim of this study was to investigate the molecular mechanisms of REST-mediated repression and its potential role in hypertrophy. This was achieved by interrogating protein-DNA interactions and chromatin modifications at REST binding sites by chromatin immunoprecipitation and RT-PCR. REST represses its target genes by recruiting two distinct corepressor complexes that include histone deacetylases (HDAC1, HDAC2) and a H3 lysine 4-specific demethylase (LSD1). Inhibition of REST function resulted in increases in ANP and BNP gene expression that correlated with increases in histone acetylation and dimethylation of H3 lysine 4 at the ANP and BNP promoters. Additionally, increasing REST expression in these cardiomyocytes prevented increases in ANP and BNP expression by the hypertrophic agonist, endothelin-1. This data provides evidence that a therapeutic strategy aimed at augmenting REST and/or the action of its corepressors may be effective in treating HCM.

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PC586

Epigallocatechin gallate and other green tea polyphenols inhibit glucuronide transport across the endoplasmic reticulum membrane

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Toxic endogenous or exogenous compounds can be inactivated by various conjugation reactions. Glucuronidation is especially important due to the large number of drugs and chemical carcinogens that are detoxified through this pathway. Stable and harmless glucuronides can be reactivated by enzymatic hydrolysis thus inhibitors of glucuronidase activity reduce the risk of chemical carcinogenesis. The aim of this study was to reveal whether this mechanism contributes to the anti-cancer effect of green tea flavanols, which has been shown in various animal models. Therefore, we investigated the effect of these polyphenols on deglucuronidation in rat liver microsomes and in Hepa 1c1c7 mouse hepatoma cells, using 4-methylumbelliferyl glucuronide as model substrate. Tea flavanols inhibited beta-glucuronidase in intact vesicles, where glucuronide transport across the microsomal membrane is rate-limiting, but were almost ineffective in permeabilized vesicles. Epigallocatechin gallate, the major green tea flavanol was shown to have a concentration-dependent inhibitory effect on both beta-glucuronidase

activity and glucuronide transport in native vesicles. Epigallocatechin gallate also inhibited beta-glucuronidase activity in native Hepa 1c1c7 mouse hepatoma cells, while failed to affect the enzyme in alamethicin-permeabilized cells, where the endoplasmic membrane barrier was eliminated. Our findings indicate that tea flavanols inhibit deglucuronidation in the endoplasmic reticulum at the glucuronide transport stage. This phenomenon might potentially contribute to the cancer-preventing dietary or pharmacological effect attributed to these catechins.

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PC587

TAK-778 stimulates osteogenesis activity of alveolar bone-derived osteoblasts

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The aim of this study was to investigate the effect of TAK-778 on human osteoblasts derived from alveolar bone. Primary cells were obtained by enzymatic digestion of human alveolar bone fragments and cultured in osteogenic medium. First passage cells were cultured in 24-well culture plates (2×10^4 cells/well) or in culture flasks (8×10^4 cells/flask) in osteogenic medium containing TAK-778 (10^{-5} M) or vehicle. At days 7, 14 and 21, cell proliferation and viability, total protein content and alkaline phosphatase (ALP) activity were evaluated. Bone-like formation was evaluated at day 21. Quantitative polymerase chain reaction (RealTime-PCR) to investigate ALP, osteopontin (OP) and Cbfa1 gene expression was performed at day 7. All experiments were done in quintuplicate with exception of gene expression that was carried out in triplicate. Data were compared by ANOVA or Mann Whitney test. TAK-778 did not affect cell viability ($p > 0.05$). Cell proliferation was reduced by TAK-778 at 14 and 21 days ($p < 0.05$). ALP activity, total protein content and bone-like nodule formation were increased by TAK-778 ($p < 0.05$). Expression of OP and ALP genes were up-regulated by TAK-778 ($p < 0.05$), while Cbfa1 gene was down-regulated ($p < 0.05$). TAK-778 enhanced key parameters of osteoblast differentiation including reduced cell proliferation, increased both ALP activity and bone-like formation and up-regulated the expression of OP and ALP genes. It means that TAK-778 could be a useful drug to enhance new bone formation in clinical situations that require rapid restoration of physiologic function.

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PC588

Heart rate responses to spontaneous arousals from sleep in autonomic patients and controls

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Arousal from sleep produces an acute increase in heart rate and blood pressure (O'Driscoll et al 2004). We tested the hypothesis that this increase is mediated by the sympathetic nervous system, by measuring the acute heart rate response to arousal from sleep in autonomically challenged patients; specifically Multiple System Atrophy (MSA) patients who have both central sympathetic and parasympathetic neuropathy, and Pure Autonomic Failure (PAF) patients who have a peripheral sympathetic neuropathy. After ethical approval and written informed consent, we studied 10 autonomic failure (AF) patients (5 MSA patients; median age, (range): 59 (48–68 years), 5 PAF patients; 52 (37–72 years) and 10 Controls (58 (42–71 years)). Each subject underwent a night of polysomnography (SOMNOScreen, S-Med, UK). Sleep stages were scored according to standard rules by a researcher blinded to the subject group. Spontaneous arousals (5 to 15 seconds of wake EEG) were identified from stable sleep with no apnoeas / hypopnoeas in the preceding 3 minutes. The RR interval was quantified pre and post arousal. To test the effect of arousal on the RR interval in AF patients compared to Controls, a mixed-model regression analysis was used; data were expressed as median (range). Please refer to Table 1.

The number of arousals analysed per patient was: MSA, 4 (1–7); PAF, 4 (2–5); Controls, 3 (1–6). The RR interval at baseline (60 s pre arousal) was: MSA, 1041 (762–1097) s; PAF, 938 (570–999) s, Controls, 995 (758–1179) s. There were no significant differences in the response to arousal across the 3 time periods (AF patients vs. Controls: coefficient of variation (CV) 14.8, 95% confidence interval (CI) -8.9–38.6; $p=0.22$, MSA patients vs. Controls: CV 10.6, 95% CI -18.9–39.9; $p=0.48$, and PAF patients vs. Controls: CV 17.3, 95% CI -14.4–49.1; $p=0.29$). In conclusion, although our sample size was small, our data indicates that the RR response to arousal from sleep was greater in the controls compared to the autonomically challenged patients, with the smallest response occurring in the peripherally de-innervated PAF patients.

3 time periods.

Table 1. Change in RR interval from baseline at 3 time periods

RR-I (in ms) Median (Range)	0-3s post arousal	3.1-9.9s post arousal	0-20s post arousal
Age Matched Controls (N=10)	-94 (-196-130)	-94 (-525-133)	28 (-244-147)
Autonomic Patients (N=10)	-37 (-134-29)	-42 (-253-61)	-2 (-259-79)
MSA Patients (N=5)	-40 (-134-29)	-48 (-253-61)	-27 (-259-79)
PAF Patients (N=5)	-29 (-69-12)	-21 (-129-35)	0 (-90-33)

O'Driscoll et al (2004). J Appl Physiol 96:865-870

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PC589

Antagonistic response of *Calotropis procera* on ovarian steroid hormone

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PRINCIPLE

The primary function of the ovarian hormone is to prepare the structural component of the female reproductive system for reproduction by maturing the primordial germ cell and providing the hormonal timing for ovulation. Gonadotropin releasing hormones (GnRH) which are required for the maturation of follicles in the ovaries is regulated by a feed back action of oestrogen and progesterone which is secreted by the granulosa cells of the follicles, act on the pituitary to inhibit the release of follicle stimulating hormone (FSH). Previous reports show that extract of *Calotropis procera* has a significant effect on the reproductive organ of Wister rat. This study shows how ethanoic extract of *Calotropis procera* antagonize the effect of ovarian steroid hormone based on the principle of inhibition of the feed back mechanism.

LABORATORY ANALYSIS

100 rabbit used for this research were grouped into five of (20) based on the dose per body weight, the last group served as the control. The extract was administered at different rate/dose for 10 days, after which Biochemical/Hormonal assay were done.

STATISTICAL ANALYSIS

The analyses were done between the groups and their respective control. Independent t- test with $p < 0.05$ was considered significant in all cases.

CONCLUSION

The ethanoic extract of *Calotropis procera* proved to be Abortifacient plant by decreasing of progesterone and causes hormonal imbalance needed for the maturation of follicles in the ovaries by antagonising the feed back action for such processes.

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PC590

Physiological mechanoadaptation of smooth muscle during pregnancy is associated with changes in expression and activation of dense plaque-associated proteins

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Smooth muscle is known to be a malleable tissue in that it can adapt *in vitro* to maintain contractile capacity over a wide range of imposed lengths. Uterine smooth muscle *in vivo* is exposed to considerable mechanical stimuli during pregnancy that necessitates complex adaptive responses so that premature delivery of the fetus is not precipitated but that, at term, contractile effort is maximized. The molecular and physiological nature of any such responses is unknown but may involve plasmalemmal dense plaque-associated molecules acting as sensors and transducers of the extracellular mechanical stimuli into intracellular signals. Our aim, therefore, was to assess the form of gestational-dependent mechanoadaptation of uterine smooth muscle, in terms of active and passive length-tension profiles, and if it was associated with changes in expression and activation of dense plaque-associated proteins. Methods: CD-1 mice (non-pregnant and gestational days (D) 7, 13, 15, 17 and 19, term d19) were humanely killed according to national guidelines, each the uterine horn weighed, the horns opened and the pups and placentas weighed, the pups humanely killed and uterine smooth muscle tissue processed for *in vitro* contractility measurements or Western blotting. Small uterine strips were bathed in physiological saline solution at 37°C, bubbled with 95% air-5% CO₂ mixture and mounted in standard organ baths for isometric force measurement. Using a calibrated micrometer arm, tissues were initially set at slack length and passive tension noted, stimulated with high K PSS (60mM KCl substituted for NaCl) and active tension measured, washed in PSS and stretched by 10-20% and restimulated with high K PSS. The process was repeated to construct incremental passive and active length-tension curves. Tissue was probed by SDS-PAGE and Western blotting for α -actin, focal adhesion kinase (FAK), paxillin, c-Src and Erk1/2. Results: Pregnancy was positively correlated (Pearson's correlation coefficient) with pup and uterine weight indicative of *in vivo* increased mechanical stimulation of the uterus. Equally, both the passive and active length-tension curves progressively shifted dramatically to the right. For example, at D19 maximum active tension was generated following 3.94 ± 0.33 -fold stretch beyond slack length compared to 1.91 ± 0.12 -fold for NP mice (mean $\pm$ sem, $p < 0.05$, Dunnett's, $n=4$). Pregnancy was also associated with elevations in the expression and activation of FAK, c-Src and Erk1/2 relative to α -actin (which was invariant).

Conclusion: These results suggest that alteration in the expression/activation of dense plaque-associated proteins may contribute to the physiological mechanoadaptation of uterine smooth muscle with pregnancy.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC591

Bretylium abolishes neurotransmitter release, without necessarily abolishing nerve terminal Ca^{2+} transients, in the mouse vas deferens

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High-resolution imaging of autonomic terminals in living organs is limited by the difficulty of determining the nerve terminal type on the microscope stage. One potentially useful tool for identifying sympathetic terminals is bretylium. By measuring the nerve terminal impulse, bretylium has been shown to abolish nerve terminal action potentials in the guinea pig vas deferens (Brock & Cunnane, 1988), but in the mouse, most nerve terminal Ca^{2+} transients are not abolished by bretylium (Jackson & Cunnane, 2002). To further characterize the action of bretylium in the mouse vas deferens, its actions on nerve terminal Ca^{2+} kinetics and neuroeffector Ca^{2+} transients (NCTs; focal smooth muscle Ca^{2+} transients rapidly triggered by local ATP release) were determined. Formaldehyde-glutaraldehyde-induced catecholamine fluorescence (FAGLU) was used in conjunction with orthograde filling of the Ca^{2+} indicator Oregon Green 488 BAPTA-1 10 kDa dextran (Brain & Bennett, 1997) to identify sympathetic terminals on the stage of a UV confocal microscope. 113 of 117 nerve terminals filled with the Ca^{2+} indicator were FAGLU positive (93–99%; $n = 7$ preparations) and are hence argued to be sympathetic. Bretylium did not abolish the field-stimulus-induced Ca^{2+} transient in any of the terminals tested in this series ($n = 7$). However, bretylium did cause both an increase in the delay between stimulation and the arrival of the action potential in the terminal (by 2 ± 0.4 ms) and the absolute refractory period to paired-pulse stimuli (by 4 ± 2 ms). In other experiments, smooth muscle cells were filled with the Ca^{2+} indicator Oregon Green 488 BAPTA-1 AM. Bretylium (10 μM) abolished NCTs at 60/61 junctions over the course of 2 hours. This suggests that sympathetic terminals can be identified by the presence of NCTs in smooth muscle cells adjacent to nerve terminal varicosities. Given that the vast majority of terminals loaded with the Ca^{2+} indicator using this protocol contain catecholamines (positive UV fluorescence after FAGLU) these findings suggests that in addition to its established action on nerve terminal action potential propagation, bretylium abolishes neurotransmitter release downstream of Ca^{2+} influx. Measurement of the bretylium-induced changes in absolute refractory period permits a simple method by which living sympathetic terminals can be identified. Brain KL & Bennett MR (1997). *J Physiol* **502**, 521–536.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC592

Resting membrane potential of rat mesenteric arterial smooth muscle correlates with vessel diameter: Relationship to myogenicity?

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It is a common observation that resistance sized arteries have the capacity to constrict in response to increases in intraluminal pressure; the myogenic response. The mechanisms that underlie myogenicity are not clearly defined although smooth muscle depolarisation and sensitivity to dihydropyridine antagonists appear strongly correlated with the myogenic response. Large arteries often lack the response, leading to the hypothesis that myogenic reactivity is inversely proportional to arterial diameter. Evidence exists to support such a relationship, for example, the arterial network of the hamster cheek pouch (Davis 1993) and rat cerebral arteries (Brekke et al 2002). In this study we tested the hypothesis that resting potentials recorded from rat mesenteric arteries at the sub-myogenic pressure of 20mmHg correlate with arterial diameter; that smaller diameter correlates with depolarised resting membrane potential.

Arteries were mounted in a pressure myograph bathed in PSS solution, bubbled with 95% O_2 :5% CO_2 , pH 7.4 at 37°C. Diameter was monitored using video microscopy and edge-detection software (Living Systems Inc). Vessels were initially pressurised to the myogenic pressure of 80mmHg, 37°C for 1 hour to record any development of tone. After which the pressure was maintained at the sub-myogenic pressure of 20mmHg and the arterial myocyte membrane potential was measured, using sharp microelectrodes (130–180M Ω), filled with 3M KCl. Quoted diameters are passive $\pm$ sem (n animals); being measured in the nominal absence of calcium, at 80mmHg.

The mean resting membrane potential of arteries below 200 μm was $-45.83 \pm 1.8\text{mV}$ (3) compared to $-49.58 \pm 1.12\text{mV}$ (3) in arteries of 250–300 μm , $-58.13 \pm 2.8\text{mV}$ (6) in arteries of 300–350 μm and $-66.63 \pm 6.4\text{mV}$ (3) in arteries of 450–500 μm . An unpaired, two-tailed t-test, showed significant difference between vessels of <200 μm and 300–350 μm ($p = 0.02$) and between vessels sizes <200 μm and 400–450 μm ($p = 0.03$). For the arteries investigated, myogenic constriction only occurred in vessels with a mean resting membrane potential of -45.83mV at the sub-myogenic pressure of 20mmHg. In summary, our data suggest that resting membrane potential of rat mesenteric arterial smooth muscle cells does correlated with passive (resting) arterial diameter.

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PC593

Messenger RNA turnover in the mammalian mitochondria

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Human mitochondrial mRNA turnover, one of the key processes governing the gene expression inside the organelle, remains so far the unsolved puzzle. Neither the possible pathway nor the enzymes involved have been identified. Mitochondrial mRNA molecules lack the 5'-cap structure, but the transcripts are constitutively polyadenylated and therefore possess stable 3'-poly(A) extensions. However the stabilising effect of polyadenylation observed in the eukaryotic cytosol has not been shown in the mammalian mitochondria and the existence of a protective poly(A)-binding protein is uncertain. To date, both in silico and biochemical studies of the mammalian mitochondrial proteome have failed to reveal any obvious candidates for the ribonucleases involved in the degradation of the mitochondrial transcripts. We present the results of our search for the proteins involved in the mt-mRNA turnover. A range of biochemical methods including 2D gel electrophoresis and zymography has been applied to analyse the ribonucleolytic activities from the mitochondrial subfractions. We have revealed a limited number of ribonuclease activities and have subsequently commenced mitochondrial protein fractionation in harness with RNA degradation assays to identify and characterise the candidates for the mRNA-metabolising enzymes.

These studies attempt to shed some light on the molecular mechanism governing mRNA metabolism and the role of polyadenylation in the human mitochondria.

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nucleotide (P2) receptors on cell membrane, resulting in activation of calcium signaling pathways. Cardiac fibroblasts (CFs) comprise approximately 90% of non-myocyte cells in the heart and are important for the regulation of cardiac function. Given the vast number of CFs in the heart, we hypothesized that CFs can be a source of ATP in the extracellular environment and that CX hemichannels may be involved in the release mechanism for ATP. Ventricular CFs were isolated from adult Sprague Dawley rats via retrograde enzymatic (collagenase) digestion. Cultured cells (12-16 days old) were plated on 60mm petri dishes or glass slides for static and fluid flow experiments, respectively. Samples exposed to selected experimental conditions were collected, and ATP levels were measured via a luminometric luciferin-luciferase ATP assay. Initial studies revealed that ATP release was increased by approximately 4-fold following 50% hypotonic challenge. ATP release was further increased when ecto-ATPase inhibitors, ARL 67156 and/or adenosine 5'-(α - β -methylene) diphosphate (AMP-CP), were present. These preliminary results suggest that the inhibition of ATP metabolism can cause further ATP release, possibly triggered by positive feedback mechanisms. Furthermore, decreasing the extracellular calcium concentration from 1 mM to 0 mM, a maneuver known to open CX hemichannels, resulted in an increase in ATP release, while 18- α -glycyrrhetic acid and carbenoxolone, CX hemichannel blockers, markedly decreased ATP release. These studies suggest that hypotonic induced ATP release from CFs may take place through CX hemichannels. In ongoing studies, the presence of CX hemichannels and P2 receptors in cardiac fibroblasts is being evaluated with PCR analysis, western blots, and fluorescence imaging, and down regulation of CX43 expression, using small interfering RNA (siRNA), is being attempted.

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PC595

Structural properties of hepatitis C virus core protein assemblingS. Lin¹, Y. Chen^{1,2}, T. Lin³, J. Liou⁴ and S. Lo²¹*Institute of life science, Tzu Chi University, Hualien, Taiwan,*²*Institute of Medical Science, Tzu Chi University, Hualien, Taiwan,*³*Department of Medical Research and Education, Veterans General Hospital, Taipei, Taiwan and* ⁴*Department of Biochemistry, Tzu Chi University, Hualien, Taiwan*

The hepatitis C virus core protein is the main consistent of nucleocapsid [1] and exhibits multiple functions involving in cellular growth, signaling and others[2-5]. In this study, we successfully overexpressed and purified the N-terminal 1-116 core protein, and assembled them into capsid-like particles. Using gel-filtration and sucrose-gradient assays, we characterized the molecular weight of the HCV core particle to be 180mers (~2400 kDa) and T=3. We studied the structural properties of the assembled core proteins using CD spectroscopy, AFM and TEM. The secondary structure of core assemblies contains 23% α -helix, 34% β -sheet and 47% random coil. The structure of this assembled core protein is hardly affected by pH, urea and ionic strength

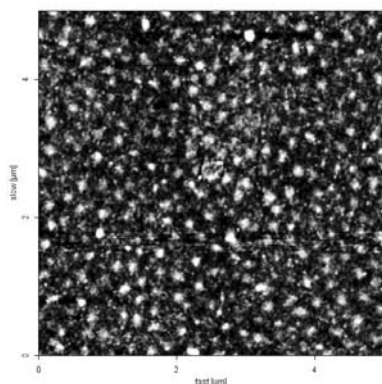
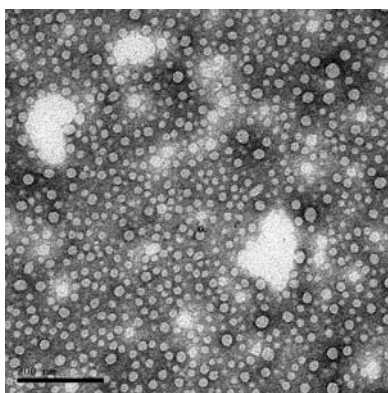
PC594

Connexin hemichannels appear to mediate ATP release from rat ventricular fibroblasts induced by hypotonic challengeS. Soleymani¹, N. Aroonsakool¹, B.S. Hu¹, H.H. Patel³, P.A. Insel² and W.R. Giles^{1,4}

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ATP is released from many cell types in response to conditions such as hypotonic challenge, mechanical stretch, or shear stress. Some of the proposed mechanisms for ATP release pathways include: flux through connexin (CX) hemichannels, exocytosis, and/or involvement of cystic fibrosis transmembrane conductance regulator (CFTR) proteins. Once released, the extracellular ATP can act as an autocrine or paracrine agent to activate

revealed by CD and AFM. Furthermore, the 3D image of virus like particle was constructed by EM.



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PC596

Decreased dehydroepiandrosterone and dehydroepiandrosterone sulphate in Alzheimer's disease

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Oxidative stress and the production of reactive oxygen species (ROS) has been implicated in a number of diseases associated with ageing, including Alzheimer's disease (AD), atherosclerosis, rheumatoid arthritis and diabetes. An increase in oxidation

products has been identified in Alzheimer's pathology including DNA damage seen as an increase in 8-oxo-dG in cortical tissue and lymphocytes (1), markers of protein oxidation including protein carbonyl formation in Alzheimer's brains (2) and plasma proteins of Alzheimer's suffers (3).

Dehydroepiandrosterone (DHEA) and its sulphate (DHEA-S) are hormones produced by the adrenal cortex. The levels of both are seen to decline in concentration with age. These levels have been associated with increased oxidative stress (4). Previous studies involving Alzheimer's disease cell models have shown DHEA has antioxidant capabilities in protection of SH-SY5Y neuroblastoma cells against both direct oxidation reactions involving hydrogen peroxide and indirect radical attack via lipid peroxidation (5).

A recent study was undertaken on 144 volunteers, (72 patients with AD, and 72 age matched healthy controls). AD sufferers had a decreased level of DHEA (4.24ng/ml $\pm$ 0.45 for control vs. 3.40ng/ml $\pm$ 0.34 in AD) which reached significance ($p=0.027$, 1-tailed Mann Whitney). In addition results showed AD sufferers had a trend towards reduced DHEAS levels (0.90 μ g/ml $\pm$ 0.109 for control vs. 0.74 μ g/ml $\pm$ 0.078 in AD). DHEA levels were significantly correlated to DHEAS levels in both control and AD conditions (pearson correlation, $r=0.641^{**}$ control and $r=0.618^{**}$ AD; $^{**}p\leq 0.01$).

This study highlighted a measurable difference in DHEA and DHEA-S concentrations in plasma from patients suffering from AD when compared to age matched controls. This decrease may contribute to oxidative stress pathology in AD. The authors and others have shown that circulating DHEA levels increase following exercise, which may present opportunities for exercise interventions in populations where DHEA is reduced.

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PC597

Quantitative analysis of dyadic environment in cardiac myocytes of mice

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Organization of organelles in muscle cells results from its ultra-structural adaptation to fulfil specific contractile functions. From the aspect of spatial relations among organelles, the organellar neighbouring can be characterized by a new stereologic parameter the environment of organelle. The environment of dyadic elements was analysed using the stereological method of vertical sections applied to electron microscopic images of ventricular muscles of mice.

The analysis of the environment of t-tubules revealed that 24% of its surface is open to the free cytosol, whereas 58% of the t-tubule has direct communication with terminal cisterns of the sarcoplasmic reticulum; 9% is in the vicinity of myofilaments and 6% of the surface presents direct contacts with mitochondria.

The analysis of the environment of terminal cisterns showed that the surface exposed to the cytosol present 32%, while 38% of their surface is in the vicinity of t-tubules. The cisternal surface interacts by 15% with mitochondria and by 13% with myofilaments.

The results of analysis showed that despite of the localization of dyadic complexes in the intermyofibrillar space there is a substantial extent of their interaction with mitochondria that can be of significance for the energetics of cardiac muscle cells.

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cells. The number of cells, the media, and the composition of the gel were optimised for maximal force production over time. To overcome the high plasmin activity of the cells, we tested 2 methods to decrease the rate of fibrin breakdown: 1) inhibition of plasmin activity through the inhibitor aprotinin; and 2) increasing the number of cross-links in the fibrin gel using the natural cross-linker genipin. Muscles were engineered with synthetic tendons set 12 mm apart to set their length and for the determination of isometric contractile properties by allowing attachment to a custom made force transducer. With aprotinin, the gel had contracted around the anchor points producing a tubular construct after 3-4 days. This occurred between 8-10 days in the genipin constructs. Standard differentiation media (DMEM with 2% horse serum (HS)) caused rapid fibrinolysis resulting in failure by 8 days at the highest concentration of aprotinin. When differentiated for only 2 days in 2% HS then moved to media supplemented with 7% FBS, aprotinin constructs could be cultured for 2 wks. Using genipin in place of aprotinin, constructs could be cultured for up to 4 wks. Aprotinin constructs produced peak twitch force (Pt) $12.6 \pm 1.8 \mu\text{N}$ and peak tetanic tension (Po) $20.1 \pm 2.1 \mu\text{N}$ after 7 days with this reducing over the next 5 days (Pt = $4.9 \pm 0.5 \mu\text{N}$ and Po = $6.5 \pm 1.4 \mu\text{N}$) prior to construct failure. Genipin constructs produced Pt = $11.5 \pm 1.9 \mu\text{N}$ and Po = $35.1 \pm 10.9 \mu\text{N}$ after 7 days and maintained force production over time so that at 3 wks constructs generated Pt = $17.8 \pm 4.8 \mu\text{N}$ and Po = $45.6 \pm 10.9 \mu\text{N}$. In conclusion we have engineered skeletal muscles from the C2C12 cell line which form in 10 days, survive in culture for 3 wks and can be used as a tool to study genetic alterations, pharmacological interventions and exercise on muscle physiology and function.

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PC598

Engineered muscle using the C2C12 cell line as a tool for studying muscle physiology and function

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The long-term goal of being able to engineer functional skeletal muscle for clinical application is decades away due to limitations such as the lack of vascularisation and innervation. In spite of this, muscle engineered from primary muscle cell cultures are functionally similar to adult skeletal muscle producing positive force frequency and normal length-tension relationships suggesting they could potentially be used as a model to study muscle physiology and function. Being able to engineer muscle from transformed cell lines would enable the study of genetic modifications (transient and/or stable) on muscle function but is currently not possible due to the short lifespan of constructs (<1 wk). The short lifespan is the result of the fibrin being rapidly digested by the transformed cells due to their high levels of plasmin (an enzyme that degrades fibrin). The aim of this study was to optimise conditions for engineering muscle from C2C12

PC599

Exo IX; A flap endonuclease stuck in reverse?

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The flap endonucleases are involved in a wide range of processes involving the structure-specific cleavage of branched DNA and RNA substrates. They are metalloenzymes, able to hydrolyse DNA and RNA substrates from the 5'-end liberating mono-, di- and polynucleotides with a 5'-phosphate. Much effort has focussed on analysis of the contribution of a number of highly conserved residues involved in metal binding, substrate interactions and catalysis (Feng et al). A group of cryptic flap endonuclease paralogues can be identified using homology searches. It has been reported that the flap endonuclease paralogue present in *E. coli* known as Exo IX is able to digest DNA in the 3'-5' direction, i.e. opposite to the usual polarity of flap endonuclease activity. We wish to present the results of our study on this unusual protein and an orthologue found in the human pathogen *Staphylococcus aureus*. The *E. coli* Exo IX protein was shown to possess a

weak 3'-5' exonucleolytic activity (Shafritz et al). It was able to interact with a range of proteins known to bind to single-stranded and branched DNA. These proteins were identified using a combination of affinity chromatography on Exo IX-Sephadrose beads with tryptic digest MALDI-TOF. The *S. aureus* enzyme was found to behave as a classical flap endonuclease and was able to hydrolyse a wide range of structured DNA substrates. We will reveal the mechanism by which Exo IX is apparently able to hydrolyse DNA in reverse!

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