C64

The effect of cholesterol depletion on insulin-like growth factor signalling in caveolin-positive and caveolin-negative cells

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Caveolae are cholesterol-enriched invaginations of the plasma membrane suggested to be important for many aspects of cellular signalling. Caveolin, a marker protein for caveolae, binds to and modulates the function of various signalling molecules including the tyrosine kinase receptor for insulin (Yamamoto *et al*, 1998). Components of the closely related insulin-like growth factor (IGF) system are important regulators of cellular function, with effects on growth, survival and metabolism mediated primarily through the type 1 IGF receptor (IGFIR).

Recent work proposed that IGFIR associated with caveolin, but the impact of lipid rafts/caveolae on IGF signalling remains controversial (Hong *et al*, 2004). Consequently, we investigated whether these membrane domains were important for IGF-mediated cell proliferation (thymidine uptake) and survival (nuclear morphology) in caveolin-positive (NWTb3) and caveolin-negative (HepG2) cells.

Subcellular fractionation and co-immunoprecipitation studies (n=3) in NWTb3 fibroblasts demonstrated that IGFIR associated with caveolin. 5nM IGF-I enhanced proliferation by 2.1±0.1-fold (mean±SEM; Mann-Whitney test; $p<0.05$; n=4) in NWTb3 cells and 1.4±0.0-fold (n=4) in HepG2 cells. When cellular cholesterol was depleted using 5mM methyl-cyclodextrin (MCD), which disrupts caveolae (Hailstones *et al*, 1998), IGF-I-stimulated cell proliferation was not impaired in either NWTb3 (IGF-I 2.1±0.1-fold; IGF-I+MCD 2.1±0.2-fold; n=4) or HepG2 cells (IGF-I 1.4±0.0-fold; IGF-I+MCD 1.2±0.1-fold; n=4).

5nM IGF-I protected NWTb3 and HepG2 cells from apoptosis induced by 24hr serum withdrawal (control 41.6±1.6% and 4.8±0.0%; IGF-I 6.1±0.4% and 1.3±0.2% apoptosis respectively; n=4). After MCD treatment, IGF-I-stimulated cell survival was significantly impaired in both NWTb3 (IGF-I 6.1±0.4%; IGF-I+MCD 27.1±1.0% apoptosis; n=4) and HepG2 cells (IGF-I 1.3±0.2%; IGF-I+MCD 2.6±0.2% apoptosis; n=4), although there was no effect from MCD alone (MCD 45.4±0.9% and 5.0±0.0% apoptosis respectively; n=4).

MCD treatment therefore induced the same effect on IGF-induced proliferation and survival in caveolin-positive NWTb3 and caveolin-negative HepG2 cells. This suggests that whilst IGFIR associates with caveolin by co-immunoprecipitation and subcellular fractionation, the interaction between IGFIR and caveolin may not be obligatory for IGF-mediated cell proliferation or survival.

Yamamoto M *et.al.* (1998) *Journal of Biological Chemistry* **273**, 26962-26968.

Hong S *et.al.* (2004) *Cell Death and Differentiation* **11**, 714-723.

Hailstones D *et.al.* (1998) *Journal of Lipid Research* **39**, 369-379.

We acknowledge BBSRC for financial support

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C65

Insulin-mediated nonspecific cation currents in control and diabetic *(ob/ob)* cardiomyocytes

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Cardiovascular disease is the highest contributor to morbidity and mortality in type 2-diabetic patients. In the heart, insulin has several effects, including altered energy metabolism, regulation of gene transcription, and activation of ion channels. Recently it has been shown that insulin activates a nonspecific cation current (NSCC) in adult cardiomyocytes (Zhang & Hancox, 2003). This NSCC could also be activated by diacylglycerol (DAG). Since DAG is known to activate some canonical transient receptor potential (TRPC) channels, these channels might be involved in the insulin-mediated NSCC.

Here we use the whole cell patch-clamp technique and Western blot analysis to study the possible role of TRPC channels in insulin-mediated NSCC in ventricular cardiomyocytes of adult mice. Moreover, we compare the NSCC and TRPC protein expression in control and insulin resistant, obese *(ob/ob)* mice. The study was approved by the local ethics committee. Mice were killed by cervical dislocation. Single cardiomyocytes were obtained by enzymatic digestion. Western blot analysis was performed on crude lysate from isolated cardiac ventricles.

Our data show that expression of TRPC3, 4 & 6 in heart are down regulated by 38%, 34% and 31%, respectively, in *ob/ob* compared with control mice (n=6 in each group). Application of the membrane permeable DAG analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG), induced an outward rectifying current both in control and *ob/ob* cardiomyocytes. This OAG-mediated NSCC was significantly smaller ($p<0.05$, Student's unpaired t test) in *ob/ob* mice (mean ± SEM; 0.73 ± 0.25 pA/pF at +50 mV, n=8) compared to controls (2.20 ± 0.77, n=6). The current was inhibited by SK&F96365 (10μM) and 2-aminoethoxydiphenyl borate (2-APB; 30μM), both frequently used to inhibit TRPC channels although their action is not specific. Application of insulin gave a dose-dependent NSCC response, which was not altered in *ob/ob* cardiomyocytes compared to controls. This insulin-mediated NSCC was insensitive to pretreatment with 2-APB but inhibited by SK&F96365 and abolished when phosphatidylinositol 3-kinase was inhibited by wortmannin (0.5μM).

In conclusion, DAG activates an NSCC in cardiomyocytes that is decreased in a mouse type 2-diabetes model. Based on pharmacological dissection and protein expression analysis, this DAG-activated NCSS might be mediated via TRPC channels. On the other hand, the insulin-activated NCSS is not different in diabetic and control cardiomyocytes and not inhibited by 2-APB, which suggests that it does not involve TRPC channels.

Zhang YH & Hancox JC (2003) Circ Res. 92, 765-768.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C66

Cross-linking CD98 heavy chain affects its trafficking from cytoplasm to plasma membrane in BeWo cells

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CD98 is a multifunctional transmembrane protein found on the surface of many activated cell types and is involved in the regulation of cellular differentiation, adhesion, growth, apoptosis as well as amino acid transport, although the exact mechanisms underlying most of these functions remain unknown.

It has been reported that some antibodies against CD98 suppress virus-induced cell fusion and CD98-mediated cell fusion of monocytes while others, cross-linking CD98, stimulate cell aggregation and growth, indicating that CD98 molecules are able to regulate cell fusion.

CD98 is expressed on placental cytotrophoblast and its involvement in syncytiotrophoblast formation has been suggested (Kudo *et al.*, 2003).

We used BeWo cells (a choriocarcinoma cell line) to study the molecular mechanism of syncytialisation in relation to the expression of CD98 and its regulation, before and after stimulation with Forskolin (cAMP treatment), incubation of anti-CD98 antibodies and antisense CD98 oligonucleotides.

BeWo cells (unlabelled or labelled with DiO or Mito-tracker deep red) were cultured in six well plates and treated, at 50-60% confluence, with Forskolin (100μM) and with or without the addition of monoclonal CD98 antibodies (mab4F2 and mabAHN18) or antisense oligonucleotide to CD98. The expression and distribution of CD98 and the degree of cell fusion were measured by western blotting, by multiple colour flow cytometry, and by immunocytochemistry and immunoelectron microscopy.

CD98 expression on the cell membrane increases by 40% in cells treated with Forskolin compared to control cells (P=0.0007, two-tailed t test), with a concomitant increase in fusion. CD98 expression is reduced to control levels by addition of antisense oligonucleotide. CD98 protein is increased significantly in both control and forskolin treated cells incubated with either monoclonal antibodies (P=0.015 in control cells, P=0.002 in forskolin treated cells, two-tailed t test). Thus flow cytometry, western blotting and confocal and electron microscopy results show recruitment and clustering of CD98 molecules from cytoplasm to cell membrane in response to cross-linking with monoclonal anti-human CD98. These data suggest that recruitment of activated CD98 to the cell membrane may be integral to the normal process of syncytialisation.

Kudo Y *et al.* (2003) *JPhysiol* **550.1**, 3-9

We thank Dr. T. Sethi (Lung Inflammation Group, Centre for Inflammation Research, University of Edinburgh, United Kingdom) for the donation of the mab4F2 antibody and The Wellcome Trust for funding this study.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C67

Mitochondrially generated oxidative stress in calf pulmonary endothelial cells

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Calf pulmonary endothelial cells (CPAE, obtained from the European Collection of Cell Cultures) respond to ATP with a biphasic increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and a transient production of reactive oxygen species (ROS), detected using fura-2 and carboxy-2',7'-dichlorodihydrofluorescein (C-DCF), the latter being a probe that is sensitive to H_2O_2. Results obtained using the mitochondrial inhibitors, antimycin A (AA), rotenone (ROT) and FCCP in intact CPAE, and FCCP in permeabilised CPAE implicated mitochondria as the source of the ROS (Wilkinson & Jacob, 2003, 2004) . Our hypothesis is that the increase in cytosolic $[Ca^{2+}]_i$ stimulates the mitochondrial electron transport chain, resulting in ROS production.

We measured either the transient increase in C-DCF fluorescence in intact CPAE in response to 100mM ATP or the maximum rate of increase of C-DCF fluorescence in CPAE permeabilised with 50μg/ml digitonin in an 'intracellular' solution of (mM) Na^+ 10, K^+ 135, Mg^{2+} 1, Cl^- 147, HEPES 20, and a nominal 100μM Ca^{2+}. This dose of digitonin selectively permeabilises the plasma membrane without releasing C-DCF trapped in the mitochondria. Data were analysed using a mixed model analysis (SAS) of the logarithms of experimental values. P values for comparison with control were calculated using Dunnett's test. Effects are quoted as a percentage, with the error estimated from the SEM of the logarithmic values. The n values quote the number of cultures; within each culture a minimum of 4 coverslips were measured.

Testing the effect of AA on intact CPAE in an additional culture revealed that the potentiation of the C-DCF response previously reported as insignificant was significant (41±18%, P<0.01, n=3). ROT and AA correspondingly stimulated the C-DCF response in permeabilised CPAE by 66±30% (P=0.027, n=3) and 93±48% (P=0.035, n=2) respectively.

The NADPH oxidase is generally recognised as a major source of ROS production in endothelial cells but its inhibitor apocynin (50mM in 0.01% DMSO) caused only a small, insignificant attenuation (13±23%, n=2) in the ATP response of intact cells.

Both these experiments provide data confirming our hypothesis that the source of the ROS is mitochondrial. We tested the effect of inhibiting nitric oxide synthase (NOS) using 10μM L-NIO. In intact CPAE, L-NIO significantly potentiated the response (68±31%, P=0.01, n=2) but in permeabilised cells its effect was insignificant (10±22%, n=2). NO could be reducing the C-DCF signal by combining with superoxide to form peroxynitrite, preventing its dismutation to H_2O_2. Furthermore, the source of the NO appears to be cytosolic since the effect of L-NIO was not seen permeabilised in cells.

Wilkinson JA & Jacob R (2003). Biochem Soc Trans 31, 960-962.

Wilkinson JA & Jacob R (2004) J Physiol 557P PC93.

Funded by Guy's and St Thomas' Charitable Foundation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C68

A role for nitric oxide in salivary gland hypofunction in Sjögren's syndrome .

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Nitric oxide is an intercellular signalling molecule which has the potential to amplify intracellular Ca^{2+} signalling in salivary gland cells by increasing cADPr levels and thus the contribution of ryanodine receptors to Ca^{2+}-induced Ca^{2+} release (CICR). Sjögren's syndrome is an autoimmune disease in which there is a failure of Ca^{2+} dependent fluid secretion. Paradoxically, NO levels are known to be elevated in the salivary glands of Sjögren's syndrome patients. (Kontinnen et al 1997)

We have investigated the effects of acute and chronic exposure to elevated levels of NO using the NO donor S-nitroso-N-acetyl penicillamine (SNAP). CD-1 mice were humanely killed by stunning and cervical dislocation. Submandibular glands were excised, acinar cells were isolated by collagenase digestion (200units/ml, Worthington) and cultured for 24 hours at 37º on matrigel (Becton Dickinson, UK) coated glass coverslips in serum free 50:50 Dulbecco's MEM:F12 medium containing antibiotics and antimycotics (Life Technologies UK). Agonist evoked changes in $[Ca^{2+}]_i$ were imaged (Cairn Research, UK) in acinar cells loaded with fura-2 AM. We found that acute application of SNAP at 100 μM caused significant amplification of threshold ACh responses, consistent with a cADPr mediated amplification of the Ca^{2+} signal (Harmer et al 2001). Sustained application of SNAP lead to a slow ramp in "resting" $[Ca^{2+}]_i$ and ultimately to insensitivity to ACh stimulation. These data suggest that prolonged exposure to elevated levels of NO render acinar cells less sensitive to ACh stimulation. These data are consistent with our previous observation that human labial gland acinar cells taken from Sjögren's syndrome patients show reduced sensitivity to stimulation by muscarinic agonists (Dawson et al 2001). The most straightforward explanation of this phenomenon is that NO leads to depletion of the intracellular Ca^{2+} stores.

Konttinen et al (1997) Arthritis Rheum. 40, 875-883

Dawson et al (2001) Clin Exp Immunol 124, 480-485

Harmer et al (2001) Biochem J. 353, 555-560.

This work was supported by the Health Foundation

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C69

Protein tyrosine phosphatase activity in lymphoid cells under conditions of whole body ionizing irradiation

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To understand the mechanisms of adaptational changes in signal transduction involving tyrosine phosphorylation, studies have been made in animals subjected to whole body ionising radiation.

Wistar rats (100-120g) were exposed once to X-rays at 0.5 Gy. After 12 h they were humanely killed. The levels of protein tyrosine phosphatase (PTP) activity were measured in spleen and thymus lymphocytes after incubation with 'cell-linked protein A', an immune stimulating factor possessing B- and T-cell blast-transforming activity. Exposure to radiation caused a decrease in the PTP activity stimulating action of mitogen incubation in the thymus, but not in the spleen (Table 1).

The catalytic features of purified CD45, the major transmembrane PTP of the lymphocytes, were evaluated using paranitrophenylphosphate and phosphotyrosine as, respectively, non-specific and specific substrates. With the former, irradiation was shown to cause a decrease in V_{max} but an increase in affinity. With phosphotyrosine both V_{max} and affinity decreased (Table 2). These data suggest that exposure to radiation causes an increase in non-specific enzyme activity with a decrease in the ability to dephosphorylate the specific substrate. A study of cooperativity parameters shows that cooperativity between two phosphatase domains increased following radiation. Analysis of inhibitor kinetics showed that radiation caused a change from competitive to mixed inhibition.

Table1. Protein tyrosine phosphatase activity (nmol Pi min^{-1} mg^{-1}), in membrane fractions and cytosol of thymus and spleen lymphoid cells of Control and 0.5 Gy-irradiated rats (mean±SEM; n=6) after mitogen incubation

Table1. Protein tyrosine phosphatase activity, nmol Pi /(min x mg), in membrane fractions and cytosol of thymus and spleen lymphoid cells of Control and 0.5 Gy irradiated rats (mean±SEM; n=6) after mitogen incubation

Sample	Control		Irradiation	
	Without mitogen incubation	With mitogen incubation	Without mitogen incubation	With mitogen incubation
Thymus membrane fractions	1.95±0.17	8.18±0.73*	9.14±0.81*	3.65±0.32*
Thymus cytosol	4.57±0.41	6.48±0.43	16.09±1.43*	16.21±1.58*
Spleen membrane fractions	10.05±0.89	16.30±1.45*	14.00±1.25	23.83±2.12*
Spleen cytosol	8.27±0.74	31.55±2.81*	10.24±0.91	6.99±0.62

Table 2. Activity of immunoaffinity chomatography purified protein tyrosine phosphatase CD45 from lymphoid cells of control and irradiated rats (mean ± SEM; n=15)

Table2. Activity of purified protein tyrosine phosphatase CD45 from lymphoid cells of control and irradiated rats (mean±SEM; n=15)

State of animals		CD45 catalytic features		
		Vmax, μmol/(min _ mg)	Km, mmol/l	K_{Hill}
PNPP as substrate				
Control		7.81± 0.64	24.72± 20.5	0.839± 0.075
0.5 Gy irradiation		1.15± 0.09*	4.29± 0.34*	0.977± 0.081
o-phospho-l-tyrosine as substrate				
Control	WITHOUT sodium vanadate cell preincubation	7.87± 0.65	2.61± 0.24	1.245± 0.121
	sodium vanadate cell preincubation	7.09± 0.58	3.4± 0.32*	1.346± 0.131
0.5 Gy irradiation	WITHOUT sodium vanadate cell preincubation	4.27± 0.35*	3.01± 0.28	1.676± 0.163*
	sodium vanadate cell preincubation	1.52± 0.12*	14.95± 1.39*	1.353± 0.147

* P≤ 0.5 compared with the control.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C70

Endothelin-1 Mediated Regulation Of Intercellular Adhesion Molecule-1 (ICAM-1) In Human Dermal Fibroblasts: Comparison with Interleukin-1β

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Endothelin-1 (ET-1), is implicated in the pathogenesis of fibrotic and inflammatory diseases including scleroderma, modulates extracellular matrix turnover and up-regulates cell surface adhesion molecules such as ICAM-1. Signal transduction under normal conditions has not been fully investigated, but is of interest as basal expression of ICAM-1 increases in cells from fibrotic lesions in scleroderma, and sensitivity to ET-1 is significantly reduced yet enhanced in response to Interleukin-1β(IL-1β).

In normal human dermal fibroblasts (HDF) ET-1 increased ICAM-1 mRNA after 1h, with protein expression increasing after 4h. Antagonists against ET-1 receptors ET_A (JKC-301, 10μM) and ET_B (BQ788, 10μM) inhibited ICAM-1 protein expression by 47±13% and 46±11%, respectively (mean±SD, n=4), whilst the dual antagonist Bosentan (10μM) abolished the ET-1 response, indicating that ET-1 signalling occurs via both receptor subtypes.

We previously reported that the signalling pathway involved in ET-1-induced ICAM-1 expression involves the activation of MEK, NFκB and PKCε(1). Involvement of PKCε was further confirmed by failure of the PKCδ-specific inhibitor Rottlerin (6μM) to prevent ET-1 induced increases in ICAM-1 mRNA. MEK and NFκB, along with PKCε, are involved in the direct effect of ET-1 on ICAM-1 expression. This was shown by rapid ET-1-induced phosphorylation of p42/p44 MAP kinase at 1min (seen via western blot), nuclear translocation of NFκB at 30 mins (NFκB EMSA/Supershift), and inhibition of ET-1-induced ICAM-1 mRNA and protein at 1h and 5h respectively by U0126 and PG490.

IL-1β signalling involves activation of NFκB and C/EBPβ in dermal microvascular endothelium, yet unknown in fibroblasts. In HDF, PG490 and PD98059 (MEK inhibitor) significantly reduced IL-1β induced protein expression, abolishing it when combined. NFκB activation (confirmed by EMSA/supershift), and ERK1/2 phosphorylation (western blot), indicates that IL-1β signalling is identical in fibroblasts vs. endothelium. The direct contribution of NFκB and ERK1/2 to IL-1-induced ICAM-1 was seen by inhibition of mRNA and protein expression by PD98059 and PG490 – as with ET-1.

An unexpected similarity in ET-1 and IL-1β-mediated ICAM-1 regulation was observed in HDF by transient transfection using a series of deleted ICAM-1 promoter constructs; ET-1 and IL-1β utilized the NFκB binding site at -180bp.

Due to the convergence of the signaling pathways, we hypothesize that the changes in sensitivity to ET-1 and IL-1β in scleroderma may be due to alterations in PKC expression and the regulation of NFκB activation.

Waters et.al., Cardiovasc Pathol Vol 13(3): 130 (2004)

Funded by the Wellcome Trust

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C71

Statins prevent lipid raft aggregation and inhibit allergen-induced mast cell activation

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Although statins lower serum cholesterol by inhibiting hepatic HMG coenzyme A reductase (HMG-CoA), this mechanism cannot explain recent observations that these drugs are immunomodulatory[1,2]. Cholesterol is an essential component of plasma membrane sphingolipid rafts and its depletion by agents such as methylβcyclodextrin (MβCD) disrupts rafts and prevents intracellular signalling by immune receptors such as the IgE receptor, FcεRI[3]. As cellular HMG-CoA is required for the de novo synthesis of plasma membrane cholesterol[4], we investigated the effect of statins on allergen-induced mast cell activation. Using the model cell line, RBL-2H3, we demonstrate that 48 h pre-treatment with either fluvastatin or lovastatin can completely inhibit FcεRI-mediated cellular degranulation. Dose-response experiments suggest these compounds are active at nanomolar concentrations (IC_{50} for fluvastatin and lovastatin of 0.5 and 29 nM, respectively). Cuvette fluorimetry of fura 2-loaded cells reveal that both fluvastatin and lovastatin pre-treatment (at 3 μM) inhibit allergen-induced calcium responses by 42 and 39%, respectively. These effects of statins were similar to those seen by physical depletion of plasma membrane cholesterol using MβCD (10 mM). The observed effects of statins are due to inhibition of HMG-CoA since they are reversed by co-incubation with mevalonic acid. Visualisation of lipid rafts using fluorescent cholera toxin B revealed that statins inhibit receptor-triggered raft aggregation equivalent to that observed following cholesterol depletion by MβCD. Furthermore statin treatment of human bone marrow-derived mast cells similarly inhibits degranulation and calcium signalling. Taken together, these data suggest that statins may uncouple FcεRI signal transduction by disrupting lipid raft function through inhibition of plasma membrane cholesterol signalling. Statins may, therefore, offer a novel therapeutic approach to the treatment of allergy.

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Funded by The Medical Reearch Council, UK and Inpharmatica

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C72

Mechanical stretch induces heme oxygenase-1 expression and elevates reduced glutathione levels in human mesangial cells: a role for p38^{MAPK} and TGFβ1

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Both metabolic (hyperglycaemia) and haemodynamic perturbations (hypertension) are involved in the pathogenesis of diabetic nephropathy. Mechanical stretch, mimicking glomerular capillary hypertension, plays an important role in diabetic glomerulopathy, which is characterized by excessive TGFβ1 production and extracellular matrix accumulation (Sharma et al., 1996). In diabetes generation of reactive oxygen species (ROS) and compromised antioxidant defenses contribute to the development diabetic complications. Previous studies have shown that mechanical stretch upregulates p38 mitogen-activated protein kinase (p38^{MAPK}) phosphorylation and increases production of TGFβ1 and fibronectin deposition in mesangial cells (Gruden et al., 2000).

We have investigated the effects of mechanical stretch (average 10% elongation, 1 cycle/s, for 2-48 h) on intracellular reduced glutathione (GSH) levels and expression of the antioxidant stress gene heme-oxygenase 1 (HO-1) in human cultured mesangial cells. GSH levels were determined using a spectrophotometric technique, while HO-1 expression was analyzed by western blotting from whole cell lysates using a specific monoclonal antiserum. We further examined whether inhibition of p38^{MAPK} with SB203580 or inhibition of TGFβ1 signalling with a TGFβ1 neutralizing antibody affects mechanical stretch-induced HO-1 expression.

Mechanical stretch upregulated HO-1 protein levels 2-3 fold as early as 3-6 h and this was sustained after 48h ($p<0.01$ students t-test). In parallel experiments, mechanical stretch for 48h caused a 2-fold increase in GSH levels (15 ± 3 vs 30 ± 3 nmol/mg protein, mean ± S.E.M., n=3, $p<0.01$). Pretreatment of cells with SB203580 (2μM) or a TGFβ1-neutralising antibody (5μg/ml) caused a further increase in stretch-induced HO-1 expression after 48h.

Mechanical stretch is a powerful stimulus for GSH production and HO-1 expression in human mesangial cells. Pathways other than TGFβ1 and p38^{MAPK} may be involved in the antioxidant response of mesangial cell to mechanical perturbations. Future studies will investigate the role of other MAPK cascades on stretch-mediated antioxidant gene expression and the potential inhibitory role of TGFβ1/ p38^{MAPK} on stretch-induced HO-1 expression.

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Supported by European Foundation for Study of Diabetes/Servier

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C73

MITOGEN ACTIVATED PROTEIN KINASES MODULATE INDUCTION OF HEME OXYGENASE-1 BY LOW DENSITY LIPOPROTEIN IN VASCULAR SMOOTH MUSCLE CELLS

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Oxidised low density lipoprotein (LDL) contribute to endothelial and smooth muscle cell (SMC) dysfunction in atherosclerosis (Ross, 1999), induce the stress protein heme oxygenase-1 (HO-1) and elevate glutathione (GSH) as an adaptive antioxidant response against oxidative injury. HO-1 catabolises heme to carbon monoxide and the antioxidant biliverdin (Siow et al., 1999). We have shown moderately oxidized LDL (modLDL), a species high in lipid peroxides, enhances HO-1 and GSH levels to a greater extent than highly oxidized LDL (oxLDL) in human aortic SMC (HASMC) (Anwar et al., 2003). The transcription factor Nrf2 mediates HO-1 induction by LDL following transactivation of the antioxidant response element of antioxidant genes (Ishii et al., 2004). In this study we investigate whether mitogen activated protein kinases (MAPK) or protein kinase C (PKC) modulates HO-1 and GSH levels in HASMC.

Cells were pretreated (30 min) in the absence or presence of MAPK inhibitors, U0126 (MEK), SB203580 (p38MAPK) and SP600125 (JNK) or PKC inhibitor GF109203 prior to treatment with native LDL (nLDL), modLDL or oxLDL (100 μg protein/ml, 2-24h). Combinations of the MAPK inhibitors were used to antagonise two or more pathways simultaneously. Phosphorylation of p42/p44MAPK (ERK), p38MAPK, JNK or CREB (cAMP-response element binding protein) and expression of HO-1 or Nrf2 in total and nuclear lysates were determined by western blot. Reduced GSH levels were assessed using a fluorescence assay. Nuclear translocation of Nrf2 was visualized by fluorescence immunohistochemistry.

Treatment of cells (2 h) with oxidised LDL, but not nLDL, significantly (unpaired Student's t test, $p<0.01$, n=3) enhanced levels of ERK, p38MAPK, JNK and CREB phosphorylation. Pretreatment with individual MAPK inhibitors[3] or PKC inhibitor significantly attenuated (47±12.2 %, $p<0.01$, n=3)HO-1 induction, but only inhibition of JNK significantly attenuated (34±2.2 %, $p<0.01$, n=7)elevation of GSH levels by modLDL(24h). Combined inhibition of JNK and p38MAPK inhibitors resulted in a further 47% attenuation in HO-1 induction by modLDL compared to either kinase individually, but did not alter GSH levels. Treatment of cells with oxidised LDL (2 h, 100 μg protein/ml) induced nuclear translocation of Nrf2.

These novel findings suggest that PKC is involved in part in HO-1 induction by oxidized LDL through activation of CREB, however, MAPK modulate HO-1 expression via activation of Nrf2. In addition, elevation of GSH levels by oxidized LDL is mediated by JNK but not PKC, ERK, or p38MAPK.

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This work was supported by a Guy's & St Thomas' Charitable Foundation Prize PhD studentship

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C74

Transcriptional regulation of pituitary tumour transforming gene-1 by the neuroprotective WldS gene in mouse cerebellar granule cells and HEK293 cell lines.

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WldS mutant mice and their transgenic equivalents show 10-fold slower axonal and synaptic degeneration in response to either peripheral or central nerve lesions, due to overexpression of an Ube4b/Nmnat-1 chimeric protein (Gillingwater & Ribchester, 2001; Mack et al., 2001). The WldS protein forms dense nuclear aggregates, suggesting that the gene exerts its neuroprotective effects indirectly (Gillingwater et al., 2004; Araki et al, 2004). Here we examined the hypothesis that the WldS phenotype is due to transcriptional regulation of other genes.

Western analysis and immunostaining of brains isolated from WldS mice killed by cervical dislocation (Home Office Schedule 1) established cerebellar granule cells as a rich source of WldS protein. We therefore extracted cerebellar mRNA from three WldS and three WT control mice and analysed gene expression using Affymetrix microarrays. Changes in the levels of candidate genes were validated by RT-PCR on the same mRNA extracts. As expected, there was a significant increase in Nmnat-1 expression in the WldS cerebellum, but several other genes were more than 5-fold up- or down-regulated, the largest change being a 10-fold downregulation in mRNA for pituitary tumour transforming gene (pttg-1). To test whether downregulation of pttg-1 was caused by WldS expression, we transfected cultures of human embryonic kidney (HEK) 293 cell lines with a fused WldS-EGFP construct, to allow for visual confirmation of protein expression. Semi-quantitative RT-PCR showed gene-dose dependent downregulation of the human PTTG-1 mRNA, 4 days after WldS transfection. Sciatic nerve section under ketamine/xylazine anaesthesia (respectively 100/10 mg/kg, IP) in nine pttg-1 (-/-) null mutant mice showed no evidence of protection of neuromuscular synapses by 48 hours. However, confocal microscopy revealed several intact axon fragments extending over 1mm in length in the distal tibial nerve.

We conclude that expression of the WldS gene results in downregulation of the pttg-1 gene. However, the neuroprotective phenotype of WldS mice is not explained by downregulation of only this gene.

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We thank the Wellcome Trust for support; and A Thomson, D Thomson and S. Ren for expert technical assistance.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C75

CALCIUM SIGNALS IN NF-κB ACTIVATION IN SKELETAL MUSCLE CELLS

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Depolarization of skeletal muscle cells in culture induced by K+ or by electrical stimulation, generates a complex Ca2+ release sequence of events both cytoplasmic and nuclear. A fast Ca2+ transient mediated by ryanodine receptors and associated to contraction, is followed by a slower Ca2+ transient induced by InsP3 receptors activation. K+-induced depolarization activates the transcription factors fos, jun and CREB, effect mediated by the slow but not by the fast Ca2+ component. These results suggest that Ca2+ signals specific properties are required for the activation of these particular factors in skeletal muscle cells.

Myotubes depolarized by K+ or by tetanic electrical stimulation (45 Hz, 400 pulses) present a transient increase in calcium/calmodulin-dependent protein phosphatase calcineurin (CaN) activity (BIOMOL kit) of 2- and 3-4 fold, respectively. The K+-depolarization of myotubes, transfected with a NF-κB luciferase-reporter vector, enhances NF-κB transcription which is partially blocked both by cyclosporin A (CsA), a CaN inhibitor, and by inhibitors of the InsP3-induced slow Ca2+ component. Further evidence for CaN activation is that the increase in ERKs and CREB phosphorylation induced by this electrical stimulation protocol, is significantly increased by CsA.

To further investigate the Ca2+ dependence over NF-κB, we have studied its activation by electrical stimulation in skeletal muscle cells kept in primary culture. Sprague-Dawley newborn rats were killed humanely, following the protocols approved by the Ethics Committee from Facultad de Medicina, Universidad de Chile. Different protocols of electrical stimulation permit the expression of fast and slow or only fast Ca2+ transients. Fast and slow Ca2+ transients are induced by 45 and 10 Hz protocols, while 1 Hz induces only the fast component. The fold induction of NF-κB-dependent transcription by 400 pulses of 45 and 10 Hz, was 2.02 ± 0.24 and 1.76 ± 0.28, respectively (n =4). Meanwhile, 1 Hz (400 pulses) activated the reporter gene by 1.30 ± 0.24 (n =4). Increasing to 1000 pulses, the reporter gene activation was 4.26 ± 0.29, 4.08 ± 0.55 and 3.50 ± 0.34 for 45, 10 and 1 Hz, respectively (n =4). These results, indicating a differential response of NF-κB to Ca2+ signals, are supported by IkBα degradation and p65 translocation experiments. Currently, we are assessing the contribution of both fast and slow Ca2+ transients on NF-κB activation by using pharmacological inhibitors. These experiments will allow to determine what kind of membrane electrical activity modulates CaN and NF-κB activation.

Supported by FONDECYT 1030988 and FONDAP-CEMC 15010006

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C76

Rapid effects of Insulin-like Growth Factor-1 on Nuclear and Cytosolic Calcium in Cultured Rat Cardiac Myocytes

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Insulin-like growth factor-1 (IGF-1) plays important roles in numerous physiological processes. IGF-1 and its receptor (IGF-1R) are present in rat heart, consistent with IGF-1 regulating growth and hypertrophy in the developing heart in an autocrine or paracrine manner. IGF-1 induces cardiac hypertrophy in vitro and in vivo; protective and anti-apoptotic properties for this growth factor have also been described.

Changes in intracellular Ca2+ concentration, [Ca2+]i after IGF-1 estimulation were visualized in single cardiomyocytes preloaded with Fluo3-AM. Increases of relative fluorescence represent an increase of free cytosolic [Ca2+].

In cultured rat cardiomyocytes, IGF-1 induced a fast and transient increase in Ca2+i levels apparent both in the nucleus and cytosol, releasing this ion from intracellular stores through an inositol 1,4,5-trisphosphate (IP3)-dependent signaling pathway. Intracellular IP3 levels increased after IGF-1 stimulation in both the presence and absence of extracellular Ca2+. The basal mass of IP3 was~20 pmol/mg of protein; IGF-1 stimulation significantly increased this value to 90 and 130 pmol/mg of protein (4.5- and 6.5-fold increase) for cardiomyocytes incubated in Ca2+-free and Ca2+-containing resting media, respectively. Data were analyzed by analysis of variance and comparisons between groups were performed using a protected Tukey's t test. A value of $p<0.05$ was set as the limit of statistical significance.

A different spatial distribution of both PLC isoforms and IP3 receptor isoforms in cardiomyocytes was found. Ryanodine did not prevent the IGF-1-induced increase of Ca2+i levels but inhibited the basal and spontaneous Ca2+i oscillations observed when cardiac myocytes were incubated in Ca2+-containing resting media. Spatial analysis of fluorescence images of IGF-1-stimulated cardiomyocytes incubated in Ca2+-containing resting media showed an early increase in Ca2+i, initially localized in the nucleus. ROI analysis clearly showed that extracellular Ca2+ slowed down the cytosolic but not the nuclear increase in [Ca2+] stimulated by IGF-1. Rates of rise of the nuclear Ca2+ transients in the presence and absence of external Ca2+ were 32.3 ± 7.6 and 38.6 ± 8.1 ($\Delta f/f$ per s) (n=4), respectively. However, rates of rise of Ca2+ transients in the cytosol were 3.9 ± 1.4 and 20.1 ± 5.3 ($\Delta f/f$ per s) (n =10), respectively.

Calcium imaging suggested that part of the Ca2+ released by stimulation with IGF-1 was initially contained in the perinuclear region. The IGF-1-induced increase on Ca2+i levels was prevented by 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid-AM, 100 μM (n= 5), thapsigargin 1 μM (n=4), xestospongins B 10 μM (n=3) and C 100 μM (n=7), 2-aminoethoxy diphenyl borate 20 μM (n=3), U-73122 50 μM (n=8), pertussis toxin 1 μg/ml (n= 7), and βARKct (an adenovirus-transduced peptide inhibitor of Gβγsignalling) (MOI 300) (n=7). Pertussis toxin and βARKct also prevented the IGF-1-dependent IP3 mass increase. Genistein (100 μM)treatment largely decreased the IGF-1- induced changes in both Ca2+i and IP3. LY29402 (but not PD98059) also prevented the IGF-1-dependent Ca2+ increase. βARKct, pertussis toxin and U73122 prevented the IGF-1-dependent induction of ERKs but not protein kinase B (PKB). We conclude that IGF-1 increases Ca2+ levels in cultured cardiac myocytes through a G βγ subunit of a pertussis toxin-sensitive G protein-PI3K-phospholipase C signaling pathway that involves participation of IP3 and perinuclear intracellular calcium store. Finally, this new signaling pathways is necessary for IGF-1-dependent ERKs activation in cardiac myocyte.

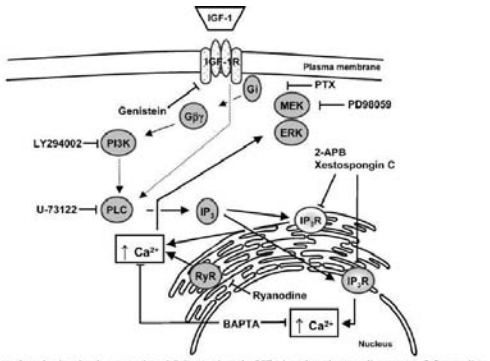

Fig. 12. Proposed mechanism for the generation of Ca^{2+} transients by IGF-1 in cultured rat cardiomyocytes. *RyR*, ryanodine receptor; *IP_3R*, IP_3 receptor (at least two different isoforms of IP_3R are considered).

Figure. Proposed mechanism for the generation of Ca2_ transients by IGF-1 in cultured rat cardiomyocytes. RyR, ryanodine receptor; IP3R, IP3 receptor (at least two different isoforms of IP3R are considered).

Ibarra C. et al (2004) J Biol Chem 279, 7554-7565.

We thank Dr. W. J. Koch for the βARKct and EV

adenovirus

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C77

The role of Ca^{2+} influx and TRPC channels in insulin-mediated glucose uptake in skeletal muscle

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Type 2-diabetes is a significant and rapidly growing health problem. Skeletal muscle is the major site of insulin-mediated glucose uptake and this process is impaired in type 2-diabetes. The signalling cascade leading to glucose uptake by skeletal muscles is not fully understood; for instance, the involvement of Ca^{2+} is a matter of debate. Here we examine the role of Ca^{2+} influx in insulin-mediated glucose uptake in skeletal muscle, specifically focusing on the possible involvement of canonical transient receptor potential (TRPC) channels.

The study was approved by the local ethics committee. Mice were killed by rapid cervical dislocation. Ca^{2+} influx was assessed in isolated single muscle cells by measuring the Ba^{2+} influx with the fluorescent indicator Calcium Green-1 and confocal microscopy. Glucose uptake was assessed by measuring the

uptake of radioactively labelled 2-deoxyglucose in isolated extensor digitorum longus (EDL) and soleus muscles.
The results show an insulin-mediated increase in Ba^{2+} influx (16 ± 2%; mean ± SEM, n = 34; P < 0.001, paired t-test), which was blocked by the TRP channel inhibitors 2-aminoethoxydiphenyl borate (2-APB, 100 μM) and Gd3+ (1 μM). 2-APB also decreased insulin-mediated glucose uptake in EDL and soleus muscles in a dose-dependent manner (~50% inhibition at 100 μM). 2-APB had no effect on basal, contraction- or hypoxia-mediated glucose uptake. The TRPC3/6/7 subfamily is known to be activated by diacylglycerol (DAG) and we observed an increased Ba^{2+} influx in response to the membrane permeable DAG analogue 1-oleyl-2-acetyl-sn-glycerol (OAG, 30 μM), which was blocked by 2-APB and Gd^{3+}. When OAG was added in the continued presence of insulin, there was an additional ~30% increase in the Ba^{2+} influx as compared to insulin alone (P < 0.001, paired t test, n = 14). In the presence of insulin plus OAG, glucose uptake in EDL muscles was increased by ~25% compared to insulin alone (P < 0.01, unpaired t test, n = 8). OAG had no effect on basal glucose uptake.
In conclusion, Ca^{2+} influx appears to have a specific role in insulin-mediated glucose uptake. Our results indicate that TRPC channels are involved in this Ca^{2+} signalling and hence these channels provide a novel target for therapeutic interventions in insulin resistant conditions.

This study was supported by the Swedish Research Council and Biovitrum Partner Fund.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C78

Intermediate steps for nuclear calcium signaling in skeletal muscle tetanic response

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The electrical stimulation of skeletal muscle cells through a tetanic type of protocol (10-90 Hz), generates in addition to the characteristic fast response due to calcium release from ryanodine type of receptors a second long-lasting rise in calcium, most prominent in the nucleus. We have characterized this second calcium signal to be originated by the activity of both the sarcolemmal dihydropyridine receptor (DHPr) and the inositol trisphosphate (IP_3) sensitive receptor activity (1). We have explored the intermediate steps that may link the DHPr and the IP_3 production to obtain this slow calcium signal.
The experiments were performed in rat myotubes kept in primary culture for 6 to 9 days. The satellite cells which originate these cultures were obtained from the hind limbs muscles of newborn animals killed humanely following methods approved by the Faculty of Medicine Ethics Committee. The standard electrical stimulation protocol used was a set of 400 pulses, 1 ms duration, delivered at 45 Hz. The field stimulation with this protocol in a whole 60 mm dish of myotubes enabled us to collect biochemical information (1). The calcium signal was monitored in individually stimulated cells preloaded with Fluo-3 (1). In particular, we studied the G protein activation by a pull down assay of the Gβγ subunit, showing a 67± 8.8% (n=3) of G protein activation over the control after the tetanic stimulation protocol. The pretreatment of the cells with a G protein inactivator (*pertussis toxin*) blocks both the calcium signal and the IP_3 transient. Also, the viral transduction of a Gβγ scavenger (Ad βARK) inhibited the slow calcium response and the IP_3 production as well. These results suggest the participation of the Gβγ subunit of the G protein as a necessary step in the signaling between DHPr and Phospholipase C (PLC) activation in skeletal muscle cells under tetanic stimulation.
Overall, these results indicate a novel mechanism of signal transduction by which the DHPr in the sarcolemma acting as a voltage sensor modulates the G protein response that is involved in the PLC activation to provide the nucleus with information about the muscle electrical activity level.

Eltit JM et al. (2004) *Biophys J* **86**,3042-3051

This work was funding by FONDAP # 15010006 and CONICYT predoctoral fellowship to JME.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C79

Effect of cholesterol extraction on $[Ca^{2+}]_i$ transients and K^+ currents in rat uterine myocytes.

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In recent years it has become clear that lipid microdomains within cellular membranes are important for cell signalling processes. Membrane lipid rafts, i.e. regions enriched in cholesterol and sphingomyelin, have been identified in many cell types including smooth muscle. Depletion of cholesterol using either methyl-β-cyclodextrin (MCD) or cholesterol oxidase causes substantial changes in phasic contractions of ureteric smooth muscle (Babiychuk et al., 2004). In the present study we analysed mechanism of stimulatory action of cholesterol extraction in freshly isolated cells. Late pregnant (19-21 days) Wistar rats were killed by cervical dislocation after CO_2 anaesthesia, in accordance with Schedule 1 of the UK Home Office. Single cells were enzymatically isolated from the longitudinal layer of the myometrium and superfused with Krebs solution. Conventional whole-cell patch clamp technique was used to measure transmembrane currents. Spontaneous and oxytocin-induced $[Ca^{2+}]_i$ transients were recorded from cells loaded with Fluo-4/AM (7mM, 10 min at 35°C) using confocal microscope (Perkin Elmer UltraView™ LCI). The amplitude of $[Ca^{2+}]_i$ transients was expressed as normalised Fluo-4 fluorescence (ΔDF/F0). Records were made from the same cell before and after cholesterol extraction, which was achieved by 10 minutes incubation of cells in Krebs solution containing 2% MCD followed by 5 min washout.
In spontaneously active cells extraction of cholesterol significantly increased the frequency of $[Ca^{2+}]_i$ transients from 0.34±0.03 to 1.46±0.11 Hz and their amplitude from 0.25±0.01

to 0.8±0.03 (t-test, $p<0.01$, n=36, data expressed as mean ± standard error). Application of 10nM oxytocin to quiescent cells caused a bi-phasic increase in $[Ca^{2+}]_i$ due to (i) release of Ca^{2+} from the SR and (ii) initiation of action potentials.

The amplitude of the oxytocin-induced SR Ca^{2+} release was not affected by extraction of cholesterol (paired t-test, 0.53±0.15 in control vs. 0.51±0.14, p=0.93, n=7). Parameters of the $[Ca^{2+}]_i$ transients evoked by oxytocin-induced action potentials were increased by cholesterol extraction similarly to that of spontaneous $[Ca^{2+}]_i$ transients.

In voltage clamped cells, cholesterol extraction led to a decrease in the potassium current density from 25.5±0.3 pA/pF to 17.1±0.12 pA/pF and increase in non-selective conductance (from 0.76±0.07 to 1.67±0.12nS) without a change in cell capacitance. These data suggest a stimulatory action of cholesterol extraction on uterine myocytes, mediated by reduction in outward K^+ current and increase in inward current possibly due to activation of non-selective cationic conductance.

Babiychuk, E.B et al (2004). J. Membrane Biol. 198 95-101.

Supported by the MRC.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C80

Calcium signalling by AMP-activated protein kinase is cell function specific

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Despite extensive study of previously favoured hypotheses, the precise mechanism of chemo-transduction in "O_2–sensing" cells remains obscure (Gonzalez et al., 2002). We now propose a novel mechanism by which hypoxia may affect cell function. Our data are consistent with the view that AMP-activated protein kinase (AMPK; Hardie et al., 2003), which monitors the AMP:ATP ratio as an index of metabolic stress, modulates O_2-sensitive Ca^{2+} signalling mechanisms.

Western blotting, immunoprecipitate kinase assays and immunocytochemistry identified AMPK in rat pulmonary artery smooth muscle. AMPK activation by phenformin (10 mM) or AICAR (1 mM) increased the Fura-2 fluorescence ratio (F340/F380) by 0.086 ± 0.017 (n = 7; mean ± S.E.M.) and 0.096 ± 0.009 (n = 22), respectively, in isolated rat pulmonary artery smooth muscle cells (for methods see Boittin et al, 2002). The increase in intracellular Ca^{2+} induced by 1mM AICAR was unaffected by removal of extracellular Ca^{2+} (1mM EGTA; n = 12). However, it was significantly inhibited by depletion of sarcoplasmic reticulum (SR) stores with 10 mM caffeine and 10 μM ryanodine (74%, n = 8, $P<0.001$, Student's t test) and by blockage of cyclic adenosine diphosphate-ribose (cADPR) with 100 μM 8-bromo-cADPR (83%, n = 23, $P<0.0001$). Thus, AMPK activation induces cADPR-dependent SR Ca^{2+} release via ryanodine receptors in pulmonary arterial smooth muscle.

The effect of AMPK activation on carotid body glomus cells was quite different (isolated as previously described; Wyatt and Peers, 1993). 1mM AICAR increased the F340/F380 ratio (0.07 ± 0.018; n=11) in a manner that was reversed by 100 μM Ca^{2+} and by removal of extracellular Ca^{2+} (n = 6). Thus, AMPK activation in carotid body glomus cells leads to voltage-gated Ca^{2+} influx.

We conclude that AMPK mediates cell-specific Ca^{2+} signalling mechanisms in carotid body glomus cells and pulmonary arterial smooth muscle cells in a manner that may be tailored to suit each cells function. Our findings suggest that AMPK may couple cell metabolism and Ca^{2+} signalling. Animals were killed humanely.

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Gonzalez et al. (2002) Respiratory Physiol. Neurobiol., 132, 17-41

Hardie et al., (2003). FEBS Lett., 546, 113-120

Wyatt, C. N and Peers, C (1993). Neuroscience, 54(1), 275-281.

This work was supported by the Wellcome Trust.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC97

Moderate exercise up-regulates the expression of antioxidant genes and of transcription factors for mitochondrial biogenesis. Oral antioxidants administration prevents it

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Exhaustive exercise generates excessive amounts of oxidative free radicals (Sastre et al, 1992) that overwhelm cellular antioxidant defences and may cause tissue damage (Gomez-Cabrera et al, 2003). They may, however, constitute signals to regulate muscle cell function. Accordingly, there is considerable interest in the potential of these mediators to regulate muscle adaptation to exercise. This is one of the oldest postulates in the field, dating back to the suggestion that free radicals produced in exercising muscle might stimulate mitochondrial biogenesis (Davies et al, 1981) and the expression of genes for antioxidant enzymes.
Moderate exercise increases lifespan in humans but this may be due to other healthy habits of the exercising population.
The aim of this study is to elucidate the role of the free radicals generated in moderate physical exercise, in the expression of antioxidant genes and of transcription factors for mitochondrial biogenesis.
Twenty male wistar rats were randomly divided into four groups: sedentary controls (n=5), exercised (n=5), exercised treated with 500 mg/Kg of vitamin C (n=5) and exercised treated with 32 mg/Kg of allopurinol (n=5). Allopurinol acts as an antioxidant because it inhibits xanthine oxidase, an important generator of free radicals in exercise (Gomez-Cabrera et al, 2003). The experimental protocol was approved by the Committee on Ethics in Research of the Faculty of Medicine Valencia. Where indicated animals were subjected to moderate exercise training (10, 17, 24 and 26 m/min for 5, 10, 10 and 20 min. each, five days a week during three weeks). Rats were anesthetized with 50 mg/kg sodium pentobarbithal by i.p. injection and gastrocnemius muscle was obtained by quick removal. Rats were killed by an ovedose of the anesthetic.
Our results, using quantitative Real-Time RT-PCR, show that moderate exercise significantly up-regulates the expression of antioxidant enzymes associated with longevity, such as Mn-SOD (3.5-fold change, $P<0.0001$) and GPx (3.4-fold change, $P<0.0001$). We also found that moderate exercise up-regulated the expression of NRF-1 (14-fold change, $P<0.0001$) that is a key transcriptional activator of nuclear genes encoding mitochondrial enzymes and Tfam (30-fold change, $P<0.0001$), which stimulates mitochondrial DNA transcription and replication. However, supplementation with vitamin C or allopurinol during training prevented all of these adaptations. Significance was determined by ANOVA followed by Tukey test relative to sedentary controls after normalization to GAPDH.
We conclude that oral antioxidant supplementation is very likely to be useful before competition when exercise is likely to be exhaustive, and damaging (Gomez-Cabrera et al, 2003), but not when training.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC98

Effects of H_2O_2 on cytochrome c, amylase release and cytosolic Ca^{2+} in the isolated rat parotid gland.

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Previously, we showed that ageing was associated with changes in the structure of the parotid gland and decreases in amylase secretion and cytosolic Ca^{2+} (Mahay *et al.* 2004). Since ageing is stress-related, we now investigate the effects of H_2O_2 on cytochrome c localisation in isolated parotid glands of young and aged male Wistar rats and amylase release and cytosolic Ca^{2+} in parotid glands of young (2-6 months) male Wistar rats. Animals were humanely killed and glands were isolated and either cut into small segments for the localisation of cytochrome c using immunocytochemistry or dissociated into acini for the measurements of amylase release and cytosolic Ca^{2+} signals (Pariente *et al.* 2000). All data are presented as mean ± SEM.
Treatment of glands from 2-6 and 12 month old rats with H_2O_2 resulted in time-dependent (2 and 4 hr) increases in cytochrome c fluorescence, compared to untreated glands. Cytochrome c was localised more in the ductal regions of the gland compared to acinar tissues. Treatment of glands from 22 to 24 month old rats for the same times showed no visible increases in cytochrome c compared to the other two age groups. Basal amylase release in acini suspensions from rats 2-6 months was 5.47± 2.09 % of total ($n = 8$). Incubation of acini with either 0.5, 1 or 2 mM H_2O_2 resulted in significant (Student's unpaired t test, $p<0.05$) increases in amylase release compared to basal. Values for amylase release with 0.5, 1 and 2 mM H_2O_2 were 10.08 ± 0.90, 11.83 ± 1.93 and 10.36 ± 0.74 % of total ($n = 8$), respectively. Stimulation of acini with either ACh, NA or ISOP (10^{-7} to 10^{-4} M) resulted in dose-dependent release of amylase above basal. Pretreatment of acini with 1 mM H_2O_2 followed by the addition of either ACh, NA or ISOP resulted in significant ($p<0.05$) decreases in amylase release compared to the responses obtained with secretagogues alone. $[Ca^{2+}]_i$ ratios (F340/F380) in fura-2 loaded parotid acinar cells isolated from 2-6 month old rats in the absence and presence of $[Ca^{2+}]_i$ was 0.845 ± 0.001, ($n = 50$ cells) 0.841 ± 0.003 ($n = 50$ cells) ratio units, respectively. Incubation of acinar cells with 1 mM H_2O_2 in either the absence or presence of $[Ca^{2+}]_o$, resulted in a gradual increase in the Ca^{2+} signal. Application of 10^{-5} M ACh after H_2O_2 incubation had no effect on these elevated Ca^{2+} signals. In contrast, ACh alone evoked a transient increase in Ca^{2+} signals followed by a plateau phase. Once, ACh-evoked Ca^{2+} signals were established, application of 1 mM H_2O_2 caused a further elevation in the Ca^{2+} signals. The results indicate that H_2O_2 can perturb cytochrome c levels, increase amylase release, alter Ca^{2+} homeostasis and antagonise the secretory response of ACh, NA or ISOP in the parotid gland.

Mahay, S *et al.*, (2004). *Mol. Cell. Biochem.* (in press).

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC99

Physiological Concentrations Of 17beta-Oestradiol And Genistein Exert Their Antioxidant Action By Induction Of Antioxidant Gene Expression In A Mammary Gland Tumour Cell Line

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Females live longer than males. The higher levels of oestrogens in females protect them against ageing, by up-regulating the expression of antioxidant, longevity-related genes (Borras et al. 2003). Oestradiol and genistein share chemical properties which confer antioxidant features to these compounds. The low concentration of them make it unlikely that they exhibit significant antioxidant capacity in the organism.

The aim of this study is to elucidate the mechanism through which oestrogens and phytoestrogens up-regulate the expression of antioxidant enzymes. We have used a mammary gland tumour cell line(MCF-7). Phosphorylation of mitogen activated protein kinase (MAPK) was determined by western blotting,translocation of p50 subunit of nuclear factor κ B(NFκB) to the nucleus in nuclear extracts by ELISA,expression of antioxidant enzymes by quantitative real-time RT-PCR,and peroxide levels was measured fluorimetrically (Barja et al. 1999).

Our results show that physiological concentrations of oestrogens (0.02 nM) and concentrations of genistein equivalent to those present in blood of oriental people (0.5 μM), activate the MAPK pathway. Oestrogens (n=6) and phytoestrogens (n=4) increase ERK1 and ERK2 phosphorylation when compared with controls (43.1±0.3 vs 41.2±1.7 arbitrary units, $p<0.01$ and 39.0±1.0 vs 37.6±0.3 arbitrary units, $p<0.05$, unpaired Student's t test, respectively). All the results are means±S.E.M. These, in turn, increase p50 subunit of NFκB in nuclear extracts from cells treated with oestrogens or phytoestrogens. The Mn-SOD and the GPx promoter region contain putative NFκB-binding motifs. Thus, activation of NFκB by oestrogens subsequently activates the expression of Mn-SOD(3.5-fold, n=3, $p<0.001$, ANOVA-Tukey test) and GPx (2.2-fold, n=3, $p<0.001$, ANOVA-Tukey test), but genistein is only capable of activating Mn-SOD expression (2.9-fold, n=3, $p<0.01$, ANOVA-Tukey test). This could be due to the fact that genistein binds preferently to oestrogen receptor β. This antioxidant protection is reflected in the lower peroxide levels we find in cells treated with oestrogens or phytoestrogens when compared with controls. The activation of signalling pathways and up-regulation of antioxidant enzyme expression is prevented when cells are co-treated with either oestrogens or phytoestrogens and the MAPK phosphorylation inhibitor UO126 (1 μM), thus reflecting the role of this signalling pathway in the antioxidant action of oestrogens and phytoestrogens. We conclude that oestrogens and phytoestrogens up-regulate expression of antioxidant enzymes via the MAPK activation, which in turn activate the NFkB signalling pathway.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC100

Sigma receptor expression in human carcinoma cell lines of ranging metastatic potential: effects of sigma receptor modulation on functional tests of metastasis performed on breast cancer cell lines

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The sigma receptor was first described as a novel opioid receptor but later found to be a distinct pharmacological entity distinguished by its unusually promiscuous ability to bind a wide variety of drugs (Martin *et al.* 1976). Binding of antipsychotic drugs such as haloperidol, along with a genetic linkage to schizophrenia, implicate sigma receptors also in psychosis (Ishiguro *et al.* 1998). In the central nervous system (CNS), sigma receptors have been shown to be involved in regulation of neurotransmitter release, modulation of neurotransmitter receptor function, learning and memory processes, and regulation of movement and posture (Su *et al.* 1993) as well as ion channel modulation (Aydar *et al.* 2002) sigma receptors have been implicated in cancer (Aydar *et al.* 2004). However the mechanisms through which sigma receptors produce their effects are still being defined.

Expression of sigma1 receptors in various human cell lines of different metastatic potential was investigated using real time (RT) PCR and by western blotting with a sigma1 receptor specific antibody. We tested the effect of sigma1 and sigma2 specific drugs on proliferation, adhesion, endocytosis and motility on two breast cell lines of different metastatic potential and a normal breast cell line. Subsequently we constructed and tested sigma1 receptor gene silencing and sigma1 receptor over-expression constructs.

Our results indicate that: 1) Metastatic cells express higher levels of sigma1 receptors than corresponding normal cells (4%), with the degree of sigma1 expression correlating with the degree of metastasis of the cell line (17-84%). 2) Silencing or over-expression of sigma1 receptor gene produced significant changes in cell proliferation for breast cancer cell lines (silencing; 6 ± 0.6%, 19 ± 1.9% and 13 ± 1.9% for MCF-10A, MCF-7 and MDA-MB-231, and over-expression; 20 ± 2.4% and 22 ± 0.4% for MCF-7 and MDA-MB-231), and less significant changes for non-cancer breast cell lines. 3) Over- expression or silencing of sigma1 receptor gene resulted in significant reductions in cellular adhesion for breast cancer cell lines (over-expression; 30 ± 5.5%, 18 ± 2.6% and 28 ± 1.6% for MCF-10A, MCF-7 and MDA-B231, knockdown; 19 ± 4.7%, 13 ± 1.7% and 13 ± 0% for MCF-10A, MCF-7 and MDA-MB-231) and a less significant reduction in adhesion in a normal breast cell line.

Overall, we conclude that sigma receptors may play significant roles in the proliferation and adhesion of breast cancer cells. Our

results are discussed in the light of recent advances in the understanding of sigma receptor biology.

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This project is funded by Wellcome Trust Career Development Grant awarded to Dr E Aydar.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC101

Human placenta P2Y nucleotide receptors contribute to blood flow regulation

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Extracellular nucleotides activate either P2X ionotropic receptors or P2Y receptors; the latter coupled to various G proteins. We assessed the distribution of $P2Y_1$ and $P2Y_2$ receptors along the human placenta vasculaure and investigated their relevance to maternal foetal blood flow regulation, extending the study of Valdecantos *et al.*, 2003.

Term Caesarean human placentas were perfused; the perfusion pressure was monitored, the nitric oxide (NO) outflow was determined by chemiluminescence. Bioassays measured vascular reactivity using rings prepared from segments of umbilical or superficial chorionic arteries or veins with or without endothelium; half-maximal effects (EC_{50}) were derived from concentration-response curves. The guidelines on ethical regulations for research with human samples were strictly adhered to.

The perfusion of cotyledons with 1-1000nM 2-MeSADP, a preferential $P2Y_1$ receptor ligand, elicited endothelium and concentration-dependent vasodilatation with an EC_{50} of 7.2±0.6nM (n=6). In addition, the 2-MeSADP EC_{50} to evoke a surge in NO outflow was 83±8nM; the maximal NO production elicited by 100nM amounted to 353±51pmol; this value was reduced 70% by eNOS inhibition and 85% by 100nM MRS2179 (n=5 each). 1-1000nM UTP, a preferential $P2Y_2$ receptor ligand, also dilated these vessels; its EC_{50} was 86±7nM. Receptor activation elicited a subsequent rise in NO outflow with an EC_{50} of 1843±124nM; 1μM UTP produced 120±17pmol NO (n=8), an effect blocked by eNOS inhibition, but not by MRS2179. In contrast, 2-MeSADP or UTP contracted the circular smooth muscle layer from umbilical or chorionic vessel rings; their EC_{50} in chorionic arteries were 4.2±1 and 5±2 nM respectively, (n=5 each). The contractile responses are mediated probably by an arachidonate metabolite since the vasomotor responses were reduced 80-90% by either 100nM indomethacin or 10 nM GR32191, a thromboxane receptor antagonist, suggesting that the response are indirect in origin and involve the synthesis of a thromboxane. RT-PCR demonstrated the presence of the mRNAs coding for the $P2Y_1$ and $P2Y_2$ receptors, in addition to $P2Y_6$ and $P2Y_{11}$ receptors. Western blot analysis of the $P2Y_1$ receptor protein confirmed its expression in both endothelial and vascular smooth muscle cells of the umbilical and chorionic vessels; however its distribution towards the cotyledons is uneven, showing a more intense expression in the endothelium of the smaller sized vessels, most likely micro vessels of the cotyledons.

The expression of multiple P2Y receptors coupled to vasocontractile or vasodilator mechanisms highlights the complexity of purinergic receptor distribution and their intracellular signalling along the human placenta vasculature. While the larger placental vessels express the $P2Y_1$ and $P2Y_2$ receptors, which promote the synthesis of an arachidonate metabolite, smaller sized vessels release NO.

Valdecantos, P., Briones, R., Moya, P., Germain, A., & Huidobro-Toro, J.P. (2003) Placenta, **24**: 17-26.

FONDAP grant 13980001 and MIFAB-Institute.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC102

REDOX POTENTIAL AND NO DONORS MODULATE THE CALCIUM DEPENDENCE OF RYANODINE RECEPTOR CHANNELS FROM RAT BRAIN CORTEX

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Ryanodine receptors/calcium release channels (RyR) from rat brain cortex, show three different responses to cytoplasmic free calcium concentration: low, moderate and high Po, depending on the oxidation state of their SH residues (Marengo et al,1998). Therefore, RyR channels have been proposed as intracellular redox sensors. Neuronal RyR channels are most likely activated by an initial increase in cytoplasmic calcium concentration [calcium induced calcium release (CICR)]. We tested the calcium dependence of single RyR channels from rat brain cortex fused with planar lipid bilayers at different redox potentials or after treatment with NO donors. Exposure of the cytoplasmic face of the channel to different ratios of the redox pair GSH/GSSG, modified the degree of channel activation, measured as fractional open time (Po). In the presence of a ratio GSH/GSSG=40/1 (redox potential=-220 mV), all RyR channels displayed the low Po behaviour. After changing the redox potential from -220 to -180 mV, the channels displayed the moderate Po response, increasing Po at all [Ca^{2+}] tested (0.1 to 500 μM). This effect was maximal at activating cytoplasmic [Ca2+]: Po increased from 0.036 ± 0.003 to 0.390 ± 0.111 (mean ± S.E.M.) at 14 μM [Ca^{2+}] (n=10). The calcium dependence of channel activity changed due to a tenfold increase in apparent affinity for activation by calcium (Ka = 110 ± 18 μM at -220 mV; Ka=12 ± 2 μM at -180 mV) and a fivefold decrease in apparent affinity for inhibition by calcium (Ki=5 ± 1 μM at -220 mV; Ki=24 ± 4 μM at -180 mV). Exposure of low Po channels to the NO donor SNAP (15 μM) induced a fivefold increase in Po at 14 μM cytoplasmic [Ca^{2+}] (n=13). The channel switched to the moderate Po behaviour due to an increase in apparent affinity for activation by calcium (Ka=189 ± 30 μM, control; Ka=39 ± 5 μM, SNAP) and a decrease in apparent affinity for inhibition by calcium (Ki=12 ± 2 μM,

control; Ki=35 ± 5 μM, SNAP). Exposure of low Po channels to the NO donor GSNO (200 μM) induced a fourfold increase in Po at 100 μM cytoplasmic [Ca^{2+}] (n=13). The channel switched to the moderate Po behaviour due to an increase in apparent affinity for activation by calcium (Ka=141 ± 29 μM, control; Ka=60 ± 18 μM, GSNO) and a decrease in apparent affinity for inhibition by calcium (Ki=8 ± 0.07 μM, control; Ki=20 ± 5 μM, GSNO). Our results suggest that SH oxidation and the modification induced by NO donors of RyR channels favours amplification of calcium signalling via CICR, and may therefore participate in a diversity of neuronal processes like neurotransmission and synaptic plasticity.

Marengo, JJ et. al.(1998) Biophys. J. 74.,1263-1277.

Supported by FONDAP grant 15010006 and FONDECYT grant 1040717.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC103

High Glucose Modifies Signalling Pathways Mediated by Bradykinin in Endothelial Cells

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The endothelium participates on the regulation of hemodynamic processes. In vascular disorders such as the ones observed in diabetes and hypertension, endothelial function is severely deteriorated. One of the substances involved in the regulation of endothelial function is bradykinin (BK), through the activation of two receptors B1KR and B2KR. While the B2KR is expressed constitutively, the B1KR is absent in physiological conditions but it is induced by inflammation and injury. We are interested in dillucidating the signalling cascade activated by BK in pathological conditions such as diabetes where both receptors have been documented. To approach this question, we determined the effects of high glucose (25 mM) on BK-activated signalling in endothelial cells in culture. We used the human cell line EAhy-926 cultured in normal (5 mM) or high glucose for 24 h prior to the stimulation with BK. Experiments were done in quadruplicate and expressed as mean±SEM. Values were analysed by the non parametric method of Kruskal-Wallis, and were considered different if $p<0.05$. BK induced an increase in Extracellular Regulated Kinases 1 and 2 (ERK1/2) phosphorylation that was maximal at 5 min (29±18% over control; n=4) whereas ERK1/2 phosphorylation was decreased (48±9% of control; n=4) at 15 min when cells were cultured in high glucose. In addition, BK induced an increase in PGE_2 concentration in the supernatants (199 pg/mg in control versus 517 pg/mg in BK stimulated; n=4) measured by ELISA, after 24 h of incubation in normal glucose but caused a decrease in the concentration of this prostaglandin (451 pg/mg in control versus 281 pg/mg in BK-stimulated; n=4) when cells were cultured in high glucose. In agreement with this observation, BK induced an increase in COX-2 protein expression determined by Western-blot that was maximal at 6 h in normal glucose, whereas it induced a decrease in COX-2 expression when cells were cultured in high glucose (n=4). High glucose induced an increase in the B1KR protein that was maximal at 12 h (200±13% over control; n=4). In addition, the B1 agonist des-Arg^9-BK, in the presence of Icatiban (a B2KR specific antagonist), induced an increase in nitric oxide (NO) release measured by the method of Greiss that was maximal at 15 min (2.5-fold over control; n=4) in high glucose. This effect was not observed when cells were cultured in normal glucose. In conclusion we have observed that high glucose increases the expression of the B1KR and modifies the effects of BK on ERK1/2 activation, PGE_2 and NO generation and these results suggest that high glucose may alter endothelial function by shifting BK signalling from the B2KR to the B1KR pathways.

Funded by FONDECYT 1040809.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC104

EPIDERMAL GROWTH FACTOR-INDUCED ACTIVATION OF A Cl^- CURRENT BY A NAD(P)H OXIDASE-DEPENDENT MECHANISM IN HELA CELLS.

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Evidence show that diverse growth factors (GF) induce an increase of reactive oxygen species (ROS) (1) suggested to participate in cell proliferation. One putative role of these ROS is to modify the phosphorylation state of GF receptors by reversible inhibition of proteins tyrosine phosphatases. It has been also demonstrated that subtoxic oxidative challenges induce cell proliferation. On the other hand, a crucial role of Cl^- channels in cell proliferation has been suggested in different cells lines (2). Recently, we have demonstrated that hydrogen peroxide derived from NAD(P)H oxidase (NOX) activation plays an important role in the activation of volume-sensitive outward rectifying (VSOR) Cl^- channels (3). Therefore, we explored the possibility that intracellular ROS produced by epidermal growth factor (EGF) could be part of the mechanism leading to the activation of Cl^- currents implicated in cell proliferation (2). Acute exposure of HeLa cells to EGF (500 ng/ml) activated a Cl^- current with similar properties to VSOR Cl^- channels. This current was inhibited (> 90%) by intracellular application of DTT (5 mM). To examine whether EGF-induced increase in ROS was dependent on NOX activation, we tested a NOX inhibitor (DPI, 10 μM). Cells pretreated with DPI showed a significant inhibition (50 - 75%) of EGF-induced Cl^- current. Because DPI is a nonspecific blocker of NOX, inhibition of the EGF-induced Cl^- current by this compound should be interpreted with caution. To address this point more directly, HeLa cells were transiently transfected with a dominant negative mutant ($p47^{S379A}$) of the p47 subunit of NOX. This mutant is unable to translocate to the membrane, resulting in a loss of oxidase activity. As expected, the dominant negative mutant showed a significant reduction (> 80%) in the EGF-induced Cl^- current. In conclusion, our results indicate that EGF-induced chloride currents are dependent on ROS. Fur-

thermore, the results presented here suggest that ROS generation induced by EGF is mediated by the activation of the oxidase subunit p47 component of the NAD(P)H oxidase.

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Supported by FONDAP 15010006. We are grateful to B. M. Babior for kindly providing the $p47^{S379A}$ dominant negative mutant and to Bo S. Jensen for the pNS2Z plasmid.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC105

Characterisation of serum-induced intracellular calcium oscillations in primary bone marrow stromal cells

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Alterations in cytosolic calcium concentration influence a vast array of cellular functions in both excitable and non-excitable cells (Berridge *et.al.* 2003). $[Ca^{2+}]_i$ oscillations provide an additional level of control, permitting modulation of Ca^{2+}-sensitive processes without the need for prolonged global elevations in $[Ca^{2+}]_i$. Ca^{2+} oscillations have also been shown to increase the efficacy and specificity of $[Ca^{2+}]$ as a stimulus for the activation of certain transcription factors (Dolmetsch *et.al.* 1998).

Bone marrow stromal cells are pluripotent precursor cells, which possess the ability to differentiate into a range of cell types including bone-synthesising osteoblasts. Studies have shown that 72% of commercially available human marrow-derived mesenchymal stem cells generate spontaneous $[Ca^{2+}]_i$ oscillations (Kawano *et.al.* 2002; Kawano *et.al.* 2003). We have studied the generation of $[Ca^{2+}]_i$ oscillations by primary rat marrow-derived stromal cells.

Femora were removed from male Wistar rats humanely killed by cervical dislocation, and the marrow cavity flushed with culture medium. Marrow stromal cells were cultured in α-minimal essential media with 15 % [v/v] fetal calf serum (FCS), penicillin, streptomycin, ascorbic acid and β-glycerophosphate, 5 % CO_2 at 37 °C for 16 to 30 days. Cells were seeded onto glass coverslips and $[Ca^{2+}]_i$ was monitored by loading cells with Calcium-Green-1.

Spontaneous oscillations were not observed in primary marrow stromal cells, (although occasional, irregular 'Ca^{2+} spikes' were seen in some cells). Exposure to 1 to 5% FCS, however, induced continuing regular Ca^{2+} oscillations in 38 ± 3.3% of cells (1703 cells; 49 experiments; ± SEM). These oscillations were blocked by disruption of Ca^{2+} stores with 100nM to 1μM thapsigargin. They were sensitive to inhibition of IP_3-receptor activity by 50μM 2-APB and to inhibition of phospholipase C with 5μM U73122. The oscillations exhibited a gradual decrease in amplitude and frequency following inhibition of Ca^{2+} influx with EGTA or La^{3+}.

Although the precise identity of the oscillation-inducing serum factor(s) are as yet unclear, preliminary experiments have revealed that the factor(s) are stable to heat (100°C; 4min), and are retained by a 30kDa molecular weight filter. Since serum is routinely present in cell culture medium at 10 to 15% [v/v], it is reasonable to assume that the observed oscillations occur constantly in these cells in culture.

All procedures accord with current UK legislation.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC106

The modulation of intracellular calcium responses by oestrogen and progesterone in human spermatozoa

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Human spermatozoa are exposed to micromolar concentrations of oestrogen and progesterone within the female reproductive tract (Osman *et.al.*, 1989). *In vitro*, 3 μM progesterone induces a biphasic Ca^{2+}-influx, generating a $[Ca^{2+}]_i$ transient followed by a plateau (Kirkman-Brown *et.al.*, 2002). 8-10% of cells generate slow $[Ca^{2+}]_i$ oscillations during the plateau phase. Harper *et.al.* (2004) reported that stimulation with a progesterone gradient induced a monotonic rise in $[Ca^{2+}]_i$, 34 ± 2% of cells generating oscillations. Much less is known about the effect of oestrogen on human spermatozoa $[Ca^{2+}]_i$. Fluorimetric population studies show a simple sustained $[Ca^{2+}]_i$ response that inhibits both transient and sustained components of the response to progesterone (Luconi *et.al.*, 1999). However, populations of human spermatozoa are heterogenous, which is reflected in their $[Ca^{2+}]_i$ signalling (Lefièvre *et.al.*, 2003). We have utilised single-cell imaging to analyse responses of individual spermatozoa $[Ca^{2+}]_i$ to oestrogen (5μM) and progesterone (3.2 μM). Values are means ± S.E.M.

Spermatozoa were harvested by swim-up into sEBSS media with 0.3% BSA and incubated (5 h) at 6 million cells ml^{-1}. $[Ca^{2+}]_i$ was monitored in cells loaded with Oregon Green-1 BAPTA and adhered to the base of a perfusion chamber.

>90% of spermatozoa responded to oestrogen with an increase in $[Ca^{2+}]_i$. 60±9% (n=7) of cells showed a plateau response and 9.2±1.0% generated transient and plateau phases, 23±3% generated a transient followed by oscillations and 7±1% generated a transient response only. Following pre-treatment with progesterone approximately 40% of spermatozoa responded with a transient response (sometimes followed by oscillations) as in control preparations. However, the plateau response to oestrogen was completely occluded. In contrast, pre-treatment of cells with oestrogen failed to alter the response to progesterone.

Oestrogen, like progesterone, initiates a range of $[Ca^{2+}]_i$ signals in human spermatozoa, including oscillations. Although the response to oestrogen was sensitive to prior stimulation with progesterone, we did not observe effects of oestrogen pre-treatment. Further study is required to establish whether progesterone

and oestrogen exert their effects through separate or shared transduction systems and how progesterone selectively inhibits the plateau component of the response to oestrogen.
All procedures were in accord with local ethical guidelines and the Declaration of Helsinki.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC107

Internalised voltage-gated sodium channel protein in human breast cancer cell lines: effects of forskolin on plasma membrane expression and cellular invasiveness

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Electrophysiological studies have suggested that strongly metastatic human breast cancer (BCa) cells express functional voltage-gated sodium channels (VGSCs) (Fraser et al. 2002; Roger et al. 2003). In prostate cancer, VGSC activity potentiates a variety of cellular behaviours involved in the metastatic cascade (e.g. Laniado et al. 1997; Mycielska et al. 2003). In fact, it has been suggested that VGSC expression alone is 'necessary and sufficient' for cellular invasiveness (Bennet et al. 2003).
The aims of this study were to investigate the subcellular localization of VGSC alpha subunit (VGSCα) protein in human BCa cells and its possible functional role.
Weakly and strongly metastatic human BCa cell lines (MCF-7 and MDA-MB-231, respectively) were adopted. Western blots and confocal microscopy identified VGSCα protein and its subcellular location (quantified by confocal densitometry). A commercial pan-VGSCα and a novel polyclonal antibody specific for the neonatal Nav1.5 (nNav1.5; expressed predominantly in metastatic BCa) were used. Invasion and proliferation were measured as before (Grimes et al. 1995). Cells were treated with 50 μM forskolin, a stimulator of adenyl cyclase, and/or 10 μM TTX in normal medium for 48 h. Data are presented as mean ± SEM. Statistical analyses were done by Mann-Whitney U test and unpaired t test.
Both BCa cell lines expressed VGSCa protein. Subcellular fractionation and confocal microscopy showed that intracellular VGSCα was present, mainly in endoplasmic reticulum and Golgi. However, only MDA-MB-231 cells expressed VGSCαs strongly in plasma membrane (PM). Forskolin had no effect on total VGSCα or nNav1.5 protein in the cells, but increased nNav1.5 expression in PM, by 126 ± 16 % in MCF-7 and 98 ± 21 % in MDA-MB-231 cells (n=37 cells/3 experiments, $p < 0.05$, for both). Forskolin treatment increased invasiveness of MCF-7 and MDA-MB-231 cells by 118 ± 29 % and 120 ± 29 %, respectively (n=9; $p<0.05$). Co-application of TTX reduced invasiveness to control levels. Regarding proliferation, only forskolin had an effect on MDA-MB-231 cells, reducing it by 16.5 ± 1.7 % (n=8; $p<0.001$). These results are consistent with forskolin inducing trafficking of intracellular nNav1.5 protein into PM, possibly via protein kinase A activation, and thus increasing the cells' invasiveness.

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This study was supported by an Amber Fellowship (PCRF) to A-MC.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC108

Activation of JNK, p38 MAPK and AP-1 in human vascular smooth muscle by xanthine oxidase

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Enhanced oxidative stress appears as a common pathogenic mechanism of vascular diseases, like coronary artery disease or diabetic vasculopathy. Increased vascular activity of the superoxide anions–releasing enzyme xanthine oxidase (XO) has been reported in such diseases. Mitogen-activated protein kinases (MAPK) are key signalling enzymes that regulate growth, inflammation and cell death in eukaryotic cells. We analysed the role of p38 MAPK and JNK, which can be activated by cellular stress, inflammatory cytokines and some mitogenic molecules as mediators of XO signalling in cultured human aortic smooth muscle cells (HASMC), obtained by enzymatic dissociation from five organ donors.
Treatment of HASMC with 100 μM xanthine together with XO (50 μU/ml) resulted in increased intracellular content of superoxide anions, as shown with the fluorescent probe dihydroethidine, which could be prevented by both superoxide dismutase (SOD; 100 U/ml) and the specific XO inihibitor allopurinol (100 μM). In addition, XO activated both p38 and JNK MAPK, with maximal effects at 10 (1.50±0.48 times vs time 0) and 30 min (1.77±0.21 times vs time 0), respectively. XO-induced MAPK activation was not observed upon co-incubation with SOD or allopurinol. XO also stimulated both de novo synthesis (151.47±35.43% vs time 0) and activity of the transcription factor AP-1 (2.24±0.27 times vs control). The p38 inhibitor SB203580 (5 μM), but not the JNK inhibitor SP600125 (10 μM), significantly reduced increased AP-1 levels. Finally, XO increased HASMC size (117.52±3.01% vs control) without affecting proliferation through a SOD-dependent mechanism. This hypertrophic effect of XO was dependent on JNK and p38 activation, as it was prevented by both SB203580 and SP600125. In summary, XO can favour, through MAPK signalling, an activated pro-inflammatory and growth-promoting state of human

VSCMC, which may in turn contribute to the development and maintanance of vascular diseases.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC109

Functional expression of voltage-gated Na^+ channel in human small-cell lung cancer: Control of proliferation *in vitro* and detection *in vivo*

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We have shown previously that metastatic prostate and breast cancer cells express functional voltage-gated Na+ channels (VGSCs) which potentiate a variety of cellular behaviours involved in the metastatic cascade (e.g. Grimes *et al.*,1995; Fraser *et al.*,2002). Small-cell lung cancer (SCLC) has the most aggressive clinical course of any type of pulmonary tumour, and human SCLC cells also have strong VGSC activity (Blandino *et al.*,1995). However, the role VGSCs in SCLC is not known.

We investigated the possible involvement of VGSC activity in proliferation and endocytosis of H510 human SCLC cells. A colorimetric MTT method was used to quantify proliferation. The possible role VGSC activity was tested using tetrodotoxin (TTX) as a specific blocker. Vesicular uptake of horseradish peroxidase (HRP) was used to quantify endocytosis. Results were compiled as the per cent mean (± SEM) of three repeats of drug versus control readings from individual multi-well plates. Data were analysed by Mann-Whitney U test. VGSC expression in human clinical biopsies was studied by immunohistochemical staining using a commercial pan-VGSC antibody.

Treatment with TTX (100 nM) for 24 h caused 57 ± 3 % reduction in proliferation. This effect was highly significant ($P \leq 0.01$) and was maintained for 48 h. Increasing the TTX concentration to 1 μM produced no further effect. TTX (100 nM) reduced HRP uptake in H510 cells by 81 ± 5 %, nearly to the level of the negative control (endogenous peroxidase activity). Although both types of cell behaviours appeared to be controlled mainly by TTX-sensitive VGSC(s), the possible involvement of TTX-resistant VGSC(s) and /or non-VGSC control, especially in proliferation, could not be ruled out. Immunohistochemical staining showed that there was little or no VGSC protein expressed in normal human lung biopsies. On the other hand, significant upregulation was seen in SCLC.

We conclude the following: 1) VGSC activity can potentiate metastatic cell behaviour (proliferation and endocytosis) in human SCLC cells *in vitro*, as previously shown for metastatic prostate and breast cancer. (2) VGSC expression is upragulated in human SCLC also *in vivo*. It is possible, therefore, that VGSC expression activity is a novel target for clinical management of SCLC.

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We would like to acknowledge PCRF for supporting this project.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC110

A microbial TRP-like polycystic kidney disease related ion channel gene: a putative mechanosensitive ion channel gene

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Ion channel genes have been discovered in many microbial organisms. We have investigated a transient receptor potential ion channel gene in fission yeast which has most similarity (46%) in the six predicted transmembrane domains to a polycystic kidney disease related ion channel gene in *Drosophila melanogaster*. We have shown that this gene (*pkd2*) is essential for cellular viability and involved in cell growth and cell shape determination. Cells in liquid culture 24 h after *pkd2* depletion appeared elongated, average cell length was increased by 31% (compared with the control cells) with 11% of cells containing more than three septae compared with less than 1% for control cells. Additionally, 22% of the cells appeared bulbous and possessed an uneven cell periphery, whilst 5% of the cells appeared to be dead, which was confirmed by trypan blue staining. Over-expression of *pkd2* resulted in cell death on liquid and solid media. Microscopic examination of these cells at 16 h following promoter induction suggested cell lysis, with many bent and kinked cells. Significant changes in cellular morphology were observed: about 12% of cells had an altered growth polarization, appearing as small buds growing parallel or at 45 deg to the long axis of cell growth. Average cell length was increased by 8% but multiple septae were not observed. This fission yeast gene appears to be a key signalling component in the regulation of cell shape and cell wall synthesis through an interaction with Rho1-GTPase which was determined by immunoprecipitation. This gene is a candidate for the mechanosensitive ion channel which has been recorded from the plasma membrane of fission yeast[1]. A model for the mode of action of this protein in a Ca^{2+} signalling pathway is hypothesized.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC111

CCK-8–evoked calcium transport and gene expressions for α-amylase and CCK-A receptor mRNA in pancreatic acinar cells of control and diabetic rats.

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In a previous study we investigated the interaction of CCK-8 with either insulin, glucagon or somatostatin in control and streptozotocin (STZ)-induced diabetic rats. Our results showed

marked reduction in amylase secretion in diabetic rats compared to control (Singh et al., 1999). The cellular mechanisms underlying reduced amylase output during diabetes is still unclear. This study measures CCK-8-evoked calcium (Ca2+) mobilisation, α-amylase and CCK-A receptor mRNA gene expressions in pancreatic acinar cells of age-matched control and STZ-induced adult male diabetic rats using established methods.
After two months of STZ treatment, diabetic animals gained significantly (Students′t-test; P<0.05) less weight and elevated blood glucose levels. Weights of diabetic and control rats were 219.8±6.8 g, (n=20) and 373.6±6.7 g, (n=20), respectively. Similarly, blood glucose levels were 380±25.9 mg dl-1 (n=20) and 73.3±3.4 mg dl-1 (n=20) in diabetic and control rats. The pancreas of diabetic rats (1.01±0.05 g, n=20) weighed significantly less (P⟨0.05) compared to controls (1.29±0.07 g, n=20). Basal [Ca2+]i in control and diabetic fura-2 loaded pancreatic acinar cells were 251.1±17.1 nM (n=25) and 151.9±8.5 nM (n=39). Stimulation of control acinar cells with 10-8 M CCK-8 resulted in a rapid increase (peak response) in [Ca2+]i followed by a decline to a plateau phase which remained above the basal level for over 10 min. In diabetic acinar cells the CCK-8-evoked peak and plateau responses were significantly (P⟨0.05) reduced compared to normal acinar cells. Typically, the CCK-8-evoked peak [Ca2+]i response was 3297.8±349.5 nM (n=25), and 2052.9±146.6 nM, (n=39). After 10 min of CCK-8 application the plateau [Ca2+]i was 351.7±30.6 nM (n=11) and 235.2±11.6 nM, (n=23) in control and diabetic acinar cells, respectively. α-amylase mRNA levels were significantly (P⟨0.05) reduced in diabetic pancreatic samples compared to controls which had a significantly lower (P⟨0.05) crossing-over value (8.54±0.131, n=8 vs, 17.96±0.272, n=7) compared to diabetic animals. Conversely, the mRNA gene expression levels of the CCK-A receptor remained unchanged between the two groups. Crossing-over values were 20.92±0.0104 (n=8) and 21.52±0.181 (n=7) for control and diabetic animals, respectively. The results indicate that STZ-induced diabetic exocrine pancreatic insufficiency may be associated with a derangement in cellular Ca2+ transport and reduced transcription of α-amylase, but not the CCK-A receptor mRNA.

Singh, J, Adeghate, E, Salido, G.M, Pariente, J.A, Yago, M.D & Juma, LMO (1999), Exp Physiol 84 23-38.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC113

Role of NAD(P)H oxidase in the activation of volume-sensitive chloride channels

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Most mammalian cells types respond to cell swelling by activating a Cl^- current termed $I_{Cl,vol}$. Although this current has been well studied (1), the physical stimuli and the signal transduction pathways connecting cell swelling and the channels underlying $I_{Cl,vol}$ are still elusive. In cells from a rat liver-derived cell line (HTC), it has been proposed that phosporylation of PLCγ is fundamental for the regulatory volume decrease (RVD) response (2), however, the upstream molecules and the role of these intracellular signalling components on the activation of $I_{Cl,vol}$ are not known. Recent evidence indicate that reactive oxygen species may function as intracellular messengers leading to a reversible inactivation of some protein tyrosine phosphatases (PTPs) (3). In agreement, several authors demonstrate an increase in the phosphorylation state of PLCγ after exogenous application of hydrogen peroxide (H_2O_2) (4,5). In this work we show that a reduction of extracellular osmolarity (30% hypotonicity) increases the rate of production of intracellular H_2O_2 15-fold. This increase was partially blocked (70 ± 11%) by pre-treatment of the cells with a non-specific inhibitor of NAD(P)H oxidase (diphenylene iodonium, DPI). To establish a correlation between the increase of H_2O_2 and the activation of $I_{Cl,vol}$, whole-cell chloride currents were studied using the nystatin perforated patch configuration. HTC cells exposed to hypotonicity developed a Cl^- current similar to that observed in other cell lines, however, pre-treatment with DPI inhibited this current by 77 ± 2%. Overexpression of a NAD(P)H oxidase dominant negative ($p47^{S379A}$) also abolished the development of this current, indicating a central role of this complex in the activation of $I_{Cl,vol}$. Extracellular application of H_2O_2 (20-200 μM) in isotonicity reversibly activated a chloride current that resembled $I_{Cl,vol}$. Consequently, internal application of DTT completely abolished the activation of this current. Application of a specific inhibitor of PLC, U73122 completely abolished the development of the current activated by hypotonicity or H_2O_2 (inhibition of 94 ± 1% and 93 ± 1%, respectively) suggesting that hydrogen peroxide may act upstream to PLC. These results indicate that hydrogen peroxide plays a central role in the activation of $I_{Cl,vol}$ in a PLC-dependent manner.

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Supported by FONDAP 15010006. We are grateful to B. M. Babior for kindly providing the $p47^{S379A}$ dominant negative mutant and to Bo S. Jensen for the pNS2Z plasmid.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC114

Effects of extracellular citrate on adhesion, motility and endocytic membrane activity of normal (PNT2-C2) and strongly metastatic (PC-3M) human prostate epithelial cells lines

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A major function of prostatic epithelial cells is production, accumulation and release of citrate which is present in large amounts in the gland and prostatic fluid (Costello and Franklin,1991). The level of citrate is reduced in prostate cancer and almost dis-

appears in metastatic disease (Costello et al. 1999). Using PNT2-C2 cells as a model, we have recently characterised a novel K+-dependent citrate transporter that appeared designed primarily to release citrate (Mycielska & Djamgoz, 2004). In contrast, in PC-3M cells, a Na+-dependent citrate uptake mechanism was also present. Here, we have determined the effects of exogenous citrate on cellular behaviours involved in the metastatic cascade. PNT2-C2 and PC-3M cells were plated on 35 mm Petri dishes (for adhesion and motility) or 24-well plates (for endocytic membrane activity) and preincubated with 0.1 to 10 mM Na+ citrate (in normal growth medium) for 24 and 48 h. Single-cell adhesion was measured using a specifically designed device ('SCAMA'), endocytic membrane activity was quantified by spectrophotometric measurement of horseradish peroxidase (HRP) uptake, and motility was determined using the 'wound heal' method (Fraser et al. 2003). Each experiment was performed at least 3 times. Data were analysed by t-test and are presented as mean percentage changes ± SEM.

Preincubation of the normal prostate epithelial PNT2-C2 cells with citrate did not result in any significant change in any of the functional assays performed. On the other hand, PC-3M cells were sensitive to extracellular citrate. After 48 h of preincubation in 10 mM Na+ citrate, their motility was enhanced by 16 ± 5 % ($p=0.026$, $n=5$), adhesion was reduced by 50 ± 8 % ($p<0.001$, $n=3$) whilst HRP uptake was increased by 45 ± 16 % ($p=0.038$, $n=3$). These effects were dose dependent. Short term incubation (35 min) had no effect on HRP uptake by either cell line, which would suggest that the effects were likely to involve cellular metabolism.

These results show, for the first time, differential sensitivity of normal and strongly metastatic prostate epithelial cells to extracellular citrate. Whilst normal cells cannot utilise the excess citrate, strongly metastatic cells can take up and use citrate probably as an additional source of energy in enhancing metastatic behaviour.

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This study was supported by The Wellcome Trust.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC115

Protein kinase A regulates functional expression of voltage-gated Na+ channels in the strongly metastatic MAT-LyLu rat prostate cancer cell line

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It has been shown previously that functional voltage-gated Na+ channels (VGSCs) are expressed in strongly metastatic rat (MAT-LyLu) and human (PC-3) prostate cancer cells and can potentiate invasion in vitro (Grimes et al. 1995; Laniado et al. 1997; Bennett et al. 2004). However, the mechanism(s) controlling VGSC expression in these cells is not known. We reported earlier that long-term (> 24 h) exposure to tetrodotoxin (TTX) can suppress VGSC activity in MAT-LyLu cells (Brackenbury and Djamgoz, 2003). In the present study, we have investigated whether protein kinase A (PKA) is also involved in VGSC regulation.

MAT-LyLu cells were pre-incubated with a PKA inhibitor (KT5720) for 48 h, starting 3 h after seeding. Expression of Nav1.7 mRNA, predominant in these cells (Diss et al. 2001), was quantified using real-time PCR and normalised to rat cytochrome b5 reductase (Livak and Schmittgen, 2001). Functional VGSC activity was assayed by whole-cell patch clamp recording (pipette Cs+ used to eliminate K+ currents). Data are presented as mean ± SEM. Statistical significance was evaluated with Student's t tests. Pre-treatment with KT5720 (500 nM) significantly reduced Nav1.7 expression by 45 ± 0.17 % ($P < 0.05$; $n = 3$). A lower (50 nM) concentration had no effect ($P = 0.47$; $n = 3$). Pre-treatment with TTX (1 μM) resulted in a similar reduction of 52 ± 0.12 % ($P < 0.01$; $n = 3$). Co-application of KT5720 (500 nM) with TTX (1 μM) reduced Nav1.7 expression by 58 ± 0.16 % ($P < 0.01$; $n = 3$), which was not significantly different to the reduction caused by KT5720 alone ($P = 0.30$; $n = 3$). Pre-treatment with KT5720 (500 nM) significantly reduced peak VGSC current density from 14.8 ± 1.7 pA/pF to 4.7 ± 0.7 pA/pF ($P < 0.001$; $n = 11$-15). A lower concentration (50 nM) of KT5720 again had no effect ($P = 0.24$; $n = 14$-15).

The results indicate that inhibition of PKA can reduce functional VGSC activity in MAT-LyLu cells, and at least a part of this regulation is transcriptional. Furthermore, these results are consistent with PKA being an intermediary component of the activity-dependent VGSC regulation in MAT-LyLu cells (Brackenbury and Djamgoz, 2003). Accordingly, from the available evidence taken together, we propose the following scheme:

$\text{VGSC}\uparrow \rightarrow \text{Na+} \rightarrow \text{PKA} \rightarrow \text{VGSC}\uparrow$.

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This work was funded by a MRC Priority Area (Prostate Cancer) PhD studentship.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC116

Nitric oxide release from strongly metastatic MAT-LyLu rat prostate cancer cells: Control by voltage-gated sodium channels

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Expression of nitric oxide synthase (NOS) and voltage-gated Na^+ channels (VGSCs) have both been linked to progression of prostate cancer (Klotz *et al.*, 1998; Grimes *et al.*, 1995). In the present study, we aimed to answer two questions: (1) Do metastatic prostate cancer cells release nitric oxide (NO). (2) Is VGSC activity involved in this process?

Electrochemical detection was used to measure NO release from MAT-LyLu cell line (incubated in Krebs solution) over a period up to 24 hours. This method involved conversion of nitrates and nitrites in solution back to NO by enzymatic reduction (Neal *et al.*, 1998). The specificity of the measurements were tested using two agents: (1) NO scavenger, cPTIO (2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium salt; 0.025, 0.25 and 2.5 μM) and (2) NOS substrate, L-arginine (0.1, 0.5 and 1 μM). The possible effect of the VGSC activity on NO production was tested using tetrodotoxin (TTX; 20 nM and 1 μM). Data are presented as means ± S.E.M. Student's t-test was used to analyse the data.

Under resting conditions, MAT-LyLu cells released NO increasingly with time. There was some variability in the time course of production, but on average, the concentration of NO in solution saturated at a mean value of 8.9 ± 2.2 μM (n = 10) by 9 h. This duration, therefore, was adopted as the sampling period. Incubation with cPTIO (2.5 μM) caused a reduction in the quantity of NO detected by 61 ± 7 % (n = 5; $p < 0.05$). Conversely, L-arginine (1 μM) induced an increase in NO production by 88 ± 27 % (n = 3; $p < 0.05$). These effects were dose-dependent. Treatment with TTX (1 μM) increased NO production, on average to 14.8 ± 2.7 μM (n = 10; $p < 0.01$). Interestingly, however, in a subpopulation (n = 5/10) of the cultures tested, TTX had noticeably little effect.

In conclusion, (1) NO is released tonically from strongly metastatic rat prostate cancer cells and (2) VGSC activity, previously shown to be associated specifically with the strongly metastatic phenotype, is involved in the NO release. The available data, taken together, are consistent with NO and VGSC systems being in equilibrium, such that under the experimental conditions used, VGSC activity suppresses NO release. Further work is required to elucidate the mode(s) of VGSC action on NO production and how this relates to metastatic cell behaviour.

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This work was supported by a BBSRC studentship.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.