PS C60

Atypical PKC isoforms in interleukin-1β-induced cyclooxygenase-2 expression in human myometrium

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Contractility of the uterus during labour is under the control of prostaglandins regulated by the enzyme cyclooxygenase-2 (COX-2). Preterm labour is responsible for 70% of neonatal deaths and one of its leading causes is intrauterine infection (Challis *et al.* 2002). The onset of both term and preterm labour has been likened to an inflammatory reaction. COX-2 expression is rapidly induced in response to a number of growth factors, tumour promoters and inflammatory cytokines including TNF-α and IL-1β (Smith *et al.* 1996). The exact mechanisms involved in COX-2 regulation by inflammatory cytokines remain unclear. We have previously demonstrated a role for p38 mitogen activated protein kinase (MAPK) and protein kinase C (PKC) in IL-1β-induced COX-2 expression in human myometrial smooth muscle cells (HMSMCs). Recent work has focused on the precise isoforms of PKC involved and possible interaction with NF-κB-regulated COX-2 expression.

HMSMCs were isolated from fresh biopsies of the lower segment myometrium taken during elective caesarean section after informed consent. Cultured cells were used to examine COX-2 expression by western blotting after 5 h of treatment with 10 ng ml^{-1} IL-β. Pre-incubation of cells with a range of doses of two PKC inhibitors, GF109203X and LY379196, suggests a role for atypical isoforms ζ and ι with complete inhibition of COX-2 expression seen at 10 μM GF109203X (the IC_{50} for ζ = 5.8 μM). Inhibition of COX-2 expression was not observed with LY379196 at 10 μM, a concentration known to inhibit all isoforms except the atypical (IC_{50} for ζ = 48 μM). Furthermore, experiments with pseudosubstrate inhibitors to α/β and ζ isoforms showed inhibition of COX-2 expression only with the ζ inhibitor. Long-term treatment of HMSMCs with 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 h down-regulated expression of the conventional and novel PKC isoforms but not the atypical. IL-1β induced an increase in COX-2 expression after this long-term PMA treatment that was inhibited by GF109203X, demonstrating that atypical isoforms are the only PKCs required for IL-1β–induced COX-2 expression in HMSMCs. Further experiments using NF-κB gel shift assays or transfection of HMSMCs with an NF-κB promoter–luciferase reporter construct suggested that atypical PKC isoforms regulate COX-2 expression in these cells by interacting with NF-κB, via a mechanism that involves transactivating the bound NF-κB subunit in the promoter rather than assisting translocation of p50/p65 to the nucleus through degradation of IκB.

Challis JR *et al.* (2002). *Reproduction* **124**, 1–17.

Smith WL *et al.* (1996). *J Biol Chem* **271**, 33157–33160.

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All procedures accord with current local guidelines and the Declaration of Helsinki

PS C63

Modulation of glutathione and haeme oxygenase-1 by moderately-oxidized low density lipoproteins in vascular smooth muscle cells

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Oxidatively modified low density lipoproteins (LDL) play a central role in atherogenesis (Steinberg, 2002) while dietary antioxidant vitamins are protective against this process (Carr *et al.* 2000). We have shown that vitamin C attenuates oxidised LDL-induced cell death by apoptosis (Siow *et al.* 1999*a*) in vascular smooth muscle cells (SMC). The antioxidant stress protein haeme oxygenase-1 (HO-1) catabolises haeme to generate the vasodilator carbon monoxide and antioxidant biliverdin, and can be induced by highly oxidised LDL (oxLDL) in SMC (Siow *et al.* 1999*b*). We have now investigated whether vitamin C modulates levels of the endogenous cellular antioxidant glutathione (GSH) and HO-1 protein expression in human aortic SMC treated with moderately-oxidized LDL (mLDL), a species containing higher levels of lipid hydroperoxides, and the involvement of mitogen activated protein kinases (MAPK) in HO-1 induction.

Human smooth muscle cells were cultured from aortic explants obtained with ethical approval. Confluent SMC cultures were pretreated with vitamin C (24 h, 100 μM) prior to treatment in the absence of vitamin C with human native LDL (nLDL), mLDL or oxLDL (0–300 μg ml^{-1}) for 0–24 h. In some experiments, cells were treated with MAPK inhibitors, SP600125 (20 μM, c-Jun NH_2-terminal kinase), U0126 (1 μM, MEK), SB203580 (2 μM, p38 MAPK) for 30 min prior to LDL treatments.

Western blot analysis revealed that mLDL and oxLDL induced HO-1 expression in a dose- and time-dependent manner. HO-1 was not detected in control or nLDL treated SMC, but mLDL enhanced HO-1 expression to a significantly greater extent than oxLDL. Intracellular GSH levels, measured using a fluorometric assay, were unaltered by nLDL but elevated by mLDL to a significantly higher level than oxLDL. Vitamin C and MAPK inhibitors significantly attenuated induction of HO-1 by mLDL. Vitamin C pretreatment also significantly attenuated elevation of GSH levels mediated by mLDL and oxLDL.

These findings suggest that HO-1 induction in SMC by mLDL is mediated by MAPK signalling cascades, that GSH levels and HO-1 expression can be differentially enhanced in response to mLDL and oxLDL and that vitamin C affords protection against the more atherogenic moderately oxidized species of LDL.

Carr AC *et al.* (2000). *Circ Res* **87**, 349–354.

Siow RCM (1999*a*). *Arterioscler Thromb Vasc Biol* **19**, 2387–2394.

Siow RCM (1999*b*). *Cardiovasc Res* **41**, 385–394.

Steinberg D (2002). *Nat Med* **8**, 1211–1217.

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All procedures accord with current local guidelines and the Declaration of Helsinki

PS C64

Differential effects of a regulator of G-protein signalling on the activation of G-protein gated inwardly rectifying K^+ channels

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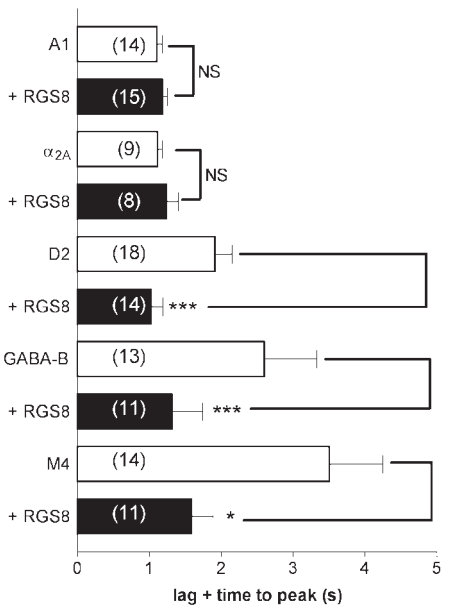
Expression of the regulator of G-protein signalling (RGS) proteins results in rapid deactivation of G-protein activated inwardly rectifying K^+ (GIRK) channels, consistent with their catalytic function as a GTPase-activating protein on active Gα subunits and their role as negative regulators of other G-protein pathways. Unexpectedly RGS proteins also accelerate receptor-stimulated activation kinetics of GIRK channels, by an unknown mechanism. The effects of neuronal RGS8 were examined in HEK-293 cell lines, stably expressing GIRK channel subunits (Kir3.1+3.2A) plus one of the following GPCRs: A_1 adenosine, α_{2A} adrenergic, D_2 dopamine, M_4 muscarinic, or the GABA-$B_{1b/2}$ heterodimeric receptor.

Whole-cell K^+ currents in symmetrical K^+ (140 mM) were recorded at a membrane potential of −60 mV. Currents were activated by fast application and removal of agonists (delay time approx. 0.25s): $GABA_B$, 100 μM baclofen; M_4, 100 μM carbachol; D_2, 10 μM dopamine; A_1, 1 μM adenosine; α_{2A}, 3 μM noradrenaline. The kinetics of receptor-stimulated currents were characterised by measuring the lag and time to peak current amplitude (lag+TTP) for activation, and by fitting a single exponential time constant (τ_{deact}) to the deactivation phase. Data are given as means ± S.E.M. and were reciprocated prior to statistical analysis with Student's unpaired *t* test.



Measured current activation data (lag TTP) from receptor cell lines. Statistical comparisons of control cells and cells expressing RGS8-YFP (RGS8)are indicated (*$P < 0.05$, ***$P < 0.001$).

The main findings were that transfection of RGS8 (tagged with the yellow fluorescent protein, RGS8-YFP) significantly accelerated current deactivation in each of the receptor cell lines (compared to control recordings) but a receptor-selective effect was seen on activation kinetics whereby activation of currents via the D_2, $GABA_B$ and M_4 receptors was significantly accelerated whilst activation via A_1 and α_{2A} receptors was not affected by RGS8-YFP expression ($GABA_B$, lag+TTP(s): 2.6 ± 0.7, $n = 13$; + RGS8: 1.3 ± 0.4, $n = 11$, $P < 0.001$; A_1, lag+TTP: 1.1 ± 0.01, $n = 14$; +RGS8: 1.2 ± 0.01, $n = 15$, NS). The lag TTP data for all receptors studied is shown in Fig. 1. These effects were dependent on the RGS catalytic domain since an N-terminally deleted construct exhibited identical kinetic effects to RGS8-YFP. The receptor selectivity of RGS8-YFP was not due to preferential regulation of certain Gα-isoforms because, when signalling was constrained to transfected PTx-insensitive GoαA(C→G) subunits in the $GABA_B$ and A_1 cell lines (treated with PTx at 100 ng ml^{-1} for >16 h), RGS8-YFP accelerated $GABA_B$-mediated activation (lag+TTP(s): +GoαA: 3.3 ± 0.6; +GoαA + RGS8: 1.2 ± 0.1, $P < 0.001$; $n = 8$) but not A_1-mediated activation of currents (lag+TTP: +GoαA: 2.2 ± 0.3; +GoαA + RGS8: 2.5 ± 0.3; $n = 6$, NS). Deactivation was significantly accelerated in both lines. Our data support current ideas of kinetic scaffolding and indicate that this occurs in a receptor-dependent fashion.

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PS C65

Effects of hyperglycaemia and diethylmaleate on L-cystine transport and intracellular glutathione levels in bovine retinal endothelial cells and pericytes

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Diabetic retinopathy is the main cause of blindness in Western countries yet the molecular basis of its pathogenesis remains poorly understood. In the present study, we have examined the effects of hyperglycaemia and the electrophilic agent diethylmaleate (DEM) on L-cystine transport, intracellular glutathione (GSH) levels and activation of p42/p44 and p38 mitogen-activated protein kinase (MAPK) in bovine retinal endothelial cells and pericytes.

Retinal endothelial cell (BREC) and retinal pericytes (BRP) were isolated from ~20 bovine eyes obtained from an abattoir and cultured in Minimal Eagle's Medium containing either 10% horse or fetal calf serum, respectively. Cells were characterized as endothelial cells or pericytes using antibodies against von Willebrand factor or α-actin, respectively. Confluent cell monolayers were exposed to normal D-glucose (5.6 mM) and elevated D-glucose (15 or 25 mM) for defined time periods (0–60 min and 24–72 h). D-Mannitol (9.4 mM + 5.6 mM D-glucose) served as an osmotic control. Intracellular GSH levels were determined using the glutathione-S-transferase (GST) catalysis reaction. Influx of L-[^{14}C]cystine (50 μM) was measured over 2 min in cells exposed to normal or elevated D-glucose or DEM. Changes in the phosphorylation of p42/44MAPK and p38MAPK were investigated by western blot analysis.

Intracellular GSH levels in BREC (43 ± 4 nmol (mg protein)$^{-1}$, mean ± S.E.M., $n = 3$) and BRP (15 ± 1 nmol (mg protein)$^{-1}$, $n = 4$) were not altered significantly following treatment with elevated D-glucose for 24 h. Exposure of BREC to 15 mM D-glucose for up to 24 h had no significant effect on basal L-cystine transport. Diethylmaleate (100 μM, 24 h), an agent known to

conjugate intracellular GSH, resulted in adaptive increases in L-cystine transport (413 ± 58 *vs.* 661 ± 78 pmol $(\mu g\ protein)^{-1}\ min^{-1}$, $n = 3$–4, Student's unpaired t test, $P < 0.05$) and GSH (43 ± 4 *vs.* 60 nmol $(mg\ protein)^{-1}$, $n = 5$, $P < 0.05$). Exposure of cells to 15 mM D-glucose or D-mannitol for 5 min to 1 h had no significant effect on phosphorylation of p42/p44MAPK in either BREC or BRP, whereas exposure of BRP to 25 mM D-glucose for 48 h resulted in activation of p38MAPK (Sato *et al.* 1999). Hyperglycaemia had negligible effects on L-cystine transport and GSH levels suggesting that, under our experimental conditions, elevated D-glucose caused minimal oxidative stress. In contrast, DEM caused a classical adaptive increase in L-cystine transport (most likely via anionic amino acid transporter xCT, see Sato *et al.* 1999) and GSH levels. Further studies are required to establish whether activation of p38MAPK in response to prolonged exposure to elevated D-glucose modulates L-cystine transport activity and intracellular GSH levels.

Sato H *et al.* (1999). *J Biol Chem* **274**, 11455–11458.

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PS C66

Extracellular calcium-sensing receptor expression in rat and human bone and intracellular signalling responses to extracellular calcium in freshly isolated fetal rat calvarial cells

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The possibility of the extracellular calcium-sensing receptor (CaR) as the sensor of external calcium (Ca_o^{2+}) in osteoblasts, the bone-forming cells, has been proposed. Nevertheless, the osteoblast expression of this receptor is still disputed. We investigated the osteoblast expression and intracellular signalling mechanisms of the CaR in freshly isolated fetal rat calvarial cells (FRC; from animals that were killed humanely) and murine clonal osteoblast cell line 2T3 cells.

We amplified and sequenced *bona fide* CaR transcripts from both FRC and 2T3 cells by reverse-transcriptase PCR. Specific (i.e. peptide-protectable) CaR immunoreactivity was detected by Western analysis and immunofluorescence microscopy using anti-CaR monoclonal and polyclonal antibodies in these cells. Furthermore we used freshly frozen, non-decalcified preparations of rat femur and human mandible (biopsies obtained with ethical approval) and demonstrated, for the first time, the CaR immunofluorescence in the cells of osteoblast origin.

We then investigated the acute (5–60 min) and short term (24 h) effects of varying $[Ca^{2+}]_o$ (0.5, 1.2, 1.8, 2.5 and 5 mM) and of the non-permeant CaR agonist gadolinium (Gd^{3+}) (10−100 μM). Treatment with the CaR agonists Ca_o^{2+} and Gd_o^{3+} both result in the phosphorylation of the extracellular signal-regulated kinases (ERK1/2). The effect of 50 μM Gd_o^{3+} was ablated by the co-incubation with PD98059, an inhibitor of the ERK-activating kinase, MEK. Furthermore this response was partially inhibited by the PI_3 kinase inhibitors wortmannin and LY294002. We also observed increased phosphorylation of the two Akt activation sites, namely Thr 308 and Ser 473 and the phosphorylation of its downstream effector glycogen-synthase kinase β (GSK3β). Together, these responses are consistent with signals of proliferation and cell survival. We then evaluated the activity of the collagen I promoter-driving green fluorescent protein (GFP) in stably transfected 2T3 cells. Our results show that the GFP fluorescence intensity was significantly increased after 24 h incubation with 2.5 mM Ca_o^{2+} (ANOVA, $n = 4$, $P < 0.05$) and 10, 50 and 100 μM Gd_o^{3+} (ANOVA, $P < 0.01$, $P < 0.001$ and $P < 0.05$ respectively, $n = 4$). Furthermore, we show by northern blotting that the 24 h incubation of FRC cells in 2.5 mM Ca_o^{2+} increases the production of collagen I mRNA.

We conclude that osteoblasts respond to acute elevations of the $[Ca^{2+}]_o$ and that this phenomenon is likely to be mediated by the calcium-sensing receptor, which is expressed in these cells.

This work was funded by the ARC and MRC.

All procedures accord with current UK legislation, local guidelines and the Declaration of Helsinki

PS C67

Effect of increases in $[Ca^{2+}]_c$ on mitochondrial membrane potential in guinea-pig colonic smooth muscle cells

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The ability of mitochondria to take up cytosolic calcium ($[Ca^{2+}]_c$) may affect overall cellular activity by modulating the amplitude and duration of Ca^{2+} signals as well as mitochondrial ATP production via an elevation of mitochondrial matrix $[Ca^{2+}]$. The principal driving force for mitochondrial ATP production is the mitochondrial membrane potential ($\Delta\psi_m$), which may be reduced by mitochondrial Ca^{2+} uptake. The present communication sought to relate changes in $[Ca^{2+}]_c$ to changes in $\Delta\psi_m$. To examine this, $\Delta\psi_m$ was measured using the membrane potential-sensitive dye tetramethylrhodamine ethyl ester (TMRE) simultaneously with fluo-4 to monitor $[Ca^{2+}]_c$ during sarcolemma-depolarisation- and inositol (1,4,5)P_3 (InsP_3)-evoked $[Ca^{2+}]_c$ increases in single voltage clamped myocytes (from guinea-pigs, ~500 g, humanely killed by stunning and exsanguination).

Treatment with either carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which uncouples oxidative phosphorylation, plus oligomycin to inhibit mitochondrial ATP hydrolysis or with the Complex I inhibitor rotenone plus oligomycin depolarised mitochondria, as assessed by the loss of punctate TMRE fluorescence. Mitochondrial depolarisation increased the time required for $[Ca^{2+}]_c$ to recover to resting values following either sarcolemma-depolarisation-induced Ca^{2+} influx or release of Ca^{2+} from the intracellular store by flash photolysis of caged InsP_3, indicating that mitochondrial Ca^{2+} accumulation occurred during these $[Ca^{2+}]_c$ transients. Despite this no alteration of TMRE fluorescence and hence $\Delta\psi_m$ was observed during the $[Ca^{2+}]_c$ transients. Infrequently the fluorescence of a single mitochondrion decreased, as if partially or completely depolarised, but the timing of these events often occurred after $[Ca^{2+}]_c$ had returned to resting levels. Oligomycin alone blocks proton re-entry into the mitochondrial matrix, causing a small $\Delta\psi_m$ hyperpolarisation. This was observed as an increased mitochondrial fluorescence, without significant alteration to either depolarisation- or InsP_3-evoked Ca^{2+} transients. The muscarinic agonist carbachol transiently increased $[Ca^{2+}]_c$ with no detectable alteration in $\Delta\psi_m$. On occasion this increase was followed by $[Ca^{2+}]_c$ oscillations and waves. During the course of these prolonged Ca^{2+} oscillations individual mitochondria could

become either partially or completely depolarised, but the timing of this occurrence was not correlated with $[Ca^{2+}]_c$ or the $\Delta\psi_m$ of surrounding mitochondria.

In conclusion, mitochondria may be functionally independent entities and under the conditions of the present experiment increases in $[Ca^{2+}]_c$ do not have an immediate effect on mitochondrial membrane potential.

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All procedures accord with current UK legislation

PS C68

Physiological changes in extracellular calcium concentration directly effect osteoblast proliferation, differentiation and mineralised nodule formation in the absence of calciotropic hormones

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Osteoblasts are bone-forming cells dependent upon the fluctuations in $[Ca^{2+}]_o$ regulated by the calciotropic hormones (e.g. parathyroid hormone). We investigated the sensing of Ca_o^{2+} by freshly isolated fetal rat calvarial cells (FRC; from animals that were humanely killed) and the involvement of the Ca_o^{2+}-sensing receptor (CaR) in this mechanism by using CaR agonists. Specifically, we investigated short- (24, 48 and 72 h) and long-term (5–23 days) effects of $[Ca^{2+}]_o$ (0.5, 1.2, 1.8 and 2.5 mM) and of the non-permeant CaR agonist gadolinium (Gd_o^{3+}; 10–100 μM) on FRC proliferation, differentiation and the production of mineralised nodules. $[Ca^{2+}]_o$ of 1.2 mM served as a control concentration.

Increasing $[Ca^{2+}]_o$ from 1.2 to 1.8 and 2.5 mM and treatment with 25 and 50 μM Gd_o^{3+} increased FRC cell proliferation at days 1–10 of the treatment with Ca_o^{2+} ($P < 0.05$ for 1.8 mM Ca_o^{2+} at day 1 and 3; $P < 0.01$ for 1.8 and 2.5 mM Ca_o^{2+} at day 5; $P < 0.001$ for 1.8 and 2.5 mM Ca_o^{2+} at days 7 and 10. All $n = 3$; ANOVA), at day 5, 7, 10 and 14 of the treatment with 50 μM Gd_o^{3+} and at day 14 for 25 μM Gd_o^{3+} (both $P < 0.05$, $n = 3$, ANOVA). Subsequently we measured changes in the mRNA expression levels of osteoblast differentiation markers in response to increasing $[Ca^{2+}]_o$ and 50 μM Gd_o^{3+}. Core-binding factor1 (cbfa1), osteocalcin (OC) and osteopontin (OP) mRNA levels increased in the presence of 1.8 and 2.5 mM $[Ca^{2+}]_o$ and 50 μM $[Gd^{3+}]_o$ at days 12 and 18. No changes in the mRNA levels were observed for tissue non-specific alkaline phosphatase (TNAP) and for the housekeeping gene β-actin. We showed that TNAP enzyme activity (nmoles of paranitrophenol per μg protein) was dependent on a narrow range of $[Ca^{2+}]_o$, with $[Ca^{2+}]_o$ lower than 1.2 mM and greater than 1.5 mM producing significant inhibitory effects at 7 days of treatment ($P < 0.01$; $n = 6$, ANOVA). Finally, both Ca_o^{2+} (1.8 and 2.5 mM) ($n = 6$) and Gd_o^{3+} (50μM) ($n = 3$) increased the production and the area of mineralised nodules ($P < 0.05$, ANOVA). The FRC responses to low $[Ca^{2+}]_o$ (0.5 mM) included an increase in proliferation at short-term time points, a decrease in proliferation and mineralised nodule formation and an increase in the expression of osteocalcin mRNA in the long-term observations.

Our results indicate that osteoblasts sense both high and low $[Ca^{2+}]_o$, and that small deviations of $[Ca^{2+}]_o$ from the physiological range have a significant impact on osteoblast proliferation and differentiation, independently of other paracrine and endocrine factors. The effects of increasing $[Ca^{2+}]_o$ are likely to be mediated by the calcium-sensing receptor, which is expressed in these cells (see Dvorak *et al.* 2003).

Dvorak MM *et al.* (2003). *J Physiol* **552.P**, C66.

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All procedures accord with current UK legislation

PS C69

Smooth muscle cells from guinea-pig portal vein have two functionally separate intracellular Ca^{2+} stores

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The organisation of InsP_3 receptors (IP_3R) and ryanodine receptors (RyR) on intracellular Ca^{2+} stores was examined in single, voltage-clamped myocytes from the portal veins of guinea-pigs.

Guinea-pigs of ~500 g were humanely killed by stunning then immediate exsanguination. Increases in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) were produced by flash photolysis of caged IP_3 (25 μM) or by pressure ejection of caffeine (10 mM). Results are shown as means ± S.E.M. and statistical significance was determined by Student's paired t test, where $P < 0.05$ was considered significant.

In ryanodine (50 μM) the amplitudes of IP_3-evoked Ca^{2+} transients were unaffected ($\Delta F/F_0 = 0.8 \pm 0.1$ units in control and $\Delta F/F_0 = 0.9 \pm 0.2$ units in ryanodine, $n = 4$) indicating that IP_3-evoked Ca^{2+} release does not evoke Ca^{2+}-induced Ca^{2+} release from RyR. Depletion of Ca^{2+} stores, which possess RyR, by caffeine which opens RyR, in the presence of ryanodine (50 μM) abolished Ca^{2+} transients evoked by IP_3, suggesting that IP_3R and RyR were located on the same intracellular Ca^{2+} store. IP_3-evoked Ca^{2+} transients were abolished in a Ca^{2+}-free extracellular bathing solution, but caffeine-evoked Ca^{2+} transients persisted at constant amplitude ($\Delta F/F_0 = 1.3 \pm 0.4$ units in control and $\Delta F/F_0 = 1.3 \pm 0.3$ units after abolition of IP_3 responses in Ca^{2+}-free solution, $n = 3$, $P > 0.05$). Thus, in addition to a store containing RyR and IP_3R, a separate store with RyR alone existed. Despite an extended time period (> 15 min) in Ca^{2+}-free extracellular solution, caffeine-evoked Ca^{2+} transients remained ($\Delta F/F_0 = 1.1 \pm 0.3$ units, $n = 3$) suggesting that the separate RyR-only store was able to refill itself from the cytosolic Ca^{2+} pool. Both IP_3- and caffeine-evoked Ca^{2+} transients were abolished by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (500 nM, $n = 5$, $P < 0.05$).

These results suggest that the guinea-pig portal vein contains two types of Ca^{2+} store; one has only RyR and can be refilled from a cytosolic Ca^{2+} source; the other has both RyR and IP_3R and is dependent for refilling on extracellular Ca^{2+}.

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All procedures accord with current UK legislation

PS C70

NAADP triggers 'Ca^{2+} bursts' by mobilising lysosome-related calcium stores in isolated rat pulmonary artery smooth muscle cells

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Our previous studies on isolated pulmonary artery smooth muscle cells have shown that inositol 1,4,5-trisphosphate (IP_3), cyclic adenosine diphosphate-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) mobilise Ca^{2+} from intracellular stores (Boittin *et al.* 2002, 2003). Our data are consistent with the view that intracellular dialysis of IP_3 and cADPR mobilises Ca^{2+} from the sarcoplasmic reticulum (SR) by activating IP_3 receptors and ryanodine receptors, respectively. In contrast, however, we found that NAADP triggered spatially restricted 'bursts' of Ca^{2+} release from a thapsigargin-insensitive intracellular store, and subsequent global Ca^{2+} waves by Ca^{2+}-induced Ca^{2+} release via ryanodine receptors in the SR. Significantly, Ca^{2+} bursts took two forms (1) a synchronous increase in Ca^{2+} around the entire perimeter of the cell or (2) a spatially restricted increase in Ca^{2+} between 2 and 10 μm in diameter (Boittin *et al.* 2002). We therefore investigated the nature of the Ca^{2+} store accessed by NAADP.

Pulmonary artery smooth muscle cells were isolated from male Wistar rats (150–200 g) that were humanely killed. Using Fura-2 fluorescence imaging as described previously (Boittin *et al.* 2002), we have now shown that both initial Ca^{2+} bursts and global Ca^{2+} waves induced by intracellular dialysis of NAADP from a patch pipette are abolished after depletion of lysosome-related Ca^{2+} stores by a vacuolar proton pump inhibitor, bafilomycin A1. Thus, under control conditions, 10 nM NAADP increased the Fura-2 fluorescence ratio (F_{340}/F_{380}) by 130 ± 34 % ($n = 5$), at the peak of the Ca^{2+} wave. In marked contrast, 10 nM NAADP was without effect in paired cells that had been pre-incubated (40 min) with 100 nM bafilomycin. Importantly, bafilomycin (100 nM) was without effect on Ca^{2+} waves induced via IP_3 receptor activation by intracellular dialysis of IP_3 ($n = 4$), or via ryanodine receptor activation by extracellular application of caffeine (10 mM; $n = 4$). Furthermore, deconvolved images of lysosomes labelled with lysotracker red (excitation 568 nm, emission 590 nm), revealed dense clusters. Consistent with the spatial distribution of the two forms of Ca^{2+} burst initiated by NAADP (Boittin *et al.* 2002), the lysosomes clustered in either (1) a ring around the perimeter of the cell, or (2) a tight, spatially restricted unit approximately 2–6 μm in diameter. We propose, therefore, that NAADP triggers Ca^{2+} bursts by mobilising lysosome-related Ca^{2+} stores in pulmonary artery smooth muscle, in a manner consistent with previous studies on the sea urchin egg (Churchill *et al.* 2002).

Boittin F-X *et al.* (2002). *Circ Res* **91**, 1168–1175.

Boittin F-X *et al.* (2003). *J Biol Chem* **278**, 9602–9608.

Churchill GC *et al.* (2002). *Cell* **111**, 1–20.

All procedures accord with current UK legislation

PS C72

The Kir6.2 mutation L147P disrupts the ATP-sensitive potassium channel function and misdirects the channel to late endosomes

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Familial hyperinsulinism (HI) is a genetic disorder characterised by unregulated insulin secretion leading to hypoglycaemia. Mutations in either of the pancreatic ATP-sensitive potassium (K_{ATP}) channel-forming subunits, Kir6.2 or SUR1, can cause HI either by disrupting normal channel function or by interfering with membrane trafficking (Partridge *et al.* 2001; Huopio *et al.* 2002). The L147P mutation in the pore-forming Kir6.2 subunit produces a severe form of the disease (Thomas *et al.* 1996). Here we investigate the consequences of this mutation on the function and trafficking of Kir6.2.

The function of K_{ATP} channels was examined by two-electrode voltage clamp analysis of *Xenopus* oocytes injected with cRNA encoding the wild-type (WT) or L147P mutant Kir6.2 subunits together with SUR1.

Contrary to the WT channels, L147P-containing channels showed no currents in response to diazoxide and azide stimulation ($n = 7$), suggesting that L147P mutation destroys the channel function. To investigate if the loss of function is due to impaired trafficking to the cell surface, we have inserted a haemagglutinin A (HA) epitope into an extracellular loop of Kir6.2. The resultant construct was transfected into COS-7 cells together with SUR1, and the surface expression examined by immunocytochemistry (by staining for the HA epitope) and confocal microscopy. The results showed that the mutant channel was able to traffic to the cell surface. However, when the cells were permeabilised, unlike the WT channel, the mutant channels were distributed in punctate structures. To investigate the nature of the punctate structures COS-7 cells were co-transfected with the WT and mutant K_{ATP} channels together with GFP-tagged markers for various endocytic compartments. The cells were permeabilised and stained for Kir6.2 and examined by confocal microscopy. The data showed almost complete co-localisation of L147P Kir6.2 with GFP-Rab7, a marker of late endosomes/lysosomes. Such co-localisation was not apparent with the WT Kir6.2 containing channels.

In conclusion, these data suggest that the L147P mutation does not impair the ability of the channel to traffic to the cell surface. However, the mutation appears to alter the structure of the channel such that it not only impairs the channel function, but misdirects the channel to a late endosomal/lysosomal compartment. These effects explain why L147P mutation causes severe form of HI.

Huopio H *et al.* (2002). *Am J Physiol* **283**, 207–216.

Partridge CJ *et al.* (2001). *J Biol Chem* **276**, 35947–35952.

Thomas P *et al.* (1996). *Human Mol Gen* **5**, 1809–1812.

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All procedures accord with current UK legislation

PS P97

SH groups and the functioning of IP_3-sensitive Ca^{2+} channels of salivary gland secretory cells

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It is very important to identify Ca^{2+}-transporting systems and to investigate their structure, properties and pharmacology, because many diseases are caused by an alteration in their functioning (Missiaen *et al.* 2000). Such studies could result in the development of new drugs that by an affect on Ca^{2+}-transporting proteins could restore proper intracellular Ca^{2+} signalling and cell functioning. It has been shown that SH groups play an important role in Ca^{2+}-transport system functioning (Marshall *et al.* 1993). Moreover, some drugs (NO, for example) could influence ion-transporting system functioning by affecting SH groups (Manko *et al.* 2002). That is why it is very interesting to determine the role of SH groups in the functioning of Ca^{2+}-transporting systems, in particular, IP_3-sensitive Ca^{2+} channels (IP_3-channels).

Experiments were performed on isolated salivary glands of *Chironomus plumosus* L. larvae. Ca^{2+} content in gland tissue was measured using arsenazo III. Saponin (0.1 mg ml^{-1}) was used for cell permeabilization. *p*-Chlormercuribenzoate (PCMB; 10 μM) was used as an SH-group blocker, eosin Y (20 μM) as a Ca^{2+} pump blocker and inositol-1,4,5-trisphosphate (IP_3; 10 μM) as an IP_3-channel activator. Data are presented as means ± S.E.M. and were compared with Student's paired *t* test.

We found the following. (1) IP_3 evoked a Ca^{2+} content decrease in gland tissue by 41.14 ± 11.75 % ($P < 0.05$, $n = 11$). This allows us to suggest that IP_3 channels are present in the endoplasmic reticulum (ER) membrane of investigated cells. (2) Incubation of glands with PCMB (1, 5 and 10 μM) did not cause a statistically significant change in the Ca^{2+} content of gland tissue. We assume the reason for this is the simultaneous effect of PCMB on different Ca^{2+}-transporting systems. (3) PCMB evoked a statistically significant ($P < 0.05$, $n = 6$) Ca^{2+} content decrease in gland tissue by 12.60 ± 11.61 % with eosin Y present in the incubation medium. This allows us to suggest that PCMB influences the IP_3 channels of exocrine secretory cell ER membrane. (4) IP_3 caused an increase in the Ca^{2+} content of gland tissue by 25.36 ± 7.42 % ($P < 0.05$, $n = 6$) with PCMB present in the incubation medium.

So, we suppose that SH groups play an important role in IP_3-channel functioning. However, because it is not known how IP_3 influences the functioning of different Ca^{2+}-transporting systems, the explanation of the PCMB effect needs additional investigation.

We conclude that SH-groups play an important role in the functioning of IP_3-sensitive Ca^{2+}-channels of salivary gland secretory cell endoplasmic reticulum membrane of *Chironomus plumosus* L. larvae.

Marshall I *et al.* (1993). *J Exp Biol* **184**, 161–182.

Manko V *et al.* (2002). *INABIS Proceed*

http://www.inabis2002.org/posters_congress/area_0

Missiaen L *et al.* (2000). *Cell Calcium* **28**, 1–21.

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PS P98

Ryanodine-induced Ca^{2+} release in secretory cells of *Chironomus plumosus* larvae salivary glands

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When a Ca^{2+} signal is generated, the intracellular Ca^{2+} rise is provided by Ca^{2+} influx across the plasma membrane and Ca^{2+} release from organelles via IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs). The role of RyRs has been proved for pancreatic (Leite *et al.* 1999; Ashby *et al.* (2003) and parotid (Zhang *et al.* 1999) acinar cells but is not well defined. Previously we identified IP_3Rs in secretory cell ER membrane of *Chironomus plumosus* L. larvae salivary glands and assumed RyRs to be present. The aim of this study was to obtain direct confirmation of the presence of RyRs in these cells.

The Ca^{2+} content in gland tissue was measured with the help of arsenazo III. For cell permeabilization glands were incubated with saponin (0.1 mg ml^{-1}, 10 min), then (30 min) in solution that contained (mM): 15.3 NaCl, 129.9 KCl, 0.35 Na_2HPO_4, 0.44 KH_2PO_4, 5.55 glucose, pH 7. Ryanodine, cAMP and IP_3 were added to this solution as appropriate. Data are presented as means ± S.E.M. and were compared using Student's *t* test.

(1) The ryanodine effect on the Ca^{2+} content of saponin-treated gland tissue depended on its concentration. It evoked a Ca^{2+} content decrease (by 37.95 ± 4.64 %; $P < 0.01$, $n = 6$) at a low concentration (5 nM), and an increase (by 40.72 ± 12.52 %; $P < 0.05$, $n = 7$) at a higher concentration (500 nM). This is possible only if RyRs are present. The effect of ryanodine at low concentration could be explained by RyR activation and its effect at high concentration by their suppression. (2) cAMP (10^{-4} M) intensified the effect of ryanodine: ryanodine (500 nM) decreased Ca^{2+} content by 89.54 ± 30.96 % in the presence of cAMP. cAMP decreased Ca^{2+} content by 37.59 ± 10.18 % in the presence of heparin (500 μM) in the incubation medium. But cAMP did not cause Ca^{2+} content changes in saponin-treated gland tissue in the simultaneous presence of heparin and ryanodine. (3) Ryanodine (5 nM) evoked a Ca^{2+} content increase in gland tissue by 48.61 ± 11.00 % ($P < 0.05$, $n = 6$) in the presence of IP_3 (10 μM). Alone IP_3 caused the Ca^{2+} content to decrease by 45.24 ± 13.85 % ($P < 0.01$, $n = 6$). The effect of IP_3 in the presence of ryanodine (5 nM) was the opposite: the Ca^{2+} content of gland tissue increased by 30.94 ± 9.39 % ($P < 0.01$, $n = 6$). So, the simultaneous presence of ryanodine and IP_3 did not affect the Ca^{2+} content in gland tissue in comparison with control.

The effect of different ryanodine concentrations on the Ca^{2+} content of saponin-treated salivary gland tissue confirms the presence of RyRs in salivary gland secretory cells of *Chironomus plumosus* L. larvae. RyRs may be regulated directly or indirectly by cAMP. We suppose there to be tight connections between RyRs and IP_3Rs in secretory cells: possibly, inhibition of these channels in the presence of their stimulators is connected with mechanisms that limit extreme emptying of Ca^{2+} intracellular stores and/or a negative reverse effect of Ca^{2+} on these channels. But this idea requires further study and additional confirmation.

Ashby MC *et al.* (2003). *J Biochem* **369**, 441–445.

Leite MG *et al.* (1999). *J Biochem* **337**, 305–309.

Zhang GH *et al.* (1999). *Arch Oral Biol* **44**, 735–744.

PS P99

Molecular identity of a G protein-gated inwardly rectifying K^+ current in primary cultured hippocampal neurones

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G protein-gated inwardly rectifying K^+ (GIRK) channels are predominantly expressed in neurones and atrial myocytes. A characteristic feature of these channels is their activation in response to stimulation of $G_{i/o}$-coupled receptors. For example, in neurones they are activated by adenosine and GABA and as such they play an important role in neurotransmitter-mediated regulation of membrane excitability. In response to stimulation of heptahelical receptors coupled to pertussis toxin (PTx)-sensitive $G_{i/o}$ proteins, the $G_{i/o}$ heterotrimer dissociates into its component $G_{i/o}$ α and $G\beta\gamma$ subunits. The $G\beta\gamma$ dimer binds directly to the GIRK channel complex activating it. The channels are tetrameric assemblies of Kir3.*x* subunits (Kir3.1–3.4 and splice variants). Atrial myocytes express Kir3.1 and 3.4 and neurones express Kir3.1, 3.2 and 3.3 subunits. It is therefore possible that greater channel diversity exists in the brain and that neuronal GIRK channel composition may differ in different brain regions. In this study I have characterised a neuronal GIRK current in primary cultured hippocampal neurones and compared this with cloned channel subunits heterologously expressed in HEK293 cells.

RT-PCR and immunocytochemistry were used to assess expression of Kir3.*x* subunits in hippocampus. Kir3.1, 3.2A, 3.2C and 3.3 were detected but not Kir3.2B or 3.2D. Cultured neurones were obtained using a protocol modified from Shah & Haylett (2000). Neonatal (P3–P5) Sprague-Dawley rats were humanely killed by cervical dislocation. Hippocampi were dissected out, cut into pieces and incubated in trypsin (1 h, 37 °C). Enzymatic action was terminated by the addition of serum and bovine serum albumin and individual cells obtained by trituration using fire-polished Pasteur pipettes. Cells were plated in Neurobasal medium (+2 % B27, 0.5 mM L-glutamine, 5 % serum) onto poly-D-lysine-coated glass coverslips. After 24 h the medium was replaced with serum-free medium plus 1 μM cytosine arabinoside. Cells were maintained at 37 °C with humidified 92.5 % O_2–7.5 % CO_2.

Whole-cell patch clamp recordings were made from neurones after 7–11 days in culture. The GIRK current was identified on the basis of its inward rectification, inhibition by tertiapin, enhancement by a number of $G_{i/o}$-coupled receptor agonists (e.g. baclofen, adenosine, somatostatin) and sensitivity to PTx. The properties of the neuronal GIRK current were compared to the cloned Kir3.1+3.2A channel currents activated by stimulation of either the $GABA_B$1a+2 or the $GABA_B$1b+2 receptors, also heterologously and stably expressed in HEK293 cells. Striking similarities between the native hippocampal GIRK current and the cloned channel current were observed in terms of channel kinetics, activation by baclofen and inhibition by tertiapin.

Shah M & Haylett DG (2000). *J Neurophysiol* **83**, 2554–2561.

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All procedures accord with current UK legislation

PS P100

The trafficking of regulators of G-protein signalling and G-protein $\beta\gamma$ subunits studied in mammalian cell lines

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The principles by which components in the G-protein cycle (Gα, G$\beta\gamma$ and regulators of G-protein signalling) reach the plasma membrane (PM) is only partially understood.

DNA constructs fused in frame to fluorescent proteins (cyan (CFP), green (GFP) and yellow (YFP) fluorescent protein) were generated (Goα-CFP/GFP/YFP, Giα3-CFP, Gβ1-YFP, Gγ2-CFP, RGS8-YFP and GAIP-YFP) using vectors pECFP-N1, pEGFP-N1 and pEYFP-N1 (Clontech). In addition, the first 35 amino acids of RGS8-YFP were deleted (ΔN-RGS8) or fused in frame with YFP (Nonly-RGS8-YFP). These constructs were transfected into HEK293 cells and their subcellular localisation was studied using confocal microscopy.

G-protein α subunits with a dual palmitoylation or a palmitoylation/myristoylation signal trafficked independently to the PM. In contrast, localisation of Gβ1-YFP and Gγ2-CFP to the PM required coexpression of Goα and Gγ2 or Gβ1, respectively. RGS8-YFP distribution was mainly to the nucleus and cytoplasm while that of GAIP-YFP was largely to the cytosol. Nonly-RGS8-YFP expressing cells showed largely nuclear staining whereas the ΔNRGS8-YFP mutant was diffusely distributed through the cell. Cotransfection of Goα, Goα-CFP and Giα3-CFP with RGS8-YFP and GAIP-YFP resulted in significant translocation of RGS to the plasma membrane. Colocalisation analysis was performed and produced the following values (colocalisation coefficients expressed as means ± S.E.M.) for RGS8-YFP: RGS8-YFP in Goα-CFP, 0.599 ± 0.04 ($n = 17$); RGS8-YFP in Mem-CFP (a membrane targeted CFP used as a control), 0.102 ± 0.024 ($n = 16$, Student's unpaired t test, $P < 0.0001$). ΔNRGS8-YFP and Nonly-RGS8-YFP did not localise to the PM with coexpression of either Goα-CFP or Goα. Colocalisation analysis was performed for GAIP-YFP: GAIP-YFP in Giα3-CFP, 0.605 ± 0.065 ($n = 11$, $P < 0.001$ *vs.* Mem-CFP, one-way ANOVA); GAIP-YFP in Goα-CFP, 0.553 ± 0.038 ($n = 19$, $P < 0.001$ *vs.* Mem-CFP); and GAIP-YFP in Mem-CFP, 0.228 ± 0.031 ($n = 16$). Finally, cotransfection of Go α-CFP with a point mutation (G184→S) that interferes with RGS binding and action also prevents translocation of RGS8-YFP to the PM.

We conclude that the Gα subunit has a central role in translocation of the other components involved in the G-protein cycle to the PM. The N-terminus of RGS8 is necessary but not sufficient for PM localisation after coexpression with Gα.

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PS P101

A whole-cell patch clamp study of citrate transport in human prostatic epithelia *in vito*

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One of the major functions of the prostate gland is the production and accumulation of high levels of citrate due to the tissue-specific Zn^{2+}-dependent activity of a rate-limiting oxidizing enzyme, *m*-aconitase (Costello *et al.* 1997). Citrate is released into the lumen by a membrane transport mechanism which at present is not well understood.

We adopted the normal human prostate cell line PNT2-C2 to characterise citrate transport electrophysiologically. Two different experimental protocols were used: citrate (sodium salt) was introduced into the cells either via the intracellular patch pipette or the cells were perfused with extracellular citrate. The membrane currents induced under voltage-clamp conditions were recorded. Data were analysed as means ± S.E.M.

At a holding potential of −45 mV, intracellular citrate (0.5 or 10 mM) induced an outward current (I_{cit}) which slowly reached values of 124 ± 17 and 180 ± 25 pA, respectively ($n > 30$). Equimolar intracellular NaCl had no effect. Other Krebs cycle intermediates like malate, succinate and isocitrate were 50–70 % less effective. Extracellular citrate (10 mM) elicited much smaller, inward currents of −42 ± 8.1 pA. Application of intracellular citrate had no effect on normal breast epithelial MCF-10A cells. Increasing the extracellular K^+ concentration from 2.5 to 118 mM caused a significant, dose-dependent decrease in I_{cit}. On the other hand, changes in extracellular Na^+, Cl^-, Ca^{2+} or Mg^{2+} concentrations had no influence. The following pharmacological agents potentiated (+) or inhibited (−) I_{cit}: phloretin, +14 %; diethyl pyrocarbonate, −18 %; LiCl, −14 %; and 4-aminopyridine, −13 %. SCH28080, ouabain and dinitrophenol did not produce any change ($n > 10$ for each). The effects of the ionic changes and the pharmacological agents were essentially the same on the current elicited by extracellular citrate, consistent with both currents being due to the activity of the same transport mechanism. We propose that normal prostatic epithelial cells express a novel citrate transporter which is electrogenic and co-transports trivalent citrate anion with K^+ outward.

Costello LC *et al.* (1997). *J Biol Chem* **272**, 28875–28881.

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PS P102

Long-term exposure to tetrodotoxin suppresses voltage-gated Na^+ channel activity in the strongly metastatic MAT-LyLu rat prostate cancer cell line

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Functional voltage-gated Na^+ channels (VGSCs) have previously been shown to be expressed in strongly metastatic rat (MAT-LyLu) and human (PC-3) prostate cancer cells where VGSC activity enhanced *in vitro* invasion (Grimes *et al.* 1995; Laniado *et al.* 1997). In the present study, we have investigated whether long-term suppression of the VGSC could ultimately influence its subsequent functional activity.

The whole-cell voltage clamp technique was used to investigate the cells' response to pre-incubation with 1 μM tetrodotoxin (TTX) for 1–3 days starting 3 h after seeding. Current–voltage relationships were analysed.

Pre-treatment with TTX for 2 or 3 days (but not 1 day) significantly reduced peak inward current density (Table 1). This effect was almost entirely reversible. On the other hand, values for activation voltage and voltage corresponding to peak current did not change. Preincubation with 20 nM TTX for 3 days did not cause any effect.

Table 1. Peak current densities (pA/pF) recorded from MAT-LyLu cells[a]

Day	1	2	3
Control	11.1 ± 2.2	11.2 ± 1.2	14.4 ± 1.2
TTX (1 μM)	6.6 ± 0.7	5.9 ± 0.8	8.1 ± 1.0
Significance (*P* value)	0.053[b]	0.001[b]	<0.001[c]

[a]Data presented as mean ± SEM ($n = 15 – 37$)
[b]Unpaired Student's t-test
[c]Mann-Whitney Rank Sum Test

The results indicate that pre-incubation with TTX can reduce functional VGSC activity in MAT-LyLu cells, and that this effect is both dose- and time-dependent. The time dependence of this phenomenon suggests a positive feedback mechanism possibly involving transcriptional regulation. As VGSCs have been shown to be involved in potentiating several components of the metastatic cascade, the data presented here raise the possibility that inhibition of the VGSC by a long-term drug treatment regime might be a useful, novel approach to combating prostate cancer progression.

Grimes JA *et al.* (1995). *FEBS Lett* **369**, 290–294.

Laniado ME *et al.* (1997). *Am J Pathol* **150**, 1213–1221.

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PS P103

Regulation of ryanodine receptor Ca^{2+} release by FK506 binding protein 12 in colonic smooth muscle

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The FK506 binding protein (FKBP) family are thought to regulate the activity of ryanodine receptors (RyR) but the mechanisms by which they do so are currently unclear.

Smooth muscle cells were isolated from the colon of guinea-pig (~500 g), which was killed humanely by stunning followed by exsanguination. FKBP12 but not FKBP12.6 co-immunoprecipitated RyR2 from these cells. The effects of FKBP12 on Ca^{2+} release in these cells was examined in single, voltage-clamped myocytes using the dye fluo-3 to monitor $[Ca^{2+}]_c$. All values are given as means ± S.E.M. and statistical significance was assessed using Student's paired *t* test.

FK506 (10 μM), which inhibits the interaction between RyR and FKBPs as well as the phosphatase action of calcineurin,

significantly increased the $[Ca^{2+}]_c$ rise evoked by the RyR agonist caffeine from $\Delta F/F_0$ of 0.8 ± 0.16 to 1.34 ± 0.19 ($n = 9$). Rapamycin (10 μM), which also inhibits the RyR–FKBP complex but does not inhibit calcineurin, also significantly increased the caffeine-evoked $[Ca^{2+}]_c$ rise from $\Delta F/F_0$ of 0.63 ± 0.14 to 1.08 ± 0.33 ($n = 6$). Cyclosporin A (CsA) inhibits calcineurin but not the interaction between RyR and FKBP and was shown to have no effect on the $[Ca^{2+}]_c$ rise evoked by caffeine, $\Delta F/F_0$ of 2 ± 0.42 for control cells and 2.14 ± 0.44 when treated with 10 μM CsA ($n = 7$, $P > 0.05$).

These results indicate that the interaction between FKBP12 and RyR reduces the ability of the receptor to release Ca^{2+} in colonic smooth muscle cells.

This work was supported by The Wellcome Trust and the British Heart Foundation.

All procedures accord with current UK legislation

PS P104

NorpA and *itpr* reveal roles for phospholipase C and inositol (1,4,5)-trisphosphate receptor in *Drosophila* renal tubule function

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NorpA, encoding phospholipase C β (PLCβ), was originally characterised as a component of the visual signal transduction pathway in *Drosophila melanogaster*. We show by fluid transport assays that together with *itpr*, encoding inositol (1,4,5)-trisphosphate receptor (IP_3R), *norpA* transduces a diuretic signal in *Drosophila* Malpighian (renal) tubules (Dow & Davies, 2003) stimulated by CAP_{2b} (Kean *et al*. 2002) and Drosokinin (Terhzaz *et al*. 1999) neuropeptides. This was confirmed by direct assay of neuropeptide-stimulated levels of IP_3 in intact tubules and *Drosophila* S2 cells. Use of an allelic series of *itpr* mutants demonstrates attenuation of the action of CAP_{2b} and Drosokinin, demonstrating a necessary role for *itpr* in the mechanism of action of these peptides. Furthermore, measurements of cytosolic calcium levels using targeted expression of the calcium reporter, aequorin, show that mutations in *itpr* attenuate calcium responses stimulated by both neuropeptides. We have thus described a role for PLCβ and IP_3R in renal epithelia and show that both CAP_{2b} and Drosokinin neuropeptides are PLCβ-dependent, IP_3 mobilising neuropeptides (Pollock *et al*. 2003).

Dow JT & Davies SA (2003). *Physiol Rev* **83**, 687–729.

Kean L *et al.* (2002). *Am J Physiol Regul Integr Comp Physiol* **282**, R1297–1307.

Terhzaz S *et al.* (1999). *J Exp Biol* **202**, 3667–3676.

Pollock VP *et al.* (2003). *J Exp Biol* **206**, 901–911.

PS P105

Cytosolic Ca^{2+} storage and release in pancreatic islets of patients with hyperinsulinism in infancy

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Insulin is released from pancreatic β-cells in a Ca^{2+}-dependent manner as a result of depolarisation of the cell membrane. Hyperinsulinism in infancy (HI) has been shown in many cases to be caused by defects in the genes that encode K_{ATP} channels – *ABCC8* and *KCNJ11*, and this leads to constantly depolarised membrane potential in HI β-cells. Since such cells would have a greater than normal need to maintain intracellular Ca^{2+} homeostasis, we investigated the handling of $[Ca^{2+}]_c$ in islets from control adult cadavers ($n = 20$ islets from 6 donors), and patients undergoing partial or subtotal pancreatectomy for HI (with informed consent and local ethical committee approval).

Islets were maintained in RPMI medium containing 5 mM glucose on poly-D-lysine-coated coverslips and were loaded with fura-2 AM prior to microfluorimetry procedures. HI islets were subdivided according to whether they came from patients with defects in K_{ATP} channels (Type 1 and Type 2 HI-K_{ATP}, 15 patients, $n = 87$ islets), from patients where there were no defects in K_{ATP} channels (Type 3, 2 patients, $n = 8$ islets) or from patients with an atypical histology (2 patients, $n = 8$ islets). Data are presented as means ± S.E.M. and were analysed using Student's unpaired t tests and Mann-Whitney tests with $P < 0.05$ being significant.

We found that basal $[Ca^{2+}]_c$ levels were 99 ± 9 nM in control islets and 92 ± 10 nM in Type 3 HI islets. However, they were significantly higher in both the HI-K_{ATP} and atypical islets being 143 ± 5 nM ($P < 0.001$) and 199 ± 15 nM ($P < 0.001$), respectively. Treatment of the islets with a Ca^{2+}-free medium containing 1 mM EGTA caused a greater fall in $[Ca^{2+}]_c$ in HI-K_{ATP}, Type 3 and atypical HI islets than controls -14 ± 5, ns; -19 ± 3, $P < 0.03$; -27 ± 6, $P < 0.01$ *vs.* -8 ± 2 nM). Release of Ca^{2+} from internal stores was provoked using a mixture of ATP, UTP and acetylcholine (0.1 mM), in the continued absence of $[Ca^{2+}]_o$ in order to stimulate capacitative Ca^{2+} entry following reintroduction of external Ca^{2+}. This was found to stimulate a significantly higher rise in $[Ca^{2+}]_c$ in HI-K_{ATP} and atypical when compared to Type 3 or controls islets (62 ± 6, $P < 0.03$; 94 ± 16, $P < 0.003$; 44 ± 10, ns; *vs.* 40 ± 6 nM, respectively). Subsequent to these manoeuvres, experiments were carried out in the presence of thapsigargin (100 nM) to block the reuptake of Ca^{2+} into intracellular stores, and evoked changes in $[Ca^{2+}]_c$ were only statistically significantly different in the atypical islets which showed the largest rise in $[Ca^{2+}]_c$ when compared to control values ($\Delta[Ca^{2+}]_c = 225 \pm 37$ *vs.* 144 ± 17). When calculated, the rates of rise of $[Ca^{2+}]_c$ seen under capacitive influx conditions were higher than controls for the HI-K_{ATP}, Type 3 and atypical islets, but reached significance only in the latter; 0.99 ± 0.15 ($n = 18$) *vs.* 1.16 ± 0.13 ($n = 55$), 1.27 ± 0.37 ($n = 8$) and 1.95 ± 0.37 ($n = 8$), $P < 0.03$, respectively.

These results show that whilst $[Ca^{2+}]_c$ in HI-K_{ATP} islets is elevated as a consequence of K_{ATP} channel defects, the dynamics of $[Ca^{2+}]_c$ handling are broadly similar to controls. Islets from patients in which hyperinsulinism is unrelated to K_{ATP} channel defects differ

little from controls, whilst islets from patients with an atypical histological origin show evidence of a perturbation of Ca^{2+} storage dynamics which may be related to the pathogenesis of this novel form of HI.

All procedures accord with the Declaration of Helsinki