C133

Oestradiol rapidly regulates calcium signals in ciliary neurons from the chick embryo

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Oestrogen plays a key role in a great variety of actions in the nervous system, either through classical or alternative pathways (Nadal *et al.* 2001). Classical pathways are initiated after binding to classical oestrogen receptors ERα or ERβ, which translocate from the cytoplasm to the nucleus acting there as transcription factors. Conversely, alternative pathways are initiated at the plasma membrane and cytoplasm, via binding to classical or nonclassical oestrogen receptors.

Using isolated ciliary ganglion neurons from humanely killed chick embryos and Ca^{2+} imaging, we have demonstrated that 17β-oestradiol rapidly reduces Ca^{2+} influx through the plasma membrane. This effect was not reproduced by oestradiol conjugated to bovine serum albumin (E2-BSA), which does not cross the plasma membrane. This indicated an intracellular action of 17β-oestradiol. The existence of ERα in cytoplasm was demonstrated by immunostaining and its involvement by the abolishment of the 17β-oestradiol action by the pure antioestrogen ICI 182, 780. The PI3-kinase inhibitor wortmannin and the NOS inhibitor L-NAME, both completely blocked oestradiol effect. The gonadal hormone action was reproduced by 8Br-cGMP and abolished in the presence of the PKG inhibitor KT5823. Remarkably, the PKA inhibitor KT5820 was without effect.

Our study clearly indicates that 17β-oestradiol can regulate Ca^{2+} influx via PI3-Kinase, NOS and PKG after activation of cytoplasmic ER. The present work provides a novel molecular mechanism used by oestrogens to control neuronal Ca^{2+} signalling.

Nadal A *et al.* (2001). *NIPS* **16**, 251–255.

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All procedures accord with current national and local guidelines

C134

Run-up of voltage-operated calcium currents occurs during whole-cell patch-clamp recording in primary bone-marrow stromal cells

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Osteoblasts, which differentiate from bone marrow stromal cells, synthesise new bone. Ca^{2+} influx modulates a range of mechanisms in osteoblasts, including matrix deposition and responses to hormones (Liu *et al.* 2000). L-type voltage operated Ca^{2+} channels (VOCCs) are crucial to the normal response of bone cells to mechanical force (Walker *et al.* 2000). L-type VOCCs are ubiquitous in osteoblastic cells and are expressed in cultured primary bone marrow cells (Publicover *et al.* 1995).

The properties of the L-type VOCCs expressed in marrow stromal cells were characterised using whole-cell patch clamp, (V_h = −60 mV; test pulse = +20 mV. Extracellular saline contained 10 mM BaCl2,10 mM HEPES and 130 mM N-methyl glucamine (pH 7.6 with NaOH). Electrodes were backfilled with a standard saline containing 150 mM CsCl, 5 mM EGTA, 10 mM HEPES and 10mM D-glucose (pH 7.3 with CsOH). 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, penicillin, streptomycin, ascorbic acid and β-glycerophosphate, in a humidified atmosphere of 5 % CO_2 at 37 °C for 16 to 30 days.

Whole-cell recordings revealed an agonist-independent enhancement of VOCC current amplitude, which reached a plateau in approximately 8 min. This 'run-up' was followed by typical current run-down. The run-up of current amplitude was highly significant ($P = <2 \times 10^{-5}$; $n = 18$; 2-tailed paired t test), increasing by 225.3 ± 51.6 % of initial current (mean ± S.E.M., $n = 10$). Dialysis occurs between the electrode saline and cytoplasm during whole-cell recording (Pusch & Neher, 1988). This may instigate removal of an inhibitor of stromal cell VOCCs. The possibility that G-proteins confer such tonic inhibition was investigated. Preliminary experiments indicate that intracellular inclusion of 0.5 mM GTP has no significant effect on run-up amplitude ($P = 0.28$; 2-tailed t test), or the initial rate of run-up ($P = 0.757$; 2-tailed t test) in stromal cells. Run-up, particularly of this magnitude, is extremely unusual and previously unreported in bone cells.

These data suggest that a large proportion of stromal cells' Ca^{2+} channels are inactive under basal conditions as a result of tonic inhibition. Such tonic inhibition, if removed by an endogenous agonist, would afford great potential for the enhancement of voltage-operated currents. Mechanical force generates Ca^{2+} influx via L-type VOCCs in osteoblasts (discussed above). Removal of tonic inhibition could therefore provide a coincidence detection system, whereby simultaneous agonist stimulation and force-induced VOCC activation could generate larger currents than force alone.

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Walker LM *et al.* (2000). *J Cell Biochem* **79**, 648–61.

All procedures accord with current UK legislation

C135

Loose intercellular coupling of spontaneous Ca^{2+} events in rat retinal arteriolar smooth muscle cells

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The goal of the present study was to image sub-cellular Ca^{2+} transients in microvascular smooth muscle (MVSM) cells of intact, non-pressurised, retinal arterioles and to quantify the spatiotemporal coupling of these signals at an intercellular level.

Sprague Dawley rats (200–300g) were anaesthetised using CO_2 and killed by cervical dislocation. Arterioles were mechanically dispersed from fresh retinae using a fire-polished Pasteur pipette and MVSM cells loaded with 10 μM Fluo-4AM for 2 hours. Changes in $[Ca^{2+}]_i$ were imaged in MVSM cell arrays (9–17 cells) using confocal scanning laser microscopy in X-Y (2 images/s) or line scan mode (500 scans/s). Raw fluorescence data were extracted in numerical format using Image J (NIH) and cell-to-cell coupling was evaluated between adjacent cells using the

cross-correlation coefficient (CCF; SPSS) applied to data derived from line scans parallel to the long axis of each vessel.

Under resting conditions X-Y scans revealed both discretely localised, spontaneous near-membrane Ca^{2+} events resembling Ca^{2+} sparks, and more global and prolonged Ca^{2+} transients. Line scan data was characterised by sporadic periods when global Ca^{2+} signals appeared to be entrained among up to six adjoining cells, interspersed with periods when little entrainment was obvious on visual inspection. A mean CCF of 0.38 ± 0.03 (± S.E.M.) was obtained in 56 MVSM cell pairs from 6 vessels, indicating that overall, intercellular coupling was weak. However, regions selected on the basis of having either: (a) one Ca^{2+} event which appeared to propagate across three or more adjacent MVSM cells, or (b) three or more temporally associated Ca^{2+} transients in a MVSM cell pair, had a mean CCF of 0.72 ± 0.04 (5 vessels; 7 regions). The lag time which gave the peak correlation for the propagating Ca^{2+} transients in these regions of strong coupling varied between 10–414 ms (mean 169.2 ± 26.1 ms). This delay was unrelated to the amplitude of the $[Ca^{2+}]_i$ rise in the lead cell ($r^2 = 0.079$; 5 vessels; 22 paired Ca^{2+} transients). When intercellular propagation was seen in a group of cells, the cell firing the initial Ca^{2+} transient could vary, suggesting the absence any fixed pacemaker.

We conclude that retinal MVSM cells exhibit both localised and global Ca^{2+} events whilst still embedded within their parent arterioles. These signals may occasionally synchronise in neighbouring cells. The mechanisms responsible for this coupling warrant further investigation.

We thank Fight for Sight and The Wellcome Trust for financial support.

All procedures accord with current UK legislation

C136

Carbachol induced calcium signals induce metalloproteinase dependent shedding of TGFα and downstream activation of mitogenic epidermal growth factor receptor signals in HT29 colonic epithelial cells

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G protein coupled receptors are coupled to extracellular signal regulated kinase (ERK) activation via stimulation of the epidermal growth factor receptor (EGFR) in a number of tissues. Regulation of ERK1/2 activity is thought to play a central role in the control of intestinal cell proliferation and differentiation. The aim of the present study was to investigate the cell signaling pathways that link muscarinic acetylcholine receptor stimulation to ERK1/2 activation and proliferation in human colonic epithelial cells.

METHODS: HT29 cells were cultured in DMEM supplemented with 5 % FCS (5 % CO_2/37 °C). Cell preparations were stimulated by carbachol (100 μM) in the presence or absence of cell signaling inhibitors. Epidermal growth factor receptor and (EGFR) and ERK1/2 phosphorylation was assessed by immunoblotting and/or immunocytochemical techniques based on using phospho-specific antibodies. Shedding of membrane associated TGFα was assessed by immunocytochemistry. Cell proliferation was assessed by 3H thymidine incorporation (during 24 hours).

RESULTS: Carbachol (100 μM, 10 mins) stimulated cytoplasmic ERK1/2 and translocation into the nucleus; maximal cytoplasmic stimulation was noted at 1 hour. These effects were mimicked by calcium ionophores (e.g. ionomycin 1 μM) and abolished by inhibitors of calcium signal generation (e.g. BAPTA-am, 25 μM; caffeine, 5 mM). Carbachol-induced ERK activation was reduced by 50 % in the presence of BB94 (5 μM) (metalloprotease inhibitor), TIMP 3 (0.5 μM) (Tissue Inhibitor of metalloproteases), anti-TGFα (10 ng/ml), anti-EGFR (10 ng/ml; to prevent EGF ligand binding to the receptor) and tyrophostin (1 μM) (EGFR kinase inhibitor). No inhibition was seen in the presence of TIMP 1 and 2 (0.5 μM). Similar observations were made regarding carbachol induced-TGFα shedding, and -EGFR phosphorylation. Carbachol induced a 40 % increase in HT29 cell 3H thymidine incorporation. This effect was abolished in the presence of BB94 (5 μM), anti-TGFa (10 ng/ml) and anti-EGFR (10 ng/ml).

Carbachol induced calcium signals mediate metalloproteinase dependent shedding of TGFα and downstream activation of mitogenic epidermal growth factor receptor signals in HT29 colonic epithelial cells. These observations suggest that neuronal-epithelial cell interactions regulate the proliferative status of the colonic epithelium.

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C137

Clustering of inositol 1,4,5-trisphosphate receptors in RBL-2H3 cells

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Cross-linking of IgE receptors (FCεRI) by antigens in rat basophilic leukemia cells (RBL-2H3), results in the production of inositol trisphosphate (IP_3) and release of Ca^{2+} from intracellular stores, triggering exocytosis of secretory granules. FCεRI activation also leads to changes in IP_3 receptor (IP_3R) distribution. At rest IP_3R distribution is relatively homogenous throughout the cell. Upon activation, IP_3Rs form discrete clusters in the cell.

In our experiments RBL-2H3 cells were primed with anti-DNP-IgE (1 μg/ml, 12 h) and activated with DNP-BSA (1 μg/ml, 1 h). Immunohistochemistry in combination with fluorescent constructs were used to investigate the redistribution of the IP_3Rs and endoplasmic reticulum (ER) in live and fixed cells.

Staining of IP_3R in fixed RBL-2H3 cells showed that 1 h after cell activation the number of IP_3R clusters increased significantly from 8.19 ± 1.29 clusters per 100 μm^2 [± S.E.M. $n = 20$ cells] to 14.13 ± 1.09 clusters per 100 μm^2 [± S.E.M. $n = 9$ cells] (Student's t test, $P < 0.05$). Furthermore the size of the clusters increased significantly from 0.15 ± 0.01 μm^2 to 0.28 ± 0.03 μm^2 (Student's t test, $P < 0.001$). We observed similar results in living RBL-2H3 cells expressing YFP-IP_3R.Fluorescence recovery after photobleaching (FRAP) of YFP-IP_3R showed that the IP_3R was mobile within the ER. The half life of recovery of the IP_3R is 39.04 ± 2.64 s [± S.E.M. $n = 24$ cells] and this decreased significantly following cell activation to 25.10 ± 2.44 s [± S.E.M. $n = 15$ cells] (Student's t test, $P < 0.001$). The immobile fraction of IP_3R was not significantly altered.

To investigate if the ER itself was responsible for IP_3R movement we stained the ER using a BiP antibody. After cell activation the gross structure of the ER changed and, like the IP_3R, becomes concentrated in the peri-nuclear region wherue the microtubule organising centre is typically found. DsRed-ER expression in live cells demonstrated the ER was highly mobile. Expression of YFP-IP_3R and DsRed-ER showed that the IP_3R redistribution

following activation was the consequence of ER redistribution. The IP_3R clusters appear and disappear dynamically at nodules in the ER over periods of less than 30 s. In conclusion, where RBL-2H3 cell activation induces the redistribution of the IP_3R, FRAP studies show an increase in IP_3R mobility which was likely to be due to increased movement and reorganization of the ER. This restructuring of the ER causes the transient formation of nodules at branch points in the ER and the IP_3R clusters in these regions. These clusters of IP_3R remain contiguous with the ER itself and increase in number and size in the 1 h following cell activation. We speculate the clustering of the IP_3R may have an effect on the calcium signal generated and functionally on the secretory response.

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C138

Voltage control of Ca^{2+} release via $P2Y_1$ and other $G\alpha_q$-coupled receptors

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Voltage modulation of IP_3-dependent Ca^{2+} release during G-protein-coupled receptor (GPCR) activation has been described in a number of cell types but most extensively studied in the non-excitable megakaryocyte (MK) (Mahaut-Smith *et al.* 1999). To gain further insight into the underlying mechanism, we have now compared the voltage-dependence to Ca^{2+} signalling during activation of different receptors and downstream signalling events in the MK.

Male Wistar rats or C57BL/6 mice were humanely killed by CO_2 inhalation and cervical dislocation. Whole-cell patch clamp and $Ca^{2+}{}_i$ recordings from marrow MKs, adapted to allow simultaneous flash photolysis, were conducted as described previously (Mahaut-Smith *et al.* 1999; Mason & Mahaut-Smith, 2001). Depolarisation-dependent Ca^{2+} mobilisation was assessed using 80 mV pulses from −75 mV.

Ca^{2+} increases evoked by several agonists acting via $G\alpha_q$-coupled receptors were voltage-dependent with the rank order: ADP>thromboxaneA_2> serotonin (55 ± 5, 33 ± 6 and 11 ± 4 %, respectively (S.E.M.), as a percentage of the response to 1 μM ADP, 300 nM U46619 and 10 μM 5-HT). Activation of IP_3 receptors following release of IP_3 ($n = 9$) or G-PIP_2 ($n = 10$) from caged precursors, alone or in combination with stimulation of diacylglycerol-dependent pathways (100 μM OAG, $n = 8$, or 1 μM PMA, $n = 4$), failed to promote voltage control of Ca^{2+} release. Similarly, activation of phospholipase-Cγ by platelet-derived growth factor (50 ng/ml) did not lead to voltage-dependent Ca^{2+} release ($n = 4$). In $P2Y_1{}^{-/-}$ MKs, agonist and voltage-dependent Ca^{2+} increases were absent during ADP application (1 μM; $n = 43$), but still observed during U46619 (300 nM; $n = 15$) or serotonin (10 μM; $n = 19$). Ca^{2+} increases induced by intracellular GTPγS (50 μM) were potentiated 5.7 fold by depolarization, however responses to both GTPγS and voltage were not clearly observed in $P2Y_1{}^{-/-}$ MKs ($n = 5$ and 9). Sensitisation of IP_3 receptors with thimerosal (100 μM) induced a small voltage-dependent Ca^{2+} release ($n = 7$), but this also required $P2Y_1$ receptors ($n = 19$).

These data indicate that signalling via a number of $G\alpha_q$-coupled receptors in the MK is voltage-dependent and that this response to ADP requires $P2Y_1$ receptors. Evidence to date (see also Martinez-Pinna, 2003) supports the hypothesis that the voltage sensor lies at the level of the GPCR or its interaction with G-proteins, rather than a downstream signalling event.

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All procedures accord with current UK legislation

C139

Lysosomes and Ryanodine receptors co-localise to form a 'trigger zone' for Ca^{2+} signalling by NAADP in isolated rat pulmonary artery smooth muscle cells

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C140

Role of sarcoplasmic reticulum and mitochondria in cytosolic Ca^{2+} clearancein airway myocytes

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Cytosolic Ca^{2+} clearance plays a key role in Ca^{2+} signalling. The aim of this study was to use both theoretical and experimental approach to determine the influence of the sarco-endoplasmic Ca^{2+}-ATPase (SERCA) and mitochondrial Ca^{2+} uptake on cytosolic Ca^{2+} clearance in airway myocytes.

Experimental studies were performed on myocytes freshly isolated from rat trachea. Rats were humanely killed according to national guidelines. $[Ca^{2+}]_i$ responses were measured by microspectrofluorimetry using the Ca^{2+}-sensitive fluorescent dye Indo-1. Data are mean ± S.E.M. Statistical comparisons were done by Student *t* tests or ANOVA for repeated measurements, and considered as significant when $P < 0.05$.

Stimulation by caffeine (CAF) for 30 s induced a concentration-graded response characterised by a transient peak followed by a progressive decay to a plateau phase. The peak and plateau values in response to 30 s stimulation by 5 mM CAF were 697 ± 59 nM and 43 ± 4.0 nM, respectively ($n = 19$). For 1 s CAF stimulation, the amplitude of the peak was not modified (695 ± 82 nM, $n = 35$), but the decay phase was accelerated, indicating ryanodine receptor (RyR) closure. A second CAF stimulation 15 s after the first one induced a second peak smaller than the first one (206 ± 26 nM, $n = 35$). In Na^+-Ca^{2+}-free medium containing 0.5 mM La^{3+}, the $[Ca^{2+}]_i$ response pattern to CAF 30 s and 1 s caffeine stimulation was not significantly modified, indicating no involvement of transplasmalemmal Ca^{2+} fluxes. On these experimental basis, we built a mathematical model describing the mechanism of Ca^{2+} handling upon RyR stimulation that included sarcoplasmic reticulum (SR), mitochondria and cytosolic proteins as Ca^{2+} buffers. Our model predicts that after Ca^{2+} release from the SR, Ca^{2+} is first buffered by cytosolic proteins and mitochondria, and pumped back into the SR after a time delay.To test the model predictions, we studied the effect of cyclopiazonic acid (CPA), a reversible inhibitor of SERCA, and FCCP, a mitochondrial inhibitor, on 1 s

CAF stimulation. 30 s exposure to 10 microM CPA or 5 microM FCCP did not significantly modified the resting $[Ca^{2+}]_i$. When cells were stimulated 30 s after the beginning of CPA exposure, the first peak was not modified (96.4 ± 6.9 % control, $n = 21$), but the Ca^{2+} response to the second stimulation was greatly decreased (49.2 ± 68.1 % control), indicating SERCA inhibition by CPA. However, the $[Ca^{2+}]_i$ decay following Ca^{2+} increase was not modified compared to control. When cells were stimulated in the presence of FCCP, the amplitude of the peak was not modified (102.4 ± 6.2 % control, $n = 25$). The subsequent $[Ca^{2+}]_i$ decay down to baseline persisted but was significantly slowed down.

This study indicate that, after Ca^{2+} release from the SR, the shape of the $[Ca^{2+}]_i$ decay does not primarily depend on SERCA activity but on mitochondrial Ca^{2+} uptake, in parallel with other buffering processes that may be, according to the model predictions, cytosolic Ca^{2+} binding proteins.

All procedures accord with current national and local guidelines

C141

Calcium buffers and binding proteins with differing kinetics differentially shape IP3-evoked calcium signals

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Cellular calcium buffers shape cytosolic calcium signals by regulating the availability and diffusional mobility of free calcium ions. Most experimental and theoretical studies of these effects have concentrated on signals arising from a fixed 'pulse' of calcium – for example, calcium entry through voltage-gated channels. A more complex case involves intracellular calcium liberation through inositol trisphosphate (IP3) receptors, which are themselves regulated by calcium. Thus, cytosolic buffers are expected to influence the successive cycles of diffusion and calcium-induced calcium release that act both between within clusters of IP3 receptors (to generate local 'puffs'), and adjacent clusters (to generate global calcium waves).

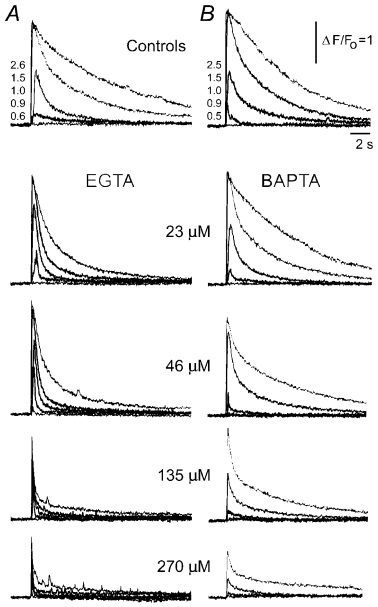

Figure 1. Modulation of IP3-evoked calcium signals by increasing concentrations of EGTA (*A*) or BAPTA (*B*). Each panel shows superimposed calcium-dependent fluorescence of Oregon Green 488 BAPTA-1 (0.04 mM intracellular concentration) monitored from 6 micron regions of linescan images in response to photolysis flashes of varying strength (indicated as normalized strength in upper panels). The upper sets of records were obtained in two oocytes before injecting buffer. Subsequent panels show recordings from the same oocyte, in response to photolysis flashes of the same strengths, after progressively loading EGTA or BAPTA to final intracellular concentrations as indicated.

We studied such interactions in *Xenopus* oocytes, using confocal line-scan microscopy together with photo-release of IP3. Intracellular injections of buffers with 'slow' calcium-binding kinetics (EGTA and parvalbumin) speeded the decay of calcium signals (Fig. 1A) and 'balkanized' IP3-evoked calcium waves into discrete puffs. Contrastingly, equivalent concentrations 'fast' buffers (BAPTA and calretinin) slowed IP3-evoked calcium responses (Fig. 1B) and promoted 'globalization' of spatially uniform calcium signals. These observations likely reflect buffer actions on calcium feedback at IP3 receptors, since the effects of EGTA and BAPTA on calcium signals evoked by influx through expressed N-type voltage gated channels were markedly less pronounced. Moreover, they point to the importance of buffer kinetics in shaping IP3-evoked calcium signals; likely because fast buffers can influence calcium feedback between individual IP3R within a cluster, whereas slow buffers are able to modulate only cluster-cluster interactions. We propose that cell-specific expression of calcium binding proteins with distinct kinetics may shape the time course and spatial distribution of IP3-evoked calcium signals for specific physiological roles.

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All procedures accord with current national and local guidelines

C142

Cyclic AMP-dependent Cl^- secretion induced by thromboxane A_2 in isolated rat colonic mucosa

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The anti-tumour drug irinotecan clinically causes severe diarrhoea as a side effect. Diarrhoea is generally accompanied with active secretion of electrolytes, especially Cl^-. We found recently that irinotecan stimulates Cl^- secretion in isolated rat colonic mucosa via the release of thromboxane A_2 (TXA_2), and that 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2), a stable TXA_2 analogue, mimics the effect of irinotecan (Sakai *et al.* 1997; 2002). TXA_2 receptor was cloned and found to link both the Ca^{2+}-and cAMP-signalling pathways (Hirata *et al.* 1996). STA_2 elevates $[Ca^{2+}]_i$ specifically via TXA_2 receptor in rat colonic crypt cells (Ikari *et al.* 1999). But it has not been clarified whether the the cAMP pathway is involved in the TXA_2-induced Cl^- secretion in the colon. Herein we investigated if the TXA_2-induced Cl^- secretion is mediated by elevation of the intracellular cAMP level.

Rats were sacrificed rapidly by stunning and cervical dislocation. Effects of the chemicals on the Cl^- secretion were examined in isolated rat distal colon mounted on Ussing chamber. The cAMP content in the HT-29 human colonic cancer cell line was measured using an enzyme immunoassay kit. Data are shown as means ± S.E.M. Differences between groups were analysed by one-way ANOVA. Comparison between the two groups was made with paired *t* test.

In HT-29 colonic carcinoma cells (1×10^5 cells), STA_2 (0.1 μM)

increased the cAMP level by 110 ± 16 fmol in the presence of indomethacin (1 μM) ($n = 3$). This increase was significantly inhibited by KW-3635 (30 μM), a selective TXA_2 receptor antagonist ($n = 3$, $P < 0.05$), suggesting that the effect is mediated specifically via the receptor. In the Ussing chamber experiments using isolated rat colonic mucosa, both the STA_2 (1 μM)-and PGE_2 (0.5 μM)-induced Cl^- secretion were inhibited in a concentration-dependent manner by chromanol 293B, a cAMP-dependent K^+ channel blocker. ($IC_{50} = 0.09$ and 0.1 μM, respectively; $n = 5$). The STA_2-induced Cl^- secretion was significantly stimulated by pre-treatment of the mucosa with 3-isobutyl-1-methylxanthine (IBMX; 100 μM), a phosphodiesterase inhibitor, in the presence of indomethacin (1 μM) and tetrodotoxin (1 μM): that is, IBMX increased the STA_2-elicited current from 45 ± 5 to 139 ± 13 μA cm^{-2} ($n = 5$, $P < 0.01$).

Our results suggest that the TXA_2-induced Cl^- secretion is mediated by the elevation of intracellular cAMP in the colonic mucosa.

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All procedures accord with current national and local guidelines

C143

Dynamic changes in near membrane cAMP in non-excitable cells

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Advances in measuring real-time cAMP dynamics have provided direct evidence for spatial and temporal heterogeneity of cAMP signals in both excitable and non-excitable cells (Zaccolo & Pozzan, 2002; Rich *et al.* 2000). Such compartmentalization of cAMP signals is thought to be due to physical or enzymatic barriers limiting diffusion of cAMP into the bulk cytosol. These cAMP microdomains may function to differentiate the downstream effects of the numerous agonists acting via adenylyl cyclase (AC). In excitable cells further differentiation may result from the frequency-encoding of cAMP transients, or oscillations (Cooper *et al.* 1995; Gorbunova & Spitzer, 2002).

We have used an adenovirus expressed cyclic nucleotide-gated (CNG) channel to determine whether cAMP oscillations can be detected in a non-excitable cell line and identify some of the signalling components involved. Human embryonic kidney cells (HEK-293) were infected with mutant olfactory CNG channel α subunit as described previously (Rich *et al.* 2001). Channel activation was monitored using either patch-clamp to detect changes in whole-cell current or fluorescent imaging of Fura-2 loaded cells to detect Ca^{2+} influx.

Application of 100 nM prostaglandin E_1 (PGE_1) produced a large cAMP transient that in ~9 % of cells ($n = 134$) was followed by one or more smaller cAMP transients. Pharmacological inhibitors of type 4 phosphodiesterase (PDE4, 10 μM Rolipram), protein kinase A (PKA, 10 μM H-89) and A-kinase anchoring protein (AKAP, 20 μM Ht31) were used to assess the role of each signalling component in the initial return of cAMP levels to baseline. Our results suggest that a PDE4/PKA/AKAP complex provides local feedback regulation of cAMP levels within the near membrane 'microdomain'. Furthermore, low doses of PDE4 inhibitor enhanced detection of single cell cAMP 'oscillations'. Following pretreatment with 0.01 μM rolipram or 0.01 μM RO-20–1724 ~22 % of cells ($n = 171$) produced more than one cAMP transient in response to 100 nM PGE_1. It is possible that low doses of rolipram or RO-20–1724 inhibit high affinity PDE4 activity induced by local increases in cAMP and unveil near-membrane cAMP oscillations. However, basal PDE activity and CNG channel expression levels (probe sensitivity) are likely to be key factors in determining whether cAMP oscillations will be observed in non-excitable cells.

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C144

A sustained increase in intracellular Ca^{2+} is required to regulate Ca^{2+}-CaM-dependent phosphodiesterase (PDE1)

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Regulation of Ca^{2+}-sensitive adenylyl cyclases (AC) by Ca^{2+} requires capacitative Ca^{2+} entry (CCE) (Mons *et al.* 1998), but whether Ca^{2+}-sensitive phosphodiesterases (PDEs) are similarly discriminating has not been addressed. Activation of muscarinic receptors inhibits isoprenaline (ISO)-evoked cAMP accumulation in 1321N1 human astrocytoma cells (Gross and Clark 1977; Meeker and Harden 1982). The effect is mediated by Ca^{2+}-CaM-dependent phosphodiesterase (PDE1), which is activated by an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Tanner *et al.* ,. 1986). Here, we attempt to determine whether PDE1 exhibits selectivity for either Ca^{2+} released from intracellular stores or Ca^{2+} entering the cell.

In fura-2-loaded 1321N1 cells, carbachol (CCh, 1 mM) evoked a rapid and transient increase in $[Ca^{2+}]_i$ (t = 24 ± 2 s, $n = 5$) followed by a slower, sustained phase of Ca^{2+} entry ($t_{½} = 93 \pm 29$, $n = 5$). The peak increase in $[Ca^{2+}]_i$ by the two phases were 1143 ± 152 nM ($n = 5$) and 712 ± 109 nM ($n = 5$), respectively. In analogous experiments, cells were stimulated with ISO (10 μM, 5 min) in the presence and absence of CCh. The inhibition of cAMP response by CCh was largely dependent on extracellular Ca^{2+} (15 ± 8 % ($n = 17$) of the total inhibition was dependent on Ca^{2+} from intracellular stores and 86 ± 8 % ($n = 17$) on Ca^{2+} entry). Both effects were reversed by the PDE inhibitors IBMX and 8-methoxymethyl-3-isobutyl-1-methylxanthine (PDE1) but not rolipram (PDE4). We conclude that PDE1 activation requires a sustained elevation in intracellular free Ca^{2+} concentration which is only achieved though by an influx of Ca^{2+} into the cell. Gd^{3+} (10 μM, 5 min) and 2-APB (100 μM, 5 min) inhibited CCE in thapsigargin (1 μM, 15 min) pre-treated cells by 89 ± 2 % ($n = 16$) and 86 ± 5 % ($n = 10$), respectively. In parallel experiments, CCE inhibited ISO-evoked cAMP accumulation; the effect was reversed by IBMX and CCE blockers. In ionomycin pre-treated cells (10 μM, 15 min), the presence of 30 μM Ca^{2+} in the extracellular medium produced a similar rise in $[Ca^{2+}]_i$ as 3 mM Ca^{2+} in thapsigargin pre-treated cells (576 ± 89 nM, $n = 9$ and 527 ± 86 nM, $n = 10$, respectively). However, only the latter effect was blocked by CCE inhibitors. Ionomycin-mediated Ca^{2+} entry also inhibited ISO-evoked cAMP accumulation and the effect also was reversed by IBMX. Thus PDE1 requires sustained Ca^{2+} entry for activation, but unlike ACs, it does not discriminate between the different routes of Ca^{2+} entry.

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C145

The glucocorticoid dexamethasone rapidly up regulates cAMP dependent protein kinase and MAP Kinase activity in human and cystic fibrosis lung epithelia

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A non-genomic anti-secretory role for glucocorticoids has been described in human bronchial epithelia. Dexamethasone has been shown to cause a rapid and non-genomic reduction of intracellular calcium concentration in these epithelia, resulting in a reduction of calcium dependent chloride secretion (Urbach *et al.* 2002).

In this study we demonstrate rapid effects of dexamethasone on cAMP dependent protein kinase (PKA) activity (using a non-radioactive PepTag Assay™ system), MAP Kinase activity (ERK1/2 western blotting) and intracellular calcium concentration (Fura-2 fluorimetry) in cell lines from normal lung epithelia (16HBE14o-) and cystic fibrosis lung epithelia (CFTE-29o-).PKA activity was rapidly up-regulated (< 5 min) three-fold over basal ($n = 3$) in response to low concentrations of dexamethasone (1–10 nM) in human bronchial epithelia. The PKA antagonist, RPcAMP (20 μM), was shown to inhibit this effect. Pertussis toxin, a Gi-protein inhibitor (10 ug/ml overnight), was demonstrated to inhibit the activation of PKA, while the ERK1/2 inhibitor, PD98059 (50 μM), had no effect. In addition, we demonstrate ERK1/2 activity was rapidly up-regulated in response to dexamethasone in human bronchial epithelia. This response was inhibited by RPcAMP, indicating PKA activates MAP Kinase.

Low concentrations of dexamethasone (1–10 nM) caused a rapid and non-genomic reduction of intracellular calcium concentration in cystic fibrosis tracheal epithelia similar to that of normal bronchial epithelia. F340/F380 relative fluorescence ratio was reduced by 0.26 ± 0.05 (mean ± S.E.M., $n = 3$; Student's t test $P < 0.000001$). The steroid-induced reduction in intracellular calcium concentration was inhibited by pre-treatment with RPcAMP. In addition, we demonstrate PKA activity was up-regulated (< 5 min) three-fold over basal ($n = 2$) by dexamethasone (1–10 nM) in cystic fibrosis tracheal epithelia and that RPcAMP inhibited this effect.

Our results show that rapid glucocorticoid activation of PKA and MAP Kinase is transduced via a Gi coupled receptor. One of the end targets of this signalling cascade is a reduced calcium mobilization in both normal and cystic fibrosis lung epithelia. Calcium is a potent regulator of secretion and MAP Kinase (ERK1/2) is anti-apoptotic in epithelia. Our results have important implications in the further development of steroid based treatments for inflammatory lung disease e.g. Cystic Fibrosis and Asthma.

Urbach V *et al.* (2002). *J Physiol* **537**, 267–75.

C146

Gender-specific expression and activation of protein kinase C isoforms and protein kinase D by 17β-oestradiol in rat distal colonic crypts

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The steroid hormone 17β-oestradiol (E2) rapidly affects K^+ transport in human colon via signal transduction involving Na^+/H+ exchange and the activation of protein kinase C (PKC) isoforms (McNamara *et al.* 2000). In rat distal colonic crypts E2 is known to inhibit Cl^- secretion under basal conditions and when secretion has been activated by cAMP or Ca^{2+} agonists (Condliffe *et al.* 2001). The anti-secretory effect of E2 is female gender-specific and is dependent on the activation of PKCα and PKCδ (Harvey *et al.* 2002). Downstream targets of PKC maybe ion transporters or other kinases. For example, the protein kinase D (PKD/PKCμ) family of serine-threonine kinases is a downstream target for classical (PKCα) and novel (PKCδ, ϵ and η) PKC isoforms. In this study we investigated rapid protein kinase signalling events stimulated by E2 in rat distal colonic crypts and, in particular, the rapid activation of PKD. We also sought to identify any gender differences in the basal expression levels in non-activated crypts of the PKC isoforms known to be activators of PKD.

Sprague-Dawley rats (3 months old) were killed humanely by cervical dislocation and distal colonic crypts were isolated as described previously (Harvey *et al.* 2002). To ensure heterogeneity, crypt samples were pooled for basal expression studies ($n = 3$) and activation studies ($n = 7$). PKC basal expression levels were determined by Western blotting. PKD activation was determined by western blotting.

Gender-specific differential expression of PKC isoforms was observed in non-activated distal colonic crypts. We observed higher levels of basal expression of PKCδ (3–5 fold) and PKCϵ (5–10 fold) in crypts isolated from female rats as compared to males. We have, for the first time, demonstrated the expression of PKD in rat distal colonic crypts with females showing a greater level (2-fold) of basal expression of PKD. No difference was observed in the basal expression levels of PKCα in crypts isolated from males and females. Also, no difference was observed in PKC isoform expression in crypts from female rats at different stages of the oestrus cycle. Treatment with E2 (10 nM) produced a rapid (< 2 min) activation of PKD in rat colonic crypts.

In conclusion, we provide the first evidence for differential female gender-specific expression of PKC isoforms and expression of PKD in the rat colon. The physiological significance of PKD activation in the rapid responses to steroid hormones remains to be elucidated, however the known PKD effectors; NHE-1, ERK1/2 and cJun maybe important in the anti-secretory and proliferative effects of oestrogen.

Condliffe *et al.* (2001). *J Physiol* **530**, 47–54.
Harvey *et al.* (2002). *Steroids* **67**, 483–91.
McNamara B *et al.* (2000). *Br J Pharmacol* **131**, 1373–1378.

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All procedures accord with current national and local guidelines

C147

Concentrations of signal transduction proteins mediating adaptation to exercise in fast, slow and electrically stimulated rat muscles

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The output of a signal transduction pathway depends both on the strength of the input signal and the signal amplification by the pathway. The amplification, in turn, depends largely on the signalling protein concentrations within a pathway and their control coefficients, to use the metabolic control analysis terminology. The aim was to investigate a) whether signal transduction protein levels differ with a muscle's phenotype and b) whether the levels in fast muscle respond to chronic electrical stimulation.

Signalling proteins that have previously been shown to regulate phenotype (calcineurin; extracellular signal regulated kinase, ERK1/2), mitochondrial biogenesis (AMP-activated kinase, AMPK; p38 kinase, p38), translation (protein kinase B, PKB; glycogen synthase kinase, GSK3beta; 4E-binding protein, 4E-BP1; p70 S6kinase, p70 S6k), myostatin growth regulation (SMAD2/3) and cachexia (nuclear factor kappaB, NF-kappaB) were measured in rat soleus (Sol; 84 % I fibres), extensor digitorum longus (EDL; 3 % I fibres) and EDL electrically stimulated for 6 weeks at 10 Hz ($n = 4$ per muscle). For stimulation, miniature stimulators were implanted into the peritoneal cavity under isoflurane/nitrous oxide anaesthesia with their integral electrodes placed close to the common peroneal nerve. Intramuscular buprenorphine (0.1–0.5 mg/kg) was used to provide post-operative analgesia. Rat EDL muscles were chronically stimulated at 10 Hz for 6 weeks. Animals were humanely killed and protein levels were determined by densitometry of Western blots.

The levels of most of the signal transduction proteins differed consistently between slow soleus and fast EDL. Chronic electrical stimulation of EDL also produced repeatable changes in the level of these proteins. The levels of signal transduction proteins involved in phenotype regulation (ERK1/2, calcineurin) or mitochondrial biogenesis (AMPK, p38) differed but there was no obvious relation to phenotype or mitochondrial content. Proteins mediating translation were all higher in EDL than soleus with the exception of the inhibitory GSK3beta, suggesting higher growth signal sensitivity in EDL. This greater sensitivity could be a partial explanation for the greater size of fast type II fibres compared to type I fibres. Chronic electrical stimulation led to a concentration change in fast-to-slow direction with the exception of PKB and GSK3beta, which also mediate insulin-regulated carbohydrate metabolism. The increase in PKB and decrease in GSK3beta in response to stimulation could be a contributory factor to the increased insulin-mediated glucose uptake in response to exercise observed in trained muscle (James *et al.* 1973). Both myostatin-activated SMAD2/3 and pro-inflammatory-cytokine activated NF-kappaB have been shown to promote muscle loss and cachexia. These proteins are expressed at higher levels in soleus and stimulated muscle suggesting that these muscles display a higher sensitivity to growth-inhibitory stimuli.

James DE *et al.* (1973). *J Histochem Cytochem* **21**, 51–55.

All procedures accord with current UK legislation

C148

Real-time measure of exocytosis in mouse submandibular acinar cells

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Most assays of secretion in exocrine tissues involve the collection and measurement of secretory products, such as amylase. The methods are slow, have poor time resolution and give no insight into the exocytotic process of single-vesicle events. We now describe initial results obtained using 2-photon microscopy to identify and follow single vesicle events in submandibular acinar cells.

Mice were humanely killed and the submandibular gland removed. The gland was then minced and incubated in collagenase (Worthington CLSPA) for 5–10 min at 37 °C. The tissue was then resuspended in extracellular solution (containing [mM] NaCl 135, KCL 5, $MgCl_2$ 1, $CaCl_2$ 2, Glucose 10, Hepes 10 −pH 7.4 NaOH) and gently triturated to produce a preparation of large clusters of acinar cells. The clusters were then placed on Poly-l-ornithine coated coverslips and Oregon Green (100 μM, Molecular Probes) added to the extracellular solution. The cell clusters were imaged using 2-photon microscopy (Leica) with light excitation at 890 nm and emitted lighted, longer than 505 nm, collected. Images were then processed using Metamorph software (Universal Imaging).

In unstimulated cells the fluorescent dye in the extracellular media labels the ductal system of the acinus but is excluded from cell interiors. We stimulated cells with 1 μM isoproterenol (b adrenergic agonist) and with a latency of 64.85 s (± 31.1 s mean ± S.E.M., $n = 6$ records) observed the appearance of small spots of fluorescence exclusively in the apical pole of individual acinar cells. These spots had a diameter of 1.04 μm (± 0.07 mean ± S.E.M., $n = 15$ events). The time-course, size and location of these spots are consistent with the entry of extracellular dye through the fusion pore of single secretory vesicles undergoing exocytosis. In parallel experiments we measured the intracellular calcium response of submandibular cells to stimulation with the same concentration of isoproterenol. Resting calcium was found to be 43 nM (± 4.6 mean ± S.E.M., $n = 6$ records), calcium measured 65 s after application of agonist (same time as latency to secretory response) was 45 nM (± 5.7 mean ± S.E.M.). Subsequent application of 500 nM Acetylcholine raised calcium to 390 nM (± 225 mean ± S.E.M.) demonstrating our ability to detect a calcium response.

Using our new assay we are able to visualize single-vesicle events in response to isoproterenol in submandibular acinar cells. Our evidence indicates that secretion is relatively slow and that there is no dependence on a rise in intracellular calcium. Future experiments will aim to describe the response more fully.

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All procedures accord with current UK legislation

C149

Distinct characteristics of exocytosis in mouse pancreatic acinar cells

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Exocytosis, the fusion of a vesicle with the plasma membrane is the principal way a cell can release lipophobic substances to the outside environment. It is probable that the basic machinery of exocytosis is similar across different cell types. But recent studies have shown the process of exocytosis may be differently regulated in different cells. Here we describe novel characteristics of the prolonged (many min) exocytotic events in exocrine cells of the mouse pancreas.

Mice were humanely killed and the pancreas gland removed. The gland was then incubated in collagenase (Worthingto, n CLSPA) for 5–10 min at 37 °C. The tissue was then resuspended in extracellular solution (containing [mM] NaCl 135, KCL 5, $MgCl_2$ 1, $CaCl_2$ 1, Hepes 10 − pH 7.4 NaOH) and gently triturated to produce a preparation of large clusters of acinar cells. The clusters were then placed on Poly-l-lysine coated coverslips. The cell clusters were imaged using a custom-built 2-photon microscope. Images were then processed using Metamorph software (Universal Imaging).

We imaged lobules and smaller fragments of mouse pancreatic tissue that retained the typical morphology of the intact exocrine glands. Inclusion of a fluorescent probe (Sulphorhodamine B or Oregon Green, Molecular Porbes) in the extracellular bathing medium labelled acinar ducts and the extracellular space between cells, but dyes were excluded from the cell interior. Uncaging of caged CCh with a flash of UV light rapidly evoked fluorescence spots in the apical, but not basal, regions of cells. In many cases we observed examples of compound exocytosis, where secondary vesicles fuse with a primary vesicle and become filled with dye passing from the duct via the primary vesicle. Using fluorescence recovery after photobleaching (FRAP) techniques we show that the fusion pore remains open for protracted periods of time (minutes) to allow free exchange between the aqueous vesicle lumen and the outside. Using lipophillic dyes we show no evidence for interchange of lipid between the plasma membrane and the vesicle membrane during the lifetime of the vesicle. We propose that the unusually long fusion pore lifetime is an adaptation permitting compound exocytosis, whereby the lingering ghost of an empty primary vesicle acts as a conduit through which the contents of a secondary vesicle can be released. The lack of lipid intermixing across the fusion pore may then facilitate the selective recycling of vesicle membrane and preservation of apical membrane integrity.

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All procedures accord with current national and local guidelines

C150

PKC α is downstream and PKC ε is upstream of mitoK$_{ATP}$ channel in the signal transduction pathway of preconditioning in the human myocardium

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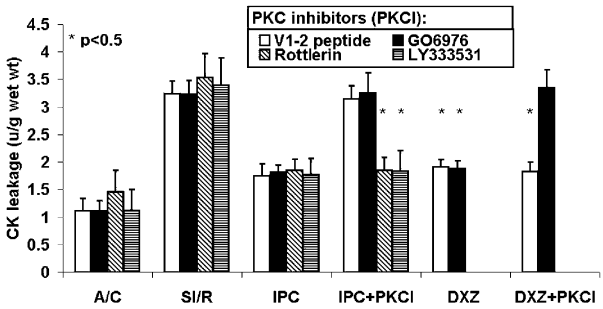
PKC isoforms play a major role in ischaemic preconditioning (IPC) of the human heart (Speechly-Dick *et al.* 1995). Using specific PKC isoforms inhibitors we aim to investigate which PKC isoforms are involved in IPC of the human myocardium and to determine their relation to mitoK$_{ATP}$ channels.

The study was approved by the local ethical committee and was conducted with the consent of patients. Right atrial muscles obtained from patients undergoing elective cardiac surgery were equilibrated according to our model (Zhang *et al.* 2000). Tissue sections were randomized to receive any of the following protocols ($n = 6$ per group): (1) aerobic control, (2) simulated ischemia for 90 min /reoxygenation for 120 min (SI/R), (3) IPC using five min SI followed by five min reoxygenation, (4) the addition of PKC isoforms inhibitors 10 min before and 10 min during IPC. PKC isoforms inhibitors investigated were V1–2 peptide [10 μM], GO6976 [100 nM], Rottlerin [100 μM] and LY333531 [100 nM] for PKC ε, PKC α, PKC δ and PKC β respectively.To investigate the relation of PKC isoforms to mitoK$_{ATP}$ channels, PKC inhibitors found to be involved in IPC were added 10 min before and 10 min during preconditioning by Diazoxide (DXZ)[100 μM] in a second experiment ($n = 6$ per group). CK leakage and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetiazolium bromide (MTT) cell viability were measured. All the values were expressed as mean ± S.E.M., $^*P < 0.05$ paired t test). Phosphorylation of PKC isoforms following activation of the sample by either DXZ or IPC was detected using western blotting and then analysed using Scion image software.



In the Figure, PKC ε and PKC α inhibitors blocked IPC whereas PKC δ and PKC β inhibitor did not. The protection elicited by mitoK$_{ATP}$ channels opening with DXZ was blocked by the inhibition by PKC α isoform but not by inhibition of PKC ε isoform. The MTT results were a mirror image of the CK results. In addition DXZ caused increased phosphorylation of PKC α similar to IPC but failed to cause significant increase of PKC ε phosphorylation.

PKC α and PKC ε are involved in IPC of the human myocardium with PKC ε upstream and PKC α downstream of mitoK$_{ATP}$ channels.

Speechly-Dick ME *et al.* (1995). *Circ Res* **77**, 1030–1035.

Zhang JG *et al.* (2000). *Clin Sci* **99**, 443–453.

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C151

Short heterodimer partner enhances glucose-stimulated insulin secretion in pancreatic β cells overexpressing uncoupling protein 2

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An acute insulin secretory response to glucose by pancreatic β cells is critical for preventing sustained post-prandial elevation of blood glucose and free fatty acids, which are detrimental factors in development of diabetes. Short heterodimer partner (SHP) is an orphan nuclear receptor of unknown function in diabetes (Lee *et al.* 1998). We investigated the potential effect of SHP on glucose responses in β cells (Shin *et al.* 2001) overexpressing mitochondrial uncoupling protein (UCP2) (Patane *et al.* 2002).

Islets of Langerhans were isolated from the pancreas of male Sprague-Dawley rats by collagenase digestion technique. Animals were Nembutal (1 mg/kg, I.P.) anaesthetized and then humanely killed by exsanguinations. Separate single β cells or islets were infected with adenoviruses expressing Ad-Null, Ad-SHP, Ad-UCP2, or Ad-UCP2+SHP. Virus-treated dishes were incubated for 2 h in a humidified incubator at 37°C in 5% CO_2 and balanced air. They were maintained for 48 h after infection for the experiments. The cell-attached configuration of the conventional patch-clamp technique was used. The extent of K_{ATP} channel activity was expressed as Po (open probability). The relative channel activity in the presence of glucose or methylpyruvate was described as Po/Poc, where Poc is the Po recorded just before drug administration. Insulin secretory capacity was measured using the batch incubation method and RIA. Microfluorescent imaging of $[Ca^{2+}]c$ was performed on β cells loaded with the calcium indicator dye Fura-2 acetoxymethyl (Fura-2/AM) ester. Statistical significance was evaluated using an unpaired Student's t test, and $P < 0.05$ was considered significant.

In the cell-attached mode, inhibition of K_{ATP} channel activity at 10 mmol/l glucose application was much greater in SHP-overexpressing (Ad-SHP) β cells than in Ad-Null control. Glucose-stimulated $[Ca^{2+}]c$ increase and insulin secretion were also greater in Ad-SHP cells. Impairment of glucose responses through K_{ATP} channel-dependent pathway in Ad-UCP2 cells was remarkably recovered by the concomitant overexpression of SHP (Ad-UCP2+SHP). The enhanced insulin secretion was not affected by the pretreatment of a PPAR-γ antagonist GW 9662. Methylpyruvate-stimulated K_{ATP} channel inhibition and insulin secretion were also enhanced in Ad-UCP2+SHP compared to Ad-UCP2, suggesting that SHP may act on downstream of glycolysis.

Our results show that up-regulation of SHP gene in β cells may increase acute insulin secretory response to glucose.

Lee HK *et al.* (1998). *J Biol Chem* **273**, 14398–14402.

Patane G *et al.* (2002). *Diabetes* **51**, 2749–2756.

Shih DQ *et al.* (2001). *Diabetes* **50**, 2472–2480.

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PC47

Hydrogen peroxide generation is required for store-mediated Ca^{2+} entry by the activation of $pp60^{src}$ in human platelets

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Store-mediated Ca^{2+} entry (SMCE), a mechanism modulated by the filling state of the Ca^{2+} stores, is an important mechanism for Ca^{2+} influx in non-excitable cells. Although the mechanism involved in the activation of SMCE is still not clearly described, the secretion-like coupling is the most likely model to explain SMCE in several cell types (Patterson *et al.* 1999; Redondo *et al.* 2003), including human platelets (Rosado *et al.* 2000b). This mechanism involves a physical and reversible interaction between the endoplasmic reticulum and the plasma membrane where the actin cytoskeleton and tyrosine kinases, such as $pp60^{src}$, play an important role (Rosado *et al.* 2000a; Redondo *et al.* 2003). Reactive oxygen species, including H_2O_2, have been recently presented as intracellular messengers required for a large number of cellular events, especially those mediated by tyrosine kinases. Hence, we have investigated the involvement of H_2O_2 generation in the activation of SMCE in human platelets.

Blood was drawn from volunteers with local ethical committee approval. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement and Western blotting were performed as previously described (Rosado *et al.* 2000a; Redondo *et al.* 2003). Treatment of human platelets with H_2O_2 resulted in a time-and concentration-dependent activation of $pp60^{src}$. Incubation for 30 min with 5 μM GF 109203X, a PKC inhibitor, prevented $pp60^{src}$ activation induced by 10 μM H_2O_2. In contrast, dimethyl-BAPTA loading did not affect this response, suggesting that activation of $pp60^{src}$ by H_2O_2 does not require rises in $[Ca^{2+}]_i$. Treatment of platelets for 40 min with 10 μM cytochalasin D, a inhibitor of actin polymerization, significantly reduced $pp60^{src}$ activation induced by 10 μM H_2O_2 by 85% ($n = 6$; $P < 0.01$, Student's t test). We found that platelet stimulation with 1 μM thapsigargin (TG) plus a low concentration of ionomycin (Iono; 50 nM) or with the physiological agonist thrombin (0.5 U/ml) induced rapid H_2O_2 production as detected by using CM-H2DCFDA. Treatment of human platelets for 5 min with 300 U/ml catalase, an enzyme that activates the decomposition of H_2O_2 into water and oxygen, abolished TG plus Iono-and thrombin-induced activation of $pp60^{src}$. In addition, catalase inhibited TG plus Iono-and thrombin-induced SMCE by 59.3 ± 4.5 and 37.4 ± 3.9%, respectively (S.E.M.; $n = 6$, $P < 0.01$, paired Student's t test), without having any effect on the maintenance of SMCE. Our findings suggest that platelet activation with TG plus Iono or thrombin is associated with H_2O_2 production, which acts as a second messenger involved in the activation of SMCE by $pp60^{src}$ activation in these cells.

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Redondo PC *et al.* (2003). *Biochem J* **370**, 255–263.

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

PC48

Magnesium signalling in rat parotid acinar cells: Effects of ACh and membrane transport inhibitors

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Magnesium (Mg^{2+}) is an abundant divalent cation in cells and it plays a vital role in the regulation of a number of biochemical and physiological processes including ion transport and digestive enzyme secretion (Yago *et al.* 2000). Moreover, a perturbation of extracellular Mg^{2+} ($[Mg^{2+}]_o$) has profound effects on secretagogue-evoked calcium (Ca^{2+}) mobilisation and on amylase secretion in the pancreas (Francis *et al.* 1990) and parotid salivary glands (Yago *et al.* 2002). This study investigated the effects of acetylcholine (ACh), several membrane transport inhibitors and substituting $[Na^+]_o$ with N-Methyl-D-glutamine (NMDG) on cytosolic Mg^{2+} concentration ($[Mg^{2+}]_i$) in magfura-2-loaded single rat parotid acinar cells using microspectrofluorimetric techniques to measure $[Mg^{2+}]_i$ (Mooren *et al.* 2001). The cells used came from humanely killed rats.

Basal $[Mg^{2+}]_i$ in zero mM, 1.1 mM, 5 mM and 10 mM $[Mg^{2+}]_o$ was (mean ± S.E.M.) 0.263 ± 0.0116; 0.304 ± 0.0101; 0.425 ± 0.0317 and 0.0495 ± 0.0161 ratio units (n = 25–30 cells taken from 10–15 animals). Stimulation of magfura-2-loaded parotid acinar cells with 10^{-5} M ACh resulted in a marked decrease in the $[Mg^{2+}]_i$. Typically, $[Mg^{2+}]_i$ decreased from its respective basal to (mean ± S.E.M.) 0.241 ± 0.047; 0.277 ± 0.009; 0.403 ± 0.03 and 0.453 ± 0.011 ratio units in zero mM, 1.1 mM, 5 mM and 10 mM $[Mg^{2+}]_o$, respectively. Superfusion of parotid acinar cells with different (zero mM, 1.1 mM, 5 mM and 10 mM) $[Mg^{2+}]_o$ resulted in a gradual increase in $[Mg^{2+}]_i$. In the continuous presence of 10 mM [$[Mg^{2+}]_o$ ACh evoked a rapid decrease in $[Mg^{2+}]_i$. Superfusion of parotid acinar cells with either 10^{-3} M lidocaine, NMDG instead of $[Na^+]_o$, 10^{-3} M amiloride, 10^{-3} M quinidine, 10–4 dinitrophenol or 10^{-3} M bumetanide resulted in a significant (Student's t test, ($P < 0.05$)) elevation in $[Mg^{2+}]_i$ compared to basal $[Mg^{2+}]_i$. Typically, $[Mg^{2+}]_i$ was increased from a basal of 0.304 ± 0.010 ratio units to 0.402 ± 0.022; 0.509 ± 0.004; 0.563 ± 0.033; 0.662 ± 0.015; 0.778 ± 0.073 and 0.931 ± 0.058 ratio units (n = 8–12) for lidocaine, amiloride, NMDG, quinidine, dinitrophenol and bumetanide, respectively. In the continuous presence of each of these membrane transport inhibitors, ACh (10^{-5} M) elicited a marked decrease in $[Mg^{2+}]_i$ compared to the respective control in the presence of each inhibitor. The decrease in $[Mg^{2+}]_i$ was much larger in the presence of bumetanide compared to the responses obtained in the presence of the other transport inhibitors or Na^+ substitution.

The results indicate that the ACh-evoked decrease in $[Mg^{2+}]_i$ in parotid acinar cells is insensitive to sodium removal and to a number of membrane transport inhibitors which themselves can increase $[Mg^{2+}]_i$.

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All procedures accord with current national and local guidelines

PC49

Low doses of endocrine disruptors imitate rapid actions of 17β-estradiol on Ca^{2+} signals in pancreatic α-cells within intact islets of Langerhans

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Blood glucose is maintained at the appropriate concentration by insulin released from β-cells and glucagon released from α-cells, both integrated within the pancreatic islet of Langerhans. The secretory processes in both types of cells are triggered by a rise in intracellular calcium concentration ($[Ca^{2+}]_i$). Endocrine disrupting chemicals (EDC) are hormone-like agents present in the environment that alter the endocrine system of wildlife and humans. Most EDC have potencies far below those of the natural hormone 17β-E2 when acting through the classic estrogen receptors (ERs).

Swiss albino OF1 male mice (8–10 weeks old) were humanly killed by cervical dislocation according to national guidelines. Pancreatic islets of Langerhans were isolated by collagenase digestion and loaded with 5 μM Fluo-3 AM for at least 1 h at room temperature. Calcium records in individual cells within the islet were obtained by imaging intracellular calcium under a Zeiss LSM 510 confocal microscope using a Zeiss × 40 oil immersion lens, numerical aperture 1.3. Experiments were performed at 34 °C. α and β-cells within the islets were identified by their $[Ca^{2+}]_i$ oscillatory pattern in 0.5 mM and 8 mM glucose, respectively (Nadal *et al.* 1999).

Using the technique described above, we have shown that the environmental estrogens Bisphenol-A, the synthetic oestrogen diethylstilbestrol (DES) and the native hormone 17β-E2 abolished $[Ca^{2+}]_i$ oscillations in glucagon releasing α-cells within intact islet of Langerhans. The pesticide o.p'-DDT which has a slightly different chemical structure presented a more reduced effect. This action was rapid in onset and it was reproduced by the membrane impermeable molecule E2 conjugated to horseradish peroxidase (E-HRP) and were not blocked by the antioestrogen ICI182, 780. Therefore, low-dose of EDC regulates $[Ca^{2+}]_i$ signals via an alternative pathway, initiated at the plasma membrane. Competition studies performed with E-HRP binding in single isolated α-cells supported the idea that the membrane binding site involved is nonclassical oestrogen membrane receptor (ncmER). This receptor has the characteristics of that previously described in pancreatic β-cells (Nadal *et al.* 2000; Ropero *et al.* 2002).

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All procedures accord with current national and local guidelines

PC50

Expression of a novel splicing variant deleting exon 4 of ATP1AL1, a nongastric proton pump, in human colorectum

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The K^+-dependent ATPase gene family is divided into three subgroups including Na^+,K^+-ATPase, gastric H^+,K^+-ATPase and non-gastric H^+,K^+-ATPases. ATP1AL1 is a human nongastric H^+,K^+-ATPase, and its gene has been reported to be expressed in brain, skin and kidney (Grishin *et al.* 1994). However, the identification of physiological roles of ATP1AL1 is still in its infancy. Recently, we found that ATP1AL1 mRNA was overexpressed in 12 out of 20 human colorectal carcinomas compared with the level in the accompanying normal mucosa (Takahashi *et al.* 2002).

Herein we have cloned a wild-type and a novel splicing valiant deleting exon 4 of ATP1AL1 from human colorectum. Then, the stable cell lines (HEK-293) expressing gastric H^+,K^+-ATPase β-subunit were transfected with the pcDNA4/His-ATP1AL1 cDNA (wild-type or the variant) construct. Exon4 encodes amino acids 77–144 of ATP1AL1, and this region includes the M1 transmembrane domain and the extracellular loop between M1 and M2. The specimens of colorectal adenocarcinomas and accompanying normal mucosa were obtained from surgical resection of patients in accordance with the recommendations of the Declaration of Helsinki. Informed consents were obtained from all patients at Toyama Medical and Pharmaceutical University Hospital. Data are shown as means ± S.E.M.

Messenger RNA of the splicing variant deleting the exon 4 of ATP1AL1 was expressed in both the colorectal adenocarcinomas and accompanying normal mucosa. The mRNA was also expressed in human kidney and brain. Protein expression of ATP1AL1 in the cell lines was monitored by using anti-Xpress antibody which reacts with the epitope at the N-terminus of the construct. Interestingly, the variant protein could be expressed in the membrane fraction of the cells, although the level of expression was (0.28 ± 0.05)-fold that of wild-type protein ($n = 3$) in the same experimental condition. The enzyme activity of ATP1AL1 in the transfecting cells was estimated by subtracting 5 μM ouabain-sensitive activity from 1 mM ouabain-sensitive activity. The enzyme activities of wild-type and the variant were 0.45 ± 0.09 and 0.04 ± 0.04 μmol Pi (mg protein)$^{-1}$ h^{-1}, respectively ($n = 3$). The variant seems to be ouabain-insensitive, because exon 4 contains the region which is one of the important major ouabain-binding sites of Na^+,K^+-ATPase.

These results suggest that the splicing variant of ATP1AL1 which is resistant to ouabain may be functional in the human colorectum, kidney and brain.

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Takahashi Y *et al.* (2002). *Jpn J Physiol* **52**, 317–321.

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

PC51

Leukotrienes-mediated effect of water extracts from *Sargassum horneri*, a marine brown alga, on Cl^- absorption in isolated rat colon

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Edible marine algae have been highlighted recently as multifunctional foods for maintaining human health. They are rich in minerals, vitamins and dietary fibers. *Sargassum horneri* is one of the edible brown marine algae and is distributed along the seacoast of Japan, and the slightly boiled alga is used as a savory food in Japan (Preeprame *et al.* 2001). Interestingly, water extracts of *Sargassum horneri* has a stimulatory effect on bone formation in rat *in vitro* (Uchiyama & Yamaguchi, 2002), and shows an antiviral activity against herpes simplex virus type I, human cytomegalovirus and immunodeficiency virus type I (Hoshino *et al.* 1998; Preeprame *et al.* 2001).

Herein we tested if the water extracts from *Sargassum horneri* affects K^+ and Cl^- transports in isolated rat colonic mucosa. Rats were sacrificed rapidly by stunning and cervical dislocation. Effects of the extracts on the short-circuit current (*I*sc), the potential difference across the mucosa (Pd) and the tissue conductance (Gt) were examined in isolated rat distal colon mounted on Ussing chamber. Data are shown as means ± S.E.M. Differences between groups were analysed by one-way ANOVA. Comparison between the two groups was made with paired *t* test.

Two kinds of water-soluble (ethanol-insoluble) extracts (EIS-1 and EIS-2) were prepared from the alga. The non-polysaccharide fraction (EIS-2; 100 μg ml^{-1}) significantly decreased *I*sc by 7.9 ± 1.6 μA cm^{-2}, and increased Gt by 0.8 ± 0.3 mS cm^{-2} ($n = 4$, $P < 0.05$). The half maximal effect of EIS-2 was obtained at 20 μg ml^{-1}. In contrast, polysaccharide fraction (EIS-1; 100 μg ml^{-1}) had little effects on *I*sc and Gt ($n = 3$, $P > 0.05$). The effect of EIS-2 depended on the presence of Cl^- and HCO_3^- but not K^+ in the bathing solution ($n = 4$–5). Increase in *I*sc by EIS-2 (100 μg ml^{-1}) was inhibited by 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2, 2-dimethyl-propanoic acid sodium (MK-886; 10 μM), a 5-lipoxygenase-activating protein inhibitor, and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; 100 μM), a Cl^- channel blocker ($n = 4$). EIS-2 attenuated the prostaglandin E_2 (0.5 μM)-increased *I*sc ($n = 5$, $P < 0.01$), and the half maximal effect of EIS-2 was obtained at 50 μg ml^{-1}.

These results suggest that the EIS-2 stimulates Cl^- absorption mediated by basolateral leukotrienes-sensitive Cl^- channels and apical Cl^- /HCO_3^- exchanger in the rat colonic mucosa.

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Preeprame S *et al.* (2001). *Chem Pharm Bull* **49**, 484–485.

Uchiyama S & Yamaguchi M (2002). *J Health Sci* **48**, 148–153.

All procedures accord with current national and local guidelines

PC52

The role of the cyclooxygenase signalling pathway in rapid responses to estrogen in MCF-7 cells

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An increased incidence of breast cancer has been observed following hormone replacement therapy. However the precise link between treatment with 17β-estradiol (E2) and an increased cancer risk is unclear. The rapid responses to E2 have been well documented and these responses involve the activation of cell signalling cascades that regulate ion transport and cell proliferation. The MCF-7 human breast carcinoma cell line is E2 responsive and can serve as a model for the effects of cancer. It has been shown that phospholipase A_2 (PLA_2) and cyclooxygenase (COX) enzymes are signalling intermediates involved in a rapid response to E2 in rat colonic epithelial cells (Doolan & Harvey, 2003). In this study we have used spectrofluorescence microscopy to investigate the effects of E2 on intracellular Ca^{2+} activity in the MCF-7 cells loaded with the Ca^{2+}-sensitive dye Fura-2.

A rapid, transient increase in intracellular calcium levels occurred in E2 (10 nM) treated MCF-7 cells. The calcium influx peaked 1 to 2 min after treatment and returned to basal levels within a further 5 min. Pre-treatment with quinacrine (5 mM) for 15 min prevents the E2 induced calcium response, suggesting that the E2 calcium response is PLA_2 dependent. A plasmid encoding GFP tagged $cPLA_2$ was transfected into MCF-7 cells and the effect of E2 on $cPLA_2$ localization was investigated by confocal microscopy. The GFP tagged $cPLA_2$ was diffusely located throughout the cytoplasm in untreated cells. Preliminary experiments have shown translocation from the cytoplasm to a perinuclear location following E2 treatment. Indomethacin, an inhibitor of COX enzymes partially blocks the E2 induced calcium response in MCF-7 cells. This implies a role for the conversion of arachidonic acid (AA) to prostaglandin E2 (PGE_2) in the regulation of the rapid calcium response.

The rapid response to E2 in MCF-7 cells appears to be linked to the PLA_2 /COX pathway. The rapid nature of the effects would imply that a change in expression levels does not occur and instead a change in the availability of substrate couples the COX pathway to the calcium events. The translocation of $cPLA_2$ from the cytoplasm to the ER and perinuclear membranes can be regulated through phosphorylation by protein kinases that are activated as a result of E2 treatment. E2 treatment and COX-2 activity have been implicated in breast cancer progression making their downstream signalling cascades important avenues for investigation.

Doolan CM & Harvey BJ (2003). *Mol Cell Endo* **199**, 87–103.

PC53

Effects of chronic hypoxia on Ca^{2+} homeostasis in primary cultures of human internal mammary artery and saphenous vein endothelial cells

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Ca^{2+} signalling is a crucial form of both inter-and intracellular signalling in the vascular endothelium. We have previously shown that such signalling is dramatically altered by periods of chronic hypoxia in other cell types (e.g. Smith *et al.* 2003). Here, we have investigated the effects of chronic hypoxia (2.5 % O_2, 24h) on agonist-evoked changes of $[Ca^{2+}]_i$ in human vascular endothelial cells. We also compare responses from arterial (internal mammary artery) and venous (saphenous vein) vessels. Cells were isolated and maintained in primary culture as previously described (Budd *et al.* 1991), and $[Ca^{2+}]_i$ monitored in Fura-2 loaded cells (see Smith *et al.* 2003, for details). Tissue was obtained with Local Ethical Permission and informed consent.

Bath application of a supramaximal concentration of ATP (10 μM) in a Ca^{2+}-free perfusate (containing 1 mM EGTA) evoked transient rises of $[Ca^{2+}]_i$ that were significantly larger ($P < 0.01$, unpaired Student's t test) in arterial cells (0.208 ± 0.008 ratio units (r.u.), mean ± S.E.M., $n = 54$ cells) than venous cells (0.172 ± 0.006 r.u., $n = 41$). Chronic hypoxia significantly potentiated peak responses in arterial cells (to 0.246 ± 0.009 r.u., $n = 54$, $P < 0.01$). However, responses in venous cells were unchanged at 0.187 ± 0.007 r.u. ($n = 35$).

Following 10 min exposure of cells to thapsigargin (1 μM), ATP failed to evoke rises of $[Ca^{2+}]_i$ when the perfusate was Ca^{2+}-free. Re-admission of 2.5 mM Ca^{2+} to the perfusate caused rises of $[Ca^{2+}]_i$ presumably due to capacitative Ca^{2+} entry (CCE). CCE was significantly greater ($P < 0.01$) in arterial cells (e.g. 0.210 ± 0.013 r.u. at 120 s after Ca^{2+} re-admission, $n = 46$) than in venous cells (0.124 ± 0.008 r.u., $n = 40$). Chronic hypoxia did not alter CCE in arterial cells, but significantly increased CCE ($P < 0.01$) in venous cells.

Our results indicate that ATP-evoked Ca^{2+} signalling appears quantitatively different in human arterial endothelial cells as compared with venous cells. Furthermore, evidence for remodelling of such signalling by chronic hypoxia was found in both cell types. These observations have important implications for perpetuation or genesis of hypoxia-inducible cardiovascular events.

Smith IF *et al.* (2003). *J Biol Chem* **278**, 4875–4881.

Budd JS *et al.* (1991). *B J Surgery* **78**, 878–882.

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

PC55

The spatial properties of calcium transients modulate contraction in rat atrial myocytes

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Calcium is a versatile intracellular messenger that encodes information in frequency, amplitude, and subcellular distribution. We examined how the ultrastructure of rat atrial myocytes constrains action potential-evoked calcium responses and their ability to promote cellular contraction.

Rats were humanely killed following CO_2 anaesthesia. Myocytes were stimulated with 40V pulses (2 ms duration) using two field electrodes (0.5 cm from the cell) at 1 Hz, and calcium changes were monitored using laser scanning confocal microscopy (NORAN Oz, Bicester, UK) of Fluo-4-loaded cells.

Under control conditions, only 10 % ($n = 120$ cells) of atrial cardiac myocytes examined showed homogenous global calcium transients following electrical stimulation. In the majority of cells

calcium transients originated in sub-sarcolemmal locations, and gave rise to a sharply-defined ring of elevated calcium. Despite functional ryanodine receptors being expressed at regular (~2 μm) intervals throughout the cells, the subsarcolemmal calcium signal did not spread in a regenerative manner to the cellular interior. Rather, there was only a diminishing inward diffusion of calcium. The averaged subsarcolemmal calcium response was 1656 ± 219 nM (mean ± S.E.M.) and took 38 ± 3 ms to reach peak (n = 5 cells), whilst the calcium rise in the central region of the atrial cells was typically 400 ± 63 nM and was maximal after 91 ± 7 ms (n = 5 cells). Immunostaining atrial myocytes revealed that L-type voltage-operated calcium channels were exclusively located around on the sarcolemma around the outside of the cells (n = 30 cells). Therefore, in atrial myocytes excitation-contraction coupling takes place in subsarcolemmal regions where L-type voltage-operated calcium channels and ryanodine receptors are co-localised. Treating atrial cells with cyclopiazonic acid (10 μM) or antimycin (20 μM) + oligomycin (20 μM), to inhibit sarcoplasmic reticulum calcium ATPases and mitochondrial calcium sequestration respectively, allowed the subsarcolemmal calcium signal to propagate regeneratively into the central regions of atrial cells (n = 30 cells). Calcium ATPases and mitochondria therefore form a functional firewall in atrial myocytes, which limits the calcium signals during normal excitation-contraction coupling to the subsarcolemmal sites. Positive inotropic agents such as endothelin-1 (100 nM) and the β-adrenergic agonist isoproterenol (100 nM) also induced globalisation of action potential-evoked calcium signals (n = 20 cells). The functional consequence of globalising calcium signals was a significant increase in the contractility of the cells. For example, with endothelin-1 the twitch amplitude increased by 585 ± 52% (n = 8). We therefore suggest that atrial myocytes have two functionally distinct populations of ryanodine receptors. The subsarcolemmal population is recruited during each action potential, but produces only a spatially limited calcium signal that triggers modest contraction. The central non-junctional ryanodine receptors represent an inotropic reserve, but are located behind a calcium ATPase and mitochondrial firewall. Physiological inotropes can activate this second population of ryanodine receptors to enhance the contractility of the cells.

All procedures accord with current UK legislation

PC56

pH sensitivity of Ca^{2+}-stimulable and Ca^{2+}-inhibitable adenylyl cyclases *in vivo*

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The activities of three adenylyl cyclases (AC1, AC3 and AC8) are stimulated by calcium in a calmodulin-dependent manner, whilst AC5 and AC6 exhibit calmodulin-independent Ca^{2+}-inhibition (Guillou *et al.* 1999). It is possible that physiological shifts in both pH and calcium have significant consequences for AC activity *in vivo*. We have examined whether Ca^{2+}-regulation of AC8 (Ca^{2+}-stimulable) and AC6 (Ca^{2+}-inhibitable) can be modulated by modest changes in pH.

Adenylyl cyclase activity was assayed *in vitro* using isolated cell membranes from HEK 293 cells transfected with AC8 and C6 glioma cells expressing endogenous AC6 as previously described (Boyajian *et al.* 1991). Calcium concentration-response curves for AC8 and AC6 activities were compared at pH 7.14, 7.50 and 7.85. At pH 7.14 basal activities decreased and there was a loss of Ca^{2+}-regulation of AC6 with a marked reduction in the Ca^{2+}-sensitivity of AC8. In contrast basal activities and Ca^{2+}-sensitivities of both cyclases were enhanced at least 2-fold at pH 7.85. To examine the effects of intracellular pH (pH_i) changes on whole-cell measurements of cAMP accumulation a weak acid (10 mM propionate) and weak base (20 mM trimethylamine) were used to induce pH_i shifts of ~0.3 pH units measured using the fluorescent dye BCECF. Both AC8 and AC6 are sensitive to capacitative Ca^{2+} entry (CCE) rather than store Ca^{2+} release in transfected HEK 293 cells (Fagan *et al.* 1996) and C6 glioma cells respectively (Fagan *et al.* 1998). Hence cells were pre-incubated for 3 min with 100 nM thapsigargin to empty the calcium stores and cAMP production was assayed for 1 min following addition of extracellular Ca^{2+} to evoke CCE. Whilst Ca^{2+}-stimulation of AC8 continued to display a similar (but weaker) dependence on pH, Ca^{2+}-inhibition of AC6 was insensitive to changes in pH_i. In contrast AC6 exhibited a clear decrease in Ca^{2+}-sensitivity when extracellular pH (pH_o) was reduced to pH 7.15 and potentiation of Ca^{2+}-sensitivity at pH_o 7.85.

Our findings suggest that Ca^{2+} dependent stimulation of AC8 and inhibition of AC6 is potentially sensitive to 'physiological' changes in pH. AC8 shows enhanced sensitivity to Ca^{2+} under alkaline conditions but this effect may be minimized due to reduced CCE in HEK 293 cells when pH_i is increased. Ca^{2+} regulation of AC6 is more sensitive to shifts in pH_o than in pH_i and this may be particularly relevant in the kidney where AC6 is the major isoform.

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Fagan KA *et al.* (1996). *J Biol Chem* **271**, 12438–12444.
Fagan KA *et al.* (1998). *J Biol Chem* **273**, 9297–9305.
Guillou TL *et al.* (1999). *J Biol Chem* **274**, 35539–35545.

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PC57

Determinants of adenylyl cyclase 5 localisation to cholesterol-rich buoyant membrane fractions

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Studies directed towards the plasma membrane have provided evidence for the existence of submicrometre cholesterol-rich domains, termed lipid rafts. These specialised domains of the plasma membrane are centrally involved in the containment of macromolecular signalling complexes and evidence suggests that Ca^{2+}-sensitive adenylyl cyclases are resident in rafts. This restrictive localisation is critical for the regulation of Ca^{2+}-stimulable AC8 (Smith *et al.* 2002) and Ca^{2+}-inhibitable AC6 by capacitative calcium entry (Fagan *et al.* 2000). Interestingly however, Ca^{2+}-insensitive adenylyl cyclases appear to be excluded from lipid rafts. The mechanisms governing the specific membrane targeting of adenylyl cyclase isoforms remains unknown. To address this question, a series of myc-tagged chimaeras were produced consisting of Ca^{2+}-inhibitable AC5 with major domains exchanged exchanged major domains substituted from Ca^{2+}-insensitive AC7. The AC5-AC7 chimaeras were expressed in HEK 293 cells and lipid rafts were isolated by a non-detergent based fractionation method. Confocal imaging

was used to investigate plasma membrane targeting. Demonstrating the separation of rafts from bulk membrane, Western blotting revealed an enrichment of the raft marker proteins caveolin and flotillin in the buoyant membrane fraction. Wild type AC5 was enriched in the buoyant membrane fraction, whereas wild-type AC7 was only detectable in the bulk membrane fraction. Confocal imaging demonstrated that both AC5 and AC7 were plasma membrane bound. Exchanging the N-terminus or the transmembrane domains of AC5 with similar regions from AC7 did not affect raft targeting or Ca^{2+}-inhibition *in vitro*. Exchanging the C1b domain of AC5 produced an ER retained construct and an intact C2b domain was essential for correct protein handling at the level of the ER for AC5 but not AC7. Therefore the C1b and C2b domains are essential for enabling restrictive localisation of AC5 to lipid raft microdomains.

Fagan *et al.* (2000). *J Biol Chem* **275**, 26530–7.
Smith *et al.* (2002). *J Biol Chem* **277**, 6025–31.

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PC58

Effects of nitric oxide (NO) on synaptic transmission in the nucleus tractus solitarii (NTS)

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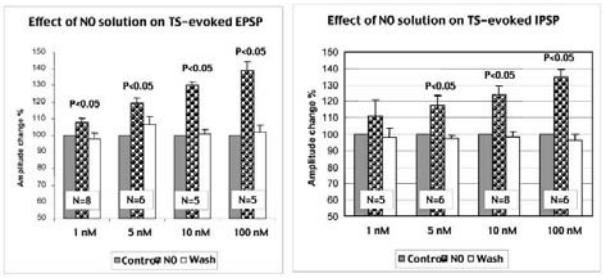
We reported previously that an NO-GABA pathway is involved in the depression of the cardiac baroreflex induced by ANGII within the NTS (Paton *et al.* 2001; Kasparov & Paton, 1999). However, the cellular mechanisms of NO action on inhibitory and excitatory synaptic transmission in NTS have not been elucidated. In this study we tested whether excitatory and inhibitory postsynaptic potentials (EPSP and IPSP, respectively) are differentially sensitive to NO.

Whole-cell patch clamp recordings were performed from NTS neurones in acutely prepared brainstem slices of humanely killed 14 day old rats as described in (Kasparov & Paton, 1999). IPSP and EPSP were evoked by electrical stimulation within solitari tract (TS). To assess the sensitivity of evoked IPSP and EPSP to NO, a wide range of concentrations of NO aqueous solutions or an NO donor (diethylamine NONOate -DEA/NO) were bath applied. All data are presented as mean ± S.E.M.



NO aqueous solution reversibly increased the amplitude of TS-evoked EPSP and IPSP in a concentration-dependent manner. The threshold concentration was 1 nM for the EPSP and for 5 nM for the IPSP (see graphs). 100 nM (DEA/NO) reversibly increased the amplitude of TS-evoked EPSP by 20 ± 4 %($n = 11$, $P < 0.05$ using paired t test) and of TS-evoked IPSP by 15 ± 3 %($n = 8$, $P < 0.05$). Integral areas of evoked EPSP and IPSP were similarly increased (41 ± 7 %, $n = 11$, $P < 0.05$; 37 ± 7 %, $n = 8$, $P < 0.05$ respectively). In contrast, lower concentrations of DEA/NO (50 and 10 nM) were without effect on evoked EPSP and IPSP. Both NO and DEA/NO could affect EPSP and IPSP without any measurable changes in input resistance or resting membrane potential.

In conclusion, our results suggest that NO potentiates both TS-evoked EPSP and IPSP in the NTS. Further experiments will clarify whether the potentiation of evoked IPSP results from a direct action of NO on GABAergic neurones or is secondary to the increased efficacy on excitatory transmission in this nucleus.

Kasparov S & Paton JFR (1999). *J Physiol* **521**, 227–238.
Paton JFR *et al.* (2001). *J Physiol* **531**, 445–458.

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All procedures accord with current UK legislation

PC59

Single channels in human osteoblast-like cells

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Osteoblasts possess both receptors and ion channels which are presumed targets for hormones, local mediators and mechanical influences. The functions of these receptors and ion channels must include regulation of cell proliferation and secretion, processes which are critical to bone remodelling. In bone tissue however such receptors and ion channels are relatively poorly characterised. electrophysiologically Whole cell measurements have shown the presence of NMDA receptors in primary rat osteoblasts (Gu *et al.* 2002) and K currents in MG63 human osteoblast-like cells or human osteogenic precursor cells (Yellowley *et al.* 1998; Weskamp *et al.* 2000). Few single channel measurements exist in these cells.

We have carried out single channel patch-clamp experiments in human MG63 osteoblast-like cells. RT-PCR showed that members of the TRP channel family (TRPM4b) and maxi-K channels were expressed in these cells. Cells with a fibroblastic appearance were selected for study. The bathing solution was mM: 150 NaCl; 3 KCl; 2 $MgCl_2$; 2 $CaCl_2$; 10 Hepes; 10 Glucose, pH 7.4. The recording pipette contained in mM: 140 KCl; 5 NaCl; 1 $MgCl_2$; 1 $CaCl_2$; EGTA 11; Hepes 10, pH 7.2. Giga-ohm seals (2–10 GΩ resistance) were formed in about 50 % of cases ($n = 155$ patches).

A number of single channel currents were observed in cell-attached recordings. A large conductance channel was present in almost every patch. The open probability (Po) was voltage-dependent increasing on depolarisation. There were up to seven active channels in a patch at positive potentials. The I-V relationship was linear up to140 mV depolarised (the mean conductance was 227 ± 41 pS ($n = 15$) thereafter drooping charactistically. The Po-voltage curve showed hysteresis, the activity of the channel being higher after depolarisation. The channel was activated by bath application of 10–4 M glutamate (Mg^{2+}-free Locke). This channel was also present in excised inside-out and outside-out patches (asymmetric K, $n = 14$), and was sensitive to both internal and external TEA (2 mM and 500 μM respectively). A proportion of patches also had another channel at resting potential and hyperpolarised voltages. This channel had a linear current-voltage relationship and a slope conductance of 54 pS ($n = 8$). There were up to six active channels in the patch. In a small percentage of patches ($n = 5$)

there was a large conductance (> 200 pS), high density, stretch-activated channel highly active at the resting potential.

Gu Y *et al.* (2002). *Calcif Tissue Int* **70**, 194–203.
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†I. Prajerová is an Erasmus Exchange Student.

PC60

Inositol 1,4,5-trisphosphate ($InsP_3$) and its receptor play a pivotal role in store operated calcium entry (SOCE)

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Depletion of calcium from intracellular stores results in the activation of calcium entry across the plasma membrane (SOCE). The mechanism by which the ER signals its state of filling to the plasma membrane however remains unclear. A direct conformational coupling between the $InsP_3R$ and plasma membrane influx channels as well as a diffusible messenger have both been hypothesised to be involved. In keeping with the conformational-coupling hypothesis, a role for both $InsP_3$ and the $InsP_3R$ has been reported. However, such studies are compromised by the poor pharmacology of the $InsP_3$ signalling cassette. Here, utilising molecular techniques to manipulate $InsP_3$ and $InsP_3R$ levels we show that both $InsP_3$ and $InsP_3Rs$ are required for maximal activation of SOCE.

Intracellular calcium levels were monitored in HEK293 cells using Fura-2. SOCE was activated by depletion of intracellular stores with thapsigargin (2 μM) in calcium-free medium. The peak calcium rise following re-addition of calcium to the extracellular medium was taken as the index of SOCE activation. Heterologous expression of proteins was achieved using standard transfection procedures. Data are presented as mean ± S.E.M. Statistical significance was calculated using unpaired Student's *t* test. Fluorescence of expressed GFP-tagged proteins or of a co-transfected plasmid was used to identify transfected cells allowing control and test cells to be simultaneously imaged.

Increasing phospholipase C (PLC) activity by agonist application (ATP 10 μM) or overexpression of the calcium sensitive PLCδ resulted in a significant enhancement of SOCE by 58.9 ± 5.2 %.($n = 103$, $P < 0.005$) and 22.7 ± 8.53 % ($n = 38$, $P < 0.05$) respectively. Inhibition of PLC by U73122 (10 μM) reduced SOCE by 78.8 ± 1.6 % ($n = 23$, $P < 0.005$). The modulation of SOCE by PLC was due to its regulation of $InsP_3$ production. Increasing $InsP_3$ levels by overexpression of phosphoinositol (PI) 4-OH kinase significantly increased SOCE by 24.0 ± 9.0 % ($n = 45$, $P < 0.02$). In addition, decreasing intracellular $InsP_3$ levels by overexpression of an $InsP_3$ sequestering 'sponge' or the $InsP_3$ metabolising 5'-phosphatase or a catalytically inactive PI4-OH kinase resulted in a significant decrease in SOCE (30.3 ± 6.3 % ($n = 18$, $P < 0.005$), 20.3 ± 5.0 % ($n = 25$, $P < 0.05$) and 28.8 ± 6.8 % ($n = 35$, $P < 0.005$) respectively of control values). To test whether the $InsP_3R$ was also involved, short interfering RNA (RNAi) was used to knockdown $InsP_3R$ expression. Stable transfection of cells with RNAi directed against the type 3 $InsP_3R$ resulted in an almost complete inhibition of its expression without affecting the expression of other calcium handling proteins SERCA and calreticulin. In these cells the magnitude of SOCE was significantly decreased by 41.2 ± 1.8 ($n = 88$, $P < 0.005$) compared to control cells. We therefore conclude that activated $InsP_3Rs$ are required for maximal activation of SOCE.

PC61

Rapid effects of steroid hormones on protein kinase A and Ca^{2+} activity in cultured epithelia from normal human bronchus and cystic fibrosis trachea

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Over the past decade, evidence has accumulated describing a rapid and non-genomic steroid hormone response (seconds-minutes) that involves novel membrane-associated receptors and fast activation of signal transduction pathways. One of the physiological roles of the non-genomic response is to increase electrolyte absorption and inhibit secretion in pluripotential epithelia. Steroid hormones such as glucocorticoids, mineralocorticoids and estradiol are involved in this anti-secretory effect. In upper airway epithelia, aldosterone and dexamethasone reduce intracellular calcium mobilization, whereas in the distal colon, estradiol inhibits basolateral K^+ channels to affect an anti-secretory response. All three steroid hormones activate the cAMP-dependent signal transduction pathway. Previous studies have demonstrated that aldosterone and estradiol down-regulate trans-epithelial chloride secretion and inhibit the action of secretagogues such as carbachol and forskolin via PKC, PKA, COX, Ca^{2+} and pH_i dependent signalling mechanisms in distal colon and lung. (Doolan *et al.* 2000).

The aim of this study was to compare the rapid effects of aldosterone, dexamethasone and estradiol on PKA and Ca^{2+} signalling response in lung epithelia.

The PepTag Assay™ for non-radioactive detection of cAMP-dependent protein kinase (PKA) was optimised to detect steroid-induced rapid up regulation of PKA activity in cultured human bronchial (16HBE14o-) and tracheal (CFTE-29o-) epithelia. Intracellular calcium concentration ($[Ca^{2+}]_i$) changes were measured in single cells using Fura-2 spectrofluorescence.

In CFTE cells, low concentrations of aldosterone (1 nM) and dexamethasone (10 nM) caused a rapid reduction of $[Ca^{2+}]_i$. We show for the first time that estradiol (1 nM) similarly caused a rapid reduction of $[Ca^{2+}]_i$ in CFTE cells. Rp-cAMP[S], the cAMP antagonist, inhibited the aldosterone, dexamethasone and estradiol effects on $[Ca^{2+}]_i$. Aldosterone caused decrease in basal Ca^{2+} as measured by a reduction in the F340/ F380 ratio of 0.44 ± 0.06, $n = 3$, mean ± S.E.M., $P < 0.001$. [Fishers *t* test]. Similar reductions in calcium were found for dexamethasone and estradiol. In both bronchial and tracheal epithelial cells PKA activity was up-regulated 3-fold over basal levels in less than 5 mins in response to low concentrations of aldosterone (1 nM) and glucocorticoids (10 nM). RP-cAMP[S] inhibited the dexamethasone activation of PKA in both epithelia.

In conclusion, we have shown rapid reduction in calcium and activation of PKA in cultured epithelia from normal human bronchial and CF tracheal airway. Intracellular calcium is a potent regulator of secretion in the lung and the anti-secretory effects of steroid hormones may be explained, in part, by a decreased calcium mobilization. Rapid non-genomic effects of estrogen on protein kinase and Ca^{2+} signalling in human lung epithelia may have relevance for the progression of cystic fibrosis lung disease, as it has been shown that female CF patients have a more severe lung disease progression than male patients (Stallings, 2003). It has been reported that during puberty, female CF patients have a marked exasperation of lung disease. (Fredriksen, 2001) which may be related to elevated estrogen levels.

Condliffe SB *et al.* (2001). *J Physiol* **530**, 47–54.
Doolan CM *et al.* (2000). *Br J Pharmacol* **129**, 1375–86.
Fredriksen PM (2001). *Heart* **85**, 259–9.
Stallings VA (2003). *J Pediatr* **142**, 15–9.

PC63

Role of poly(ADP-ribose) polymerase in TRPM2 channel-mediated oxidative stress-induced cell death

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TRPM2 is a non-selective cation channel that has been implicated in oxidative-stress mediated cell death pathways. This study aimed to address the role of poly(ADP-ribose) polymerase (PARP) in the activation of TRPM2 following exposure to hydrogen peroxide (H_2O_2).

Human embryonic kidney (HEK293) cells expressing tetracycline-inducible FLAG-tagged TRPM2 were seeded into black walled clear-based 96-well plates at 20–25, 000 cells per well and incubated at 37C / 5% CO_2 overnight. TRPM2 expression was induced by incubating cells overnight with 1 μg/ml tetracycline. Fluorimetric imaging of Fluo-3 AM loaded cells was used to determine intracellular free Ca^{2+} ion concentration ($[Ca^{2+}]_i$), changes. Cell death was assessed using a 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.

Tetracycline-induced cells expressing TRPM2 exhibited a large increase in fluo-3 fluorescence after treatment with H_2O_2 (100 μM – 1 mM) indicative of a rise in $[Ca^{2+}]_i$. This response was 10-fold greater than the corresponding fluorescence increase in non-induced cells. The calcium increase was paralleled by an increase in H_2O_2 induced, TRPM2-mediated cell death at 300 μM H_2O_2. The rises in both $[Ca^{2+}]_i$ and cell death in response to H_2O_2 treatment were inhibited by the PARP inhibitors PJ34, SB750139-B and DPQ at concentrations known to inhibit purified PARP enzyme. Complete inhibitiion of the effects of H_2O_2 were achieved by PARP inhibitor concentrations of 10μM.$_p$ EC_{50}s of 7.032 ± 0.256 (93 nM), and 7.800 ± 0.478 (16 nM) were obtained for PJ34 and SB750139-B, respectively in experiments monitoring $[Ca^{2+}]_i$. PJ34, SB750139-B, and DPQ exhibited $_p$ EC_{50} values of 6.685 ± 0.217 (206 nM), 7.318 ± 0.227 (48 nM), and 5.446 ± 0.081 (3600 nM), respectively in MTT cell death studies. Values are expressed as mean ± standard error, n = 3–5 experiments in triplicate.

These data show that HEK293 cells demonstrate an increased sensitivity to oxidative-stress following induced expression of TRPM2. Furthermore, both oxidative stress-mediated $[Ca^{+2}]_i$ increases and cell death were reduced by pretreatment of cells with PARP inhibitors. We hypothesise that PARP activation is required for oxidative stress-mediated opening of TRPM2 channels. PARP activation has been reported to play a role in a number of pathophysiological conditions. Therefore TRPM2 may be a novel therapeutic target for conditions linked to oxidative stress-mediated cell death.

E. Fonfria and K. Hill are in receipt of EU Framework V Postdoctoral Fellowship.

PC64

Rapid effects of aldosterone on human ocular ciliary body non-pigmented epithelium

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Aqueous humor is a clear fluid that circulates around the anterior segment of the eye. It is secreted by the pigmented and non-pigmented epithelial (NPE) cells of the ciliary body; these cells produce it actively and it is also passively filtered from plasma. The basal aspect of the NPE cells possesses ion channels, pumps and exchangers that are regulated by signalling pathways involving protein kinases, calcium ions (Ca^{2+}) and intracellular pH (pHi). In other secretory epithelia steroid hormones, including aldosterone, have a rapid non-genomic effect on the activity and expression of components of the signalling cascades resulting in changes in the secretory or absorptive capacity of the cell. Some ion transporters, for example the sodium-proton exchanger, are directly influenced by intracellular calcium concentrations, which in turn are dependant upon activity of protein kinases.AIM: The aim of this study was to identify rapid signalling responses to aldosterone in human NPE cells, in particular, changes in protein kinase activity and intracellular Ca^{2+} and pH_i.

Protein kinase C α (PKCα) expression was examined in cultured human NPE cells using Western Blotting techniques. A non-radioactive fluorescent assay (Promega) was used to identify Protein Kinase A (PKA) expression. Intracellular calcium and pH changes were monitored by spectrofluorescence imaging of cells loaded with the fluorescent dyes Fura2 and BCECF, respectively.

PKA expression was significantly increased after one min of incubation with physiological concentrations of aldosterone (1 nM). This increase was maximal between one and five min with expression levels of PKA returning to basal levels at ten min. Aldosterone (10 nM) also caused a rapid, transient change in PKCα expression; an increase in PKCα occurred after five min of incubation. This increase had diminished after ten min and was not evident after twenty min. Preliminary results indicate that there is an increase in intracellular Ca^{2+} and pH_i induced within min of treatment with aldosterone (1 nM).

These data demonstrate for the first time rapid (non-genomic) responses to aldosterone in human ocular ciliary body epithelium. The signalling pathways affected, PKC, intracellular Ca^{2+} and pH_i are known regulators of epithelial electrolyte secretion and may have important implications in rapid regulation of aqueous humor production by a local Renin Angiotensin Aldosterone System in the human eye (Cullinane *et al.* 2002).

Cullinane AB *et al.* (2002). *Br J Ophthalmol* **86** , 676–83.

PC65

Responses of the human spermatozoon to an increasing gradient of progesterone: simulation of approach to the egg.

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Progesterone (produced by cumulus cells around the oocyte) evokes a biphasic Ca^{2+} influx in human spermatozoa and provides communication between the sperm and the oocyte. It is well established that stepped additions of progesterone, from zero dose to μM concentrations, to human spermatozoa evoke a transient Ca^{2+} influx followed by a sustained elevation (both of which are dependent upon extracellular Ca^{2+}), and acrosome reaction. It is most unlikely that this stimulus profile occurs *in vivo* and therefore we have re-examined the role of progesterone in human gamete interaction by exposing spermatozoa to a slowly increasing progesterone gradient (3 nM–3 μM over 30 min), simulating the stimulus experienced by the sperm on approaching the oocyte.

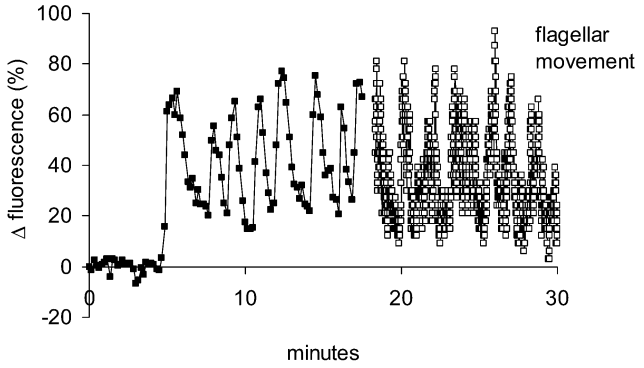

Figure 1. Cell showing $[Ca^{2+}]_i$ oscillations in response to stepped addition of 3 μM progesterone (filled squares). Subsequent assessment of flagellar motility shows 'bursts' of increased motility with a temporal pattern that matches oscillations in $[Ca^{2+}]_i$ (open squares). Motility is measured in arbitrary units.

Spermatozoa were harvested by direct swim-up into sEBSS media with 0.3% BSA and incubated for 6 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 (25°C). Upon application of progesterone nearly all cells generated a slow, steady increase in $[Ca^{2+}]_i$ at the rear of the head. Latency of this response varied between cells, but 93.6 ± 1.6% (S.E.M. 5 experiments;667 cells) of spermatozoa showed a significant response ($P < 0.05$; paired t) within 5 min (at nM concentrations of progesterone). Approximately 35% of cells, after initiation of the gradient, show slow $[Ca^{2+}]_i$ oscillations with a duration of 0.5–1 min and period 1.5–3 min. These cells show bursts of increased flagellar beating, during the oscillation peak (figure). Progesterone-induced oscillations are insensitive to thapsigargin (100 nM-5 μM) and modulators of the IP_3 signalling pathway (neomycin [100 nM-20 μM], U73122 [10 μM]), but are arrested by the intracellular Ca^{2+} channel antagonist TMB-8 (300–500 μM).We propose that the effect of progesterone on $[Ca^{2+}]_i$ in human spermatozoa *in vivo* may be markedly different to the well-characterised biphasic effect and that a significant aspect of this may be a periodic induction of increased motility controlled through store-derived oscillations in $[Ca^{2+}]_i$.

The research was carried out according to local ethical guidelines with the Birmingham Women's Hospital (HFEA no. 0119). Donors gave informed consent. This work was supported by BBSRC and The Wellcome Trust.

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

PC66

5-HT induces a calcium influx in smooth muscle cells from rat small intrapulmonary artery in normoxia and chronic hypoxia

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In normoxic conditions, 5-HT induces contraction and proliferation in pulmonary artery (MacLean *et al.* 2000). Plasmatic concentration of 5-HT is increased in patients with primary pulmonary hypertension (Kéreveur *et al.* 2000). Since intracellular calcium is strongly involved in contraction, we investigated the effect of 5-HT on intracellular calcium concentration ($[Ca^{2+}]_i$) in primary cultured smooth muscle cells from rat small intrapulmonary artery (IPA) in normoxia and chronic hypoxia.

Wistar male rats were either housed in room air at a normal atmospheric pressure (101 kPa) or kept in a hypobaric chamber for 3 weeks at a pressure of 50.5 kPa. Rats were then humanely killed according to national guidelines. IPA were isolated from the left lung by dissection. Smooth muscle cells (SMC) were enzymatically isolated and primary cultured (48–72 hours) before they were loaded with the calcium-sensitive fluorescent dye fura-PE3 (2 μM). All results are expressed as the mean ± S.E.M.; significance was tested with unpaired Student's *t* test with $P < 0.05$ and *n* represents the number of cells.

Calcium signal to 5-HT 10 μM was characterized by a transient followed by a sustained phase. The amplitude of these both phases were decreased in chronic hypoxia (change in 345/380 ratio, $\Delta R = 0.08 \pm 0.01$ *vs.* 0.2 ± 0.02 in control for the transient phase, $\Delta R = 0.02 \pm 0.003$ *vs.* 0.06 ± 0.01 in control for the sustained phase, $n = 69$ and 173 respectively). An antagonist of the 5-HT_{2A} receptor, ritanserin 0.1 μM blocked the calcium signal to 5-HT 10 μM in normoxia ($n = 22$) and R-DOI 10 μM, a 5-HT_{2A} agonist increased $[Ca^{2+}]_i$ in the same manner than 5-HT ($n = 38$). In chronic hypoxia, the calcium signal to 5-HT was also blocked by ritanserin. Calcium sources involved in the response to 5-HT were then investigated. In normoxia, the calcium response to 5-HT was not modified by thapsigargin 1 μM, which depletes intracellular calcium store ($n = 28$). The sustained phase was suppressed by bath perfusion with a calcium free solution ($n = 9$) or RHC80267 50 μM ($n = 31$), an inhibitor of the diacylglycerol lipase which thus inhibits the arachidonic acid production. In chronic hypoxia, both phases of the calcium signal to 5-HT were fully blocked by a calcium free solution ($n = 42$). Immunolabelling of the SMC with antibodies against proteins coding for non-voltage gated calcium permeable channels (TRPC or transient receptor potential channels) demonstrated the presence of TRPC1, 3, 4 and 6 and TRPC 3, 4, 5 and 6 in normoxic and hypoxic rats respectively. There was no labelling for TRPC1 in hypoxic rats.

In conclusion, 5-HT activates 5-HT_{2A} receptors and induces a calcium signal which is independent on intracellular calcium stores but strongly depends on extracellular calcium in normoxic and hypoxic rats. TRPCs appeared to be present and may be

responsible for the calcium influxes.

Kereveur A *et al.* (2000). *Arterioscler Thromb Vasc Biol* **20**, 2233–2239.
MacLean MR *et al.* (2000). *Br J Pharmacol* **131**, 161–168.

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All procedures accord with current national and local guidelines

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PC68

Intracellular localisation and functional properties of human TRPC7

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The seven mammalian TRPC isoforms have been separated into two groups (TRPC1, 4, 5 and TRPC3, 6, 7 (Trebak *et al.* 2003)) based on sequence relationships, their functional properties when over-expressed in transfected cells and their ability to assemble into heteromeric channels. Among these seven isoforms, TRPC7 is probably one of the least studied. We cloned it, expressed it transiently in the HEK-293T cell line and studied its localisation and its regulation.

The full-length cDNA sequence for htrpc7 was subcloned into the mammalian expression vector pcDNA3.1(-) (Invitrogen) and HEK 293T cells were transfected with the pcDNA3.1(-)-hTRPC7 construct using Fugene^{TM} (Roche). An epitope-tagged version of the hTRPC7 cDNA was used to transfect HEK-293T cells and the distribution of the channel examined using an anti-FLAG antibody and immunofluorescence microscopy. As with other members of the TRPC family, some fluorescence was detected at the plasma membrane, but the majority was present in an intracellular compartment.

For the functional experiments, we used the pIRES2-EGFP (Clontech, Basingstoke, UK) vector to express the full-length htrpc7 cDNA thus allowing a clear localisation of transfected cells. Ca^{2+} concentrations of individual HEK-293T cells were monitored with Fura 2 AM. Data are expressed as means ± S.E.M. Ca^{2+} elevation was assessed by measuring the area under the Ca^{2+} transient and was expressed as sum of (340/380) ratio. When a calcium re-addition protocol was used with ATP (100μM) as an agonist, we found that there was a significant increase in Ca^{2+} entry in pIRES2-htrp7-transfected cells compared to pIRES2-EGFP transfected cells (Calcium elevation = 3.44 ± 0.54 in control cells, $n = 39$, 3 transfections, compared to 12.85 ± 1.19 in htrp7-transfected cells, $n = 34$ cells, 3 transfections, $P < 0.05$ unpaired Student's t test). However, when CPA (10 μM) was used to empty the Ca^{2+} stores, there was no significant increase in calcium entry (Calcium entry = 31.92 ± 1.68 in control cells, $n = 115$ cells, 3 transfections compared to 34.70 ± 1.10 in pIRES2-EGFP-htrp7 transfected cells, $n = 125$ cells, 4 transfections. We therefore conclude that hTRPC7 is not activated by store depletion but by PLC activation.

Trebak M *et al.*(2003). *Cell Calcium* **33**, 451–461.

PC69

Bcl-2 functionally interacts with the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) to inhibit calcium release

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Central to the apoptotic cell death pathway are the Bcl-2 family members. These proteins are distributed on ER and mitochondrial membranes, where they have been reported to function in regulating calcium homeostasis. The effect of Bcl-2 overexpression on cellular calcium homeostasis however, remains controversial. To resolve this controversy we determined whether the overexpression paradigm (i.e. transient *versus*stable) was responsible. To this end, Bcl-2 fused to GFP was initially overexpressed in a transient or stable manner in HEK293 cells and its effects on cellular architecture and apoptosis determined. Data are presented as means ± S.E.M. with n indicating the number of experiments. Statistical significance was determined by a one tailed Student's t test.

Using confocal microscopy of multiple fields of view, we observed that transient over-expression of Bcl-2 significantly disrupted organelle structure, causing the mitochondria to fragment and collapse around the nucleus (93 ± 3.4 of all cells, $n = 3$, $P < 0.05$). Apoptosis was determined by quantitating sub-G1 DNA content of propidium iodide-labelled cell populations by FACS. Transiently transfected cells were sensitised to staurosporine-induced apoptosis (4 μM for 4 h). Furthermore, transient expression of Bcl-2 in the absence of staurosporine treatment induced apoptosis. In contrast, stable expression of Bcl-2 did not perturb the structure and distribution of the mitochondria and ER to the same degree as transient overexpression (33 ± 3.5 %, $n = 3$ $P < 0.05$). In addition, only 6 % of cells stably overexpressing Bcl-2 ($n = 2$) became apoptotic following staurosporine treatment whereas 25 % of non-transfected cells did ($n = 3$). Since stable transfection is the optimal paradigm for increasing Bcl-2 expression, we investigated its role in calcium homeostasis in the WEHI7.2 T-lymphocyte cell-line which is a well-characterised cellular model for apoptosis. Cytosolic and ER lumenal calcium was monitored by single-cell imaging of Fura2-AM and digitonin permeabilised Fura2FF loaded cells respectively. $InsP_3$ induced calcium release was stimulated by application of antibody to the CD3 component of the T-cell receptor (1:40 dilution) or a cell permeant $InsP_3$ ester (25 μM). The cytosolic calcium transient induced by either CD3 ligation or $InsP_3$-ester application was decreased from 97 ± 13 nM and 350 ± 20 nM in control cells to 38 ± 10 nM and 140 ± 20 nM ($n = 3$, $P < 0.005$) in Bcl-2 overexpressing cells respectively. No decrease in ER lumenal calcium content (1, 010 ± 300 nM in controls *versus*1, 000 ± 250 nM in Bcl-2 expressing cells, $n = 3$, $P = 0.815$) or $InsP_3R$ abundance ($n = 3$) was observed. By co-immunoprecipitation we detected an interaction between Bcl-2 and the $InsP_3R$ ($n = 3$). In addition, we found that Bcl-2 overexpression significantly decreased the affinity of the $InsP_3R$ for its ligand to 7.5 ± 0.2 nM from 4.8 ± 0.2 nM in control cells ($n = 3$, $P < 0.001$).

Our findings suggest that when stably expressed Bcl-2 protects cells form apoptosis by regulating $InsP_3$ induced calcium release. In addition, transient overexpression of Bcl-2, which severely disrupts organelle structure, may have abnormal consequences for calcium signalling.

PC70

Failure of endothelin receptor type A and angiotensin II type I receptor antagonists to regress hypertrophy in rat atrial myocytes in an experimental model of heart failure

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Regression of cardiac ventricular hypertrophy due to inhibition of various signalling pathways has been previously demonstrated. However, little information is available on the effect of such interventions on atrial hypertrophy. To investigate this further, the effect of endothelin receptor type A (ET-A) blockade and angiotensin II type I receptor (AT-1) blockade on ventricular and atrial cells was studied using an experimental model of heart failure due to chronic volume overload.

Chronic heart failure due to aorto-caval shunt was induced in Wistar rats (200–250 g) anaesthetised with chloral hydrate at a dose of 400 mg kg^{-1} body weight. Rats were randomised to receive either; vehicle (Veh), an ET-A antagonist (darusentan, 50 mg kg^{-1} body weight), an AT-1 antagonist (candesartan, 1 mg kg^{-1} body weight) or a combination of both ET-A and AT-1 antagonists (COM) at the same doses. Treatment by gavage was started one day after induction of aorto-caval shunt and was continued for 30 days. Sham-operated animals served as a control group.Rat atrial myocytes were isolated from all animals using a previously published method (Freestone *et al.* 2000). Individual atrial myocytes were viewed on a monitor via a camera linked to a microscope and cell dimensions recorded from randomly chosen cells. Cell dimensions are presented as mean measurement ± S.E.M.

Animals undergoing the aorto-caval shunt procedure (Veh, 22 cells from 7 animals) exhibited significantly longer (119 ± 5.0 microns *versus*89 ± 1.7 microns, $P < 0.0001$, Student's unpaired *t* test) and wider cells (17.8 ± 0.7 microns *versus*13 ± 0.3 microns, $P < 0.0001$) than sham-operated controls (18 cells from 6 animals). Despite a significant regression of hypertrophy in isolated ventricular myocytes from all treatment groups (data not shown) treatment with ET-A and AT-1 antagonists and both drugs together (COM) did not reduce either of these cell dimensions in the atrial myocytes from rats with aorto-caval shunt.Cell dimensions with the treatments were as follows, ET-A, cell length 125 ± 5.4 microns, cell width 17.2 ± 0.5 microns (20 cells from 6 animals) AT-1, cell length 128 ± 3.0 microns, cell width 17.8 ± 0.5 microns (20 cells from 7 animals) and COM, cell length 119 ± 3.5 microns, cell width 17.6 ± 0.4 microns (19 cells from 6 animals).

This data implies that the anti-hypertrophic signalling pathways in atrial myocytes are different from those in ventricular myocytes even given that both types of cells may express the same receptors. This is worthy of further investigation.All animals used in this study were sacrificed according toEU legislation.

Freestone NS *et al.* (2000). *Pflugers Archiv* **441**, 78–87.

All procedures accord with current national and local guidelines