

Quantifying the syncytialisation of a human placental trophoblast cell line grown *in vitro*

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We have generated 'green' and 'red' BeWo cell lines: the green one constitutively expresses the green fluorescent protein (GFP) attached to histone H2B (Kimura & Cook, 2001), and the red one the red fluorescent protein tagged to the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase (Mit-DsRed; Bevis & Glick, 2002). Our quantitative assay for cell fusion (syncytialisation) utilizes the fluorescence-activated cell sorter (FACS) to detect 'cells' that are both green and red.

Exponentially growing BeWo cells (gift from S. Greenwood) were transfected with vectors encoding either H2B-GFP or Mit-DsRed, and stable autofluorescent clones selected. Fluorescence microscopy revealed that one line had green fluorescent nuclei, the other red fluorescent mitochondria. The fluorescent proteins had no effect on the rates of DNA, RNA or total protein synthesis (monitored using the rate of incorporation of radiolabelled thymidine, uridine or leucine into acid-insoluble material) or on the secretion of the peptide hCG (measured by immunoassay).

When these two cell lines were mixed and cultured in forskolin, cells with green nuclei and red mitochondria appeared. Such green and red (fused) cells were readily detected amongst green or red (unfused) cells by FACS. Using this novel assay, growth in 100 μ M forskolin for 48 h increased BeWo syncytialisation approximately 5-fold over cells incubated in the absence of forskolin.

We will use this specific and rapid non-destructive assay to quantify the rate of syncytialisation and to study factors affecting the process.

Bevis, B.J. & Glick, B.S. (2002). *Nature Biotechnol.* **20**, 83–87.

Kimura, H. & Cook, P.R. (2001). *J. Cell Biol.* **153**, 1341–1353.

Insulin modulates mitochondrial membrane potential in adult rat sensory neurones

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Growth factors such as insulin, the insulin-like growth factors (IGFs) and the neurotrophins have extensive effects on the physiology of neurones. Insulin, IGF-1, IGF-II and nerve growth factor (NGF) regulate the survival, differentiation and axonal outgrowth of embryonic and adult neurones. Such effects on neuronal phenotype are mediated via global and co-ordinated alterations in gene expression. For such profound changes in gene expression to occur the cellular protein synthetic machinery must be optimised and an efficient use and supply of energy resources is a prerequisite. The mitochondrion is the main source of ATP within the cell and, therefore, alterations in neuronal phenotype will depend on the functioning of this organelle.

This study tested the hypothesis that mitochondrial function is directly modulated by growth factors, such as insulin. Real time

video microscopy of rhodamine 123 fluorescence was used to monitor mitochondrial inner membrane potential in cultured adult dorsal root ganglia (DRG) neurones. All animals were killed according to UK legislation. Acute insulin treatment (0.75 nM for 20 min) had no effect; however, 6 h and 3 day treatment significantly increased the mitochondrial membrane potential (as assessed by the normalized amplitude of CCCP-induced increase of rhodamine 123 fluorescence) of DRG neurones (control, 0.38 ± 0.05 ; 6 h insulin, 0.61 ± 0.12 ; 3 day insulin, 1.4 ± 0.19 ; mean \pm S.E.M., $P < 0.05$ vs. control, one-way ANOVA, arbitrary units). The insulin effect was maximal at a dose of 0.75 nM, higher doses of 7.5 and 75 nM had no additional effect, suggesting that signalling via the IGF type I receptor was not involved. The availability of glucose (10–50 mM) was varied but had no effect, suggesting that changes in metabolic status had no impact on mitochondrial membrane potential. The redox state of the NADH/NADPH pool was also analysed using real-time fluorescence and insulin had no effect, implying that insulin was not directly modulating electron transport within the mitochondrion.

The signal transduction pathways downstream from the insulin receptor were investigated. Inhibition of phosphoinositide 3-kinase (PI 3-kinase) by 0.001–0.01 mM LY294002 prevented the insulin effect on mitochondrial membrane potential. However, blockage of mitogen-activated protein kinase (MAPK) using the MEK inhibitor, U0126, had no effect. Western blots showed that insulin activated the protein kinase, PKB or Akt, and the transcription factor, CREB (both downstream of PI 3-kinase), but had no effect on MAPK or the related kinases – c-jun N-terminal kinase and p38 kinase.

The results show for the first time that insulin can directly modulate the membrane potential of neuronal mitochondria. Studies are now focused on the molecular mechanism of this process. Of particular interest are targets downstream from Akt, such as the bcl-2 family of proteins that are known to regulate mitochondrial properties.

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All procedures accord with current UK legislation.

Aminoglycosides induce PI₃-kinase-mediated Akt/PKB activation in proximal tubule OK cells

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The extracellular Ca²⁺-sensing receptor (CaR) has been associated with alterations in cell fate, such as protection from apoptosis in ovarian cells and proliferation in fibroblasts and osteoblasts. We have recently reported that the proximal tubular opossum kidney (OK) cell line expresses CaR and responds to CaR agonists (extracellular Ca_o²⁺ and aminoglycoside antibiotics e.g. neomycin) with increased Ca_i²⁺ mobilisation and activation of the extracellular signal-regulated kinase, ERK (Ward *et al.* 2002).

To investigate the involvement of CaR in cell-fate signalling in renal cells we studied the effects of neomycin on the phosphorylation status of the PI₃-kinase-dependent, anti-apoptotic kinase, Akt (also known as protein kinase B). Akt is activated upon the dual phosphorylation of residues Thr-308 and

Ser-473, a mechanism that was studied by semi-quantitative immunoblotting using phospho-specific polyclonal antibodies to these two residues (pAkt T308 and pAkt S473).

Treatment of OK cells (ATCC, Rockville, MD, USA) at 37°C for 5 min with either neomycin or gentamicin (300 µM) increased the phosphorylation of pAkt S473 (430 ± 117 and $486 \pm 109\%$, respectively, means \pm s.e.m.). Neomycin-induced pAkt S473 phosphorylation was inhibited by the PI₃-kinase inhibitors wortmannin (30 nM; Neo, $345 \pm 109\%$ control; wortmannin and Neo, $13 \pm 2\%$; $N = 3$; $P < 0.001$ by Student's unpaired *t* test) and LY294002 (30 µM; Neo, $510 \pm 360\%$ control; LY294002 and Neo, $17 \pm 8\%$; $N = 3$; $P < 0.001$) and by the PLC inhibitor U73122 (5 µM; Neo, $259 \pm 72\%$ control; U73122 and Neo, $6 \pm 3\%$; $N = 3$; $P < 0.001$). However, the neomycin responses were unaffected by the PKC inhibitor GF109203X (500 nM; Neo, $244 \pm 79\%$ control; GF109203X and Neo, $277 \pm 98\%$; $N = 3$; N.S.) or by the MEK (ERK kinase) activation blocker PD98059 (10 µM; Neo, $177 \pm 14\%$ control; PD98059 and Neo, $162 \pm 9\%$; $N = 3$; N.S.). Qualitatively similar observations were made in all of the above experiments using the antibody to pAkt T308.

The current data indicate that in CaR-expressing OK cells, aminoglycoside treatment causes a PLC-dependent and PI₃-kinase-dependent stimulation of Akt/PKB that does not involve either PKC or the MEK/ERK pathway.

Ward, D.T. *et al.* (2002). *J. Am. Soc. Nephrol.* **13**, 1481–1489.

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Functional coupling of FcγRI to oxidative burst and calcium is mediated through ARNO, ARF6 and PLD1

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Immunoglobulin G receptors (FcγRs) on myeloid cells are responsible for the internalisation and degradation of immune complexes. In interferon-γ primed U937 cells, FcγRI is coupled to a novel pathway that involves the sequential activation of PI3 kinases, phospholipase D (PLD) and sphingosine kinase. This study investigated the factors that couple FcγRI to PLD and the role of PLD in the FcγRI activation of NADPH oxidase.

FcγRI was aggregated by immune complexes and PLD activity was measured. Pretreatment of cells with brefeldin A had no effect on PLD activation suggesting ARFs 1–5 played no role in receptor-coupled activation of PLD. Cells were then transfected with either a dominant negative form of ARF6 or ARF6 was down-regulated using an antisense oligonucleotide. In both cases, coupling of FcγRI to PLD was significantly inhibited in the treated cells. Aggregation of FcγRI with immune complexes resulted in the translocation of ARF6 from the cytoplasm to membrane fraction as assessed by cell fractionation and Western blotting. Furthermore, following immune complex activation, ARF6 appeared in FcγRI immunoprecipitates and this was abolished in cells pretreated with 50 nM wortmannin. In order to understand the mechanisms coupling PI3 kinase to ARF6, these receptor immunoprecipitates were also probed for ARNO and were found to be positive, but not in cells pretreated with wortmannin. These same immunoprecipitates were then probed for the presence of PLD1 or PLD2. Only PLD1 was found.

Pretreatment of cells with 0.3 % butan-1-ol completely abolished the oxidase burst normally observed in these cells following aggregation of FcγRI. The role of PLD1 but not PLD2 was confirmed by the use of antisense oligonucleotides to down-regulate specifically either PLD isozyme. Thus treatment of cells with antisense to PLD1 reduced both the intracellular calcium and oxidase burst responses to FcγRI activation.

Thus FcγRI appears to be coupled to the activation of PLD1 through a PI3 kinase-dependent process that requires ARNO and ARF6. PLD1 is required for the intracellular calcium and oxidase burst responses to FcγRI aggregation.

Effects of inhibition of accessory signalling molecule expression on FcγRI-mediated phosphoinositol and intracellular Ca²⁺ signalling in U937 cells

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The high affinity receptor for immunoglobulin G (IgG), FcγRI, is constitutively expressed on cells of monocyte-macrophage lineage. It plays a central role in orchestrating both humoral and cellular arms of the immune system, mediating a number of important effector functions including phagocytosis and antibody-directed cellular cytotoxicity (Daëron, 1997). Although the cytoplasmic tail of FcγRI contains no known signalling motifs, receptor aggregation appears to cause recruitment and activation of soluble tyrosine kinases by interaction with accessory signalling molecules (ASMs) within the plasma membrane. Two candidate ASMs have been proposed: the γ chain, originally described as part of the IgE receptor (FcεRI) complex, and the low-affinity IgG receptor, FcγRII.

We have previously shown that aggregation of FcγRI, in the human monocytic cell line U937, leads to activation of very different intracellular signalling pathways depending on which ASM is involved (Melendez *et al.* 1998). In U937 cells pretreated with dibutyryl cAMP (dbcAMP), FcγRI signalling through FcγRII leads to activation of phospholipase Cγ1 generating inositol 1,4,5 trisphosphate (IP₃) and oscillations in intracellular Ca²⁺ concentration ([Ca²⁺]_i). In contrast, γ chain-mediated FcγRI signalling (as seen in interferon γ-treated U937 cells) leads to phospholipase D activation and generation of a single discrete rise in [Ca²⁺]_i by means of a sphingolipid second messenger. By inhibiting surface expression of either the γ chain or FcγRII by pretreatment with antisense oligonucleotides, we have examined the role of both ASMs in FcγRI-triggered phosphoinositol and Ca²⁺ signalling in dbcAMP-treated U937 cells.

Compared with controls, we found that inhibition of γ chain expression resulted in an increased rate of total inositol phosphate generation, increased [IP₃]_i levels and an increased proportion of cells demonstrating FcγRI-triggered Ca²⁺ oscillations. In contrast, inhibition of FcγRII expression led to complete inhibition of FcγRI-triggered [IP₃]_i rise and a reduced proportion of cells undergoing Ca²⁺ oscillations. These results suggest an absolute requirement for FcγRII in FcγRI-triggered IP₃ generation and tonic inhibition of this signalling pathway by the γ chain.

In addition, measurement at time points shortly after FcγRI aggregation of dbcAMP-treated cells revealed considerable fluctuations in [IP₃]_i. The temporal variations observed in [IP₃]_i

correlate well with summated $[Ca^{2+}]_i$ measurements of 30 cells undergoing $Fc\gamma RI$ -triggered Ca^{2+} oscillations. Furthermore, these fluctuations in $[IP_3]_i$ reflect inverse changes in levels of intracellular inositol 1,3,4,5-tetrakisphosphate and both can be inhibited by buffering rises in $[Ca^{2+}]_i$ by pre-incubation of cells with BAPTA-AM ester ($10 \mu M$ for 30 min). These results can, at least partly, be explained by intermittent Ca^{2+} -dependent activation of IP_3 kinase leading to fluctuations in $[IP_3]_i$ and subsequently $[Ca^{2+}]_i$ and provide insight into possible mechanisms involved in generating $Fc\gamma RI$ -mediated Ca^{2+} oscillations.

Daëron, M. (1997). *Ann. Rev. Immunol.* **15**, 203–234.

Melendez, A.J. *et al.* (1998). *Current Biology* **8**, 210–221.

Interleukin-1 β release requires both calcium release from intracellular calcium stores and potassium ion efflux in murine macrophages

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Interleukin-1 β (IL-1 β) is a primary mediator of immune responses to injury and infection, but the mechanism of its cellular release is unknown. IL-1 β is synthesised as an inactive precursor that lacks a signal sequence, and is cleaved and released by caspase-1. ATP induces the processing and release of IL-1 (α and β) via activation of the $P2X_7$ receptor. Experiments in primary murine macrophages derived from peritoneum (all animals were killed according to UK legislation) suggested a role for Ca^{2+} in the mechanism of IL-1 β secretion in response to ATP.

The calcium dependence of IL-1 β release in response to ATP is shown in Fig. 1. Subsequent experiments were performed to identify the source of calcium, which was shown to be thapsigargin (TG)-sensitive ER stores.

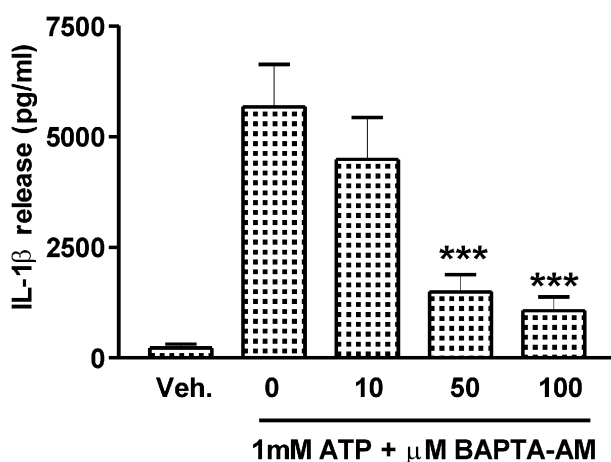


Figure 1. Effects of BAPTA-AM on ATP-induced processing and release of IL-1 β from LPS-primed macrophages. Dose-dependent inhibition of IL-1 β release by BAPTA-AM as measured by ELISA. The data are shown as means \pm S.E.M. for triplicate determinations of three separate experiments. Statistical significance was assessed by one-way ANOVA followed by the Newman-Keuls multiple comparison tests; *** $P < 0.001$. Veh, vehicle.

However, release of Ca^{2+} from intracellular stores alone is not sufficient to trigger mature IL-1 β release. For example, agents capable of inducing $[Ca^{2+}]_i$ transients such as TG, $100 \mu M$ ATP, and the purely metabotropic stimulus PAF (platelet activating factor), failed to induce IL-1 β release. In addition, treatment of the cells with ionomycin correlated with a high level of cell death, which was accompanied by the release of only pro-IL-1 β . The use of the K^+ ionophore nigericin, identified K^+ efflux as the factor required in addition to elevated $[Ca^{2+}]_i$ to promote the release of processed IL-1 β .

In conclusion, we have demonstrated that ATP-induced IL-1 β processing and release from murine peritoneal macrophages is dependent upon the release of Ca^{2+} from TG-sensitive stores. In addition, we demonstrate that this is necessary, but not sufficient to promote IL-1 β release, as it also depends on K^+ efflux.

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All procedures accord with current UK legislation.

New calibration equation for fura-2 and its application

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The measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the major experimental procedures because of its important role in various cellular events such as contraction, secretion, metabolism, excitability and proliferation, etc. To obtain the exact concentration of intracellular Ca^{2+} , the ratiometric fluorescent dye, fura-2, was used. 340 nm and 380 nm are usually selected for excitation because the emission peaks in Ca^{2+} -saturated and Ca^{2+} -free conditions, respectively. The ratio (F_{340}/F_{380}) of the emission intensities at around 500 nm by each excitation wavelength is measured. To convert ratio to $[Ca^{2+}]$, Grynkiewicz *et al.* (1985) developed an equation that needs four parameters such as the maximum ratio (R_{max}), the minimum ratio (R_{min}), dissociation constant (K_d) and the ratio of maximum and minimum ($F_{380,max}/F_{380,min}$) at 380 nm excitation. Theoretically, if we could obtain the exact values of these four parameters, we could convert the ratio to the exact Ca^{2+} concentration. Practically, it is very difficult to obtain exact values of four parameters in dye loading conditions in cells. Worse, because of the change of Ca^{2+} binding affinities by pH changes, if there are pH changes, it is almost impossible to obtain those parameters *in situ*. Fortunately fura-2 has very peculiar characteristics: the Ca^{2+} -binding form of fura-2 has virtually no emission at 400 nm excitation while the Ca^{2+} -free form produces considerable emission (Ogden *et al.* 1995). Based on this fact, we developed the following equation:

$$[Ca^{2+}] = K_d \times F_{400,max}/F_{340,max} \times (R - R_{min}).$$

K_d is a dissociation constant, R is F_{340}/F_{400} , R_{min} is a minimum ratio and $F_{400,max}/F_{340,max}$ is the ratio of maximum value at 400 nm excitation and maximum value of 340 nm excitation. The new equation has several benefits over old the equation. It needs only three parameters and all the values can be easily obtained from the experiments. Also, the error in parameter measurement can be decreased considerably. Another important benefit is that the ratio is linear to Ca^{2+} concentration, which means the rate of ratio change can be directly correlated to the rate of Ca^{2+} change. If we select the emission at isobestic excitation wavelength for the numerator, the equation can be changed into a much simpler

form, as follows: $[Ca^{2+}] = K_d \times 1/R_{min} \times (R - R_{min})$ at constant pH conditions. We verified that this new equation can be applied within reasonable Ca^{2+} concentration ranges. We obtained the changes of each parameter by pH changes which must be system specific except K_d . Finally we obtained strong evidence that the K_d values in free conditions and *in situ* are virtually the same. Based on these facts, the resting $[Ca^{2+}]$ may be measured more accurately. At present, several ion-sensitive fluoroprobes such as fura-2, bis-fura-2, fura-2-FF, pyranine, etc., can be applied to the new equation. The new development of dyes in this direction may improve its applicability.

Gryniewicz, G. *et al.* (1985). *J. Biol. Chem.* **260**, 3440–3450.

Ogden, D. *et al.* (1995). *Pflügers Arch.* **429**, 587–591.

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All procedures accord with current local guidelines.

Nitric oxide co-ordinates receptor-regulated Ca^{2+} entry pathways in A7r5 rat aortic smooth muscle cells

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Recent results challenge the long-standing idea that capacitative Ca^{2+} entry (CCE) is the major receptor-regulated pathway for Ca^{2+} entry into cells (Broad *et al.* 1999; Mignen *et al.* 2001). In A7r5 vascular smooth muscle cells, vasopressin, a potent vasoconstricting hormone, stimulates formation of IP_3 and diacylglycerol (DAG); both messengers ultimately regulate Ca^{2+} entry. IP_3 , by emptying Ca^{2+} stores, generates the signal that activates CCE, while the arachidonic acid released from DAG both stimulates non-capacitative Ca^{2+} entry (NCCE) and simultaneously inhibits CCE (Moneer & Taylor, 2002). This reciprocal regulation of the two pathways ensures that all Ca^{2+} entry is via NCCE when vasopressin is present, while CCE is transiently activated only after removal of vasopressin (Moneer & Taylor, 2002). Here we demonstrate that endogenous production of nitric oxide (NO) downstream of arachidonic acid mediates this reciprocal regulation of the two Ca^{2+} entry pathways.

Western blot analysis showed that type III NO synthase (NOS III) was expressed in A7r5 cell membranes. In A7r5 cells loaded with fura-2 to allow measurement of cytoplasmic Ca^{2+} , we used selective blockers of CCE or NCCE (Moneer & Taylor, 2002) to examine the effects of vasopressin on each pathway. L-NAME, a competitive inhibitor of NOS, reversed the inhibitory effect of vasopressin (100 nM) on CCE in a concentration-dependent manner ($IC_{50} = 39 \pm 5 \mu M$; mean \pm S.E.M., $n = 3$). With the CCE pathway blocked, vasopressin activated NCCE ($\Delta[Ca^{2+}]_i = 83 \pm 9$ nM, $n = 4$); that activation was also blocked ($\Delta[Ca^{2+}]_i = 5 \pm 3$ nM, $n = 4$) by L-NAME (700 μM). In both cases the effects of L-NAME were reversed by addition of L-arginine (2.1 mM), the endogenous NOS substrate. The effects of vasopressin on the two Ca^{2+} entry pathways were mimicked by the slow-releasing NO donor, NOC-18 and by NO solution (1 μM). We conclude that reciprocal regulation of CCE and NCCE by vasopressin is mediated by NOS, activated directly or indirectly by arachidonic acid.

The membrane-permeant analogue of cyclic GMP, 8-bromo-cyclic GMP, completely inhibited CCE (the increase in cytosolic $[Ca^{2+}]$ fell from 567 ± 63 to 12 ± 5 nM, $n = 4$), but it did not

activate NCCE ($[Ca^{2+}]_i$ rise = 7 ± 4 nM, $n = 3$). Furthermore, selective inhibitors of soluble guanylyl cyclase (ODQ, $IC_{50} = 44 \pm 8$ nM, $n = 3$) and cyclic GMP-dependent protein kinase (PKG) (KT5823, $IC_{50} = 86 \pm 10$ nM, $n = 3$) potently inhibited the ability of vasopressin to inhibit CCE, without affecting activation of NCCE. Thus the activation of soluble guanylyl cyclase by NO and subsequent activation of PKG are essential only for inhibition of CCE.

In addition to its role as endothelium-derived relaxing factor, we conclude that NO also fulfils an essential role in co-ordinating two receptor-regulated Ca^{2+} entry pathways.

Broad, L.M. *et al.* (1999). *J. Physiol.* **517**, 121–134.

Mignen, O. *et al.* (2001). *J. Biol. Chem.* **276**, 35676–35683.

Moneer, Z. & Taylor, C. W. (2002). *Biochem. J.* **362**, 13–21.

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Measurement of intra-SR $[Ca^{2+}]$ in isolated cardiac myocytes using a targeted aequorin probe

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The $[Ca^{2+}]$ within the SR of cardiac muscle ($[Ca^{2+}]_{SR}$) may modulate the activity of SR Ca^{2+} ATPase (SERCA2a) and the Ca^{2+} release channels (ryanodine receptor). However, direct measurement of $[Ca^{2+}]_{SR}$ in adult myocytes has not been possible due to the lack of a specific indicator. A mutated aequorin chimera has been used to measure free $[Ca^{2+}]_{SR}$ in cultured myotubes. Targeting of the photoprotein was achieved by generation of a construct that consisted of cDNA encoding the SR resident protein calsequestrin fused to the aequorin cDNA (p srAEQ) (Brini *et al.* 1997). To allow expression of this protein in adult cardiac myocytes, an adenovirus was generated from the aequorin-calsequestrin plasmid (Ad srAEQ). Male Wistar rats were killed by cervical dislocation and the hearts removed and perfused with collagenase/protease solution to isolate ventricular cardiomyocytes. Isolated cardiomyocytes were infected with the Ad srAEQ. Calcium dynamics were studied in populations of cells 2 days post-infection. Cardiomyocytes were permeabilised by brief exposure to 0.01 mg ml⁻¹ β -escin and subsequently bathed in a mock intracellular solution (mM): 130 K⁺, 30 Na⁺, 1 Mg²⁺, 25 Hepes, 100 Cl⁻, 0.05 EGTA, 5 ATP, 10 CrP and 0.9 Mg; pH 7.0. Aequorin was reconstituted by 15 min incubation with 5 μM coelenterazine *n*. Cardiomyocytes (5×10^5 in 200 μl) were placed in a luminometer and the aequorin light emission was monitored while the bathing $[Ca^{2+}]$ was increased from < 1 nM to 380 nM in the presence of 10 mM EGTA. Total luminescence was assayed by subsequently adding 1% Triton X-100/100 mM CaCl₂. The fractional luminescence was converted to $[Ca^{2+}]$ using a previously published calibration curve (Brini *et al.* 1997). As shown in Fig. 1, these calculations suggest that $[Ca^{2+}]_{SR}$ increases from approximately 5 to 50 μM . The increase in signal was completely inhibited by previous incubation with thapsigargin (125 μg ml⁻¹). These measurements indicate a $[Ca^{2+}]_{SR}$ of $55 \pm 6 \mu M$ (mean \pm S.E.M., $n = 14$; this corresponds to a fractional luminescence of $1.55 \pm 0.2 \times 10^{-3}$) in the presence of $[Ca^{2+}]_{cy}$ 380 nM.

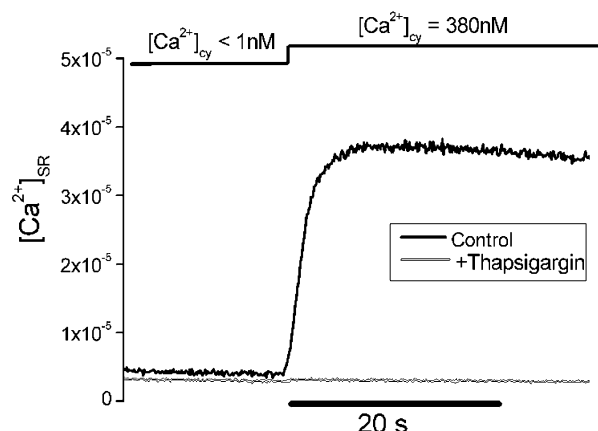


Figure 1. Records of SR $[Ca^{2+}]$ based on aequorin luminescence measurements from aggregates of permeabilised rat cardiac myocytes. The $[Ca^{2+}]$ in the bathing solution was increased at the point indicated. Records obtained under control conditions and in the presence of thapsigargin are shown.

Brini, M. *et al.* (1997). *Mol. Cell. Biol.* **8**, 129–143.

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All procedures accord with current UK legislation.

The effects of disrupting the actin network on Ca^{2+} signalling in mouse pancreatic acinar cells

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Previous studies have shown that microtubules position the endoplasmic reticulum to the apical pole region of pancreatic acinar cells, thus ensuring a polarised Ca^{2+} response that spreads from the apical pole towards the basolateral region of the cell (Fogarty *et al.* 2000). In our present studies we explore the role of the actin cytoskeleton on Ca^{2+} signalling.

Using the whole-cell patch-clamp configuration with $10 \mu M$ Ins(2,4,5) P_3 in the pipette solution, we initiated Ca^{2+} -dependent Cl^- current spikes in freshly isolated pancreatic acinar cells from humanely killed mice. The spikes reflect a Ca^{2+} response restricted to the apical pole of the cell. Latrunculin B (Lat B), an agent that inhibits actin polymerisation, at 50 – $90 \mu M$ inhibited the current spikes ($n = 10$). These effects were concentration dependent. At $90 \mu M$, Lat B had completely inhibited spiking within a minute in 3/4 cells. At $50 \mu M$ Lat B, spikes were still evident within the time range of 5–20 min in four cells, although by the end of each experiment the spikes had either been completely inhibited or reduced in amplitude and altered in profile. In further experiments where we loaded the cells with the Ca^{2+} dye Calcium Green, we demonstrated that this is due to a loss of the Ca^{2+} response and not due to an action on the Cl^- channel ($n = 3$).

We have also performed immunocytochemistry of actin and IP_3 receptors before and after the addition of Lat B. Both phalloidin and an anti-actin antibody showed actin to be present beneath

the plasma membrane throughout the cell but to predominate in the apical pole. Lat B ($100 \mu M$) over a time course of up to 1 h, diminished actin levels and disrupted actin distribution. However, in many instances even after prolonged Lat B treatment, some actin remained in the apical pole region. IP_3 receptors, type II and III, were restricted to the apical pole and type II IP_3 receptors were found to colocalise with actin. After treatment with Lat B ($100 \mu M$) over a time course of up to an hour, both type II and III IP_3 receptors showed an altered distribution. In experiments where we had labelled type II IP_3 receptors and actin and treated the cells with Lat B ($100 \mu M$) the IP_3 receptors colocalised with the remaining actin.

In conclusion, we hypothesise that either a direct or indirect attachment, mediated by an unknown protein, is present between actin and IP_3 receptors in pancreatic acinar cells. This may ensure a polarised Ca^{2+} response, which is probably required for effective secretion in these cells.

Fogarty, K.E. *et al.* (2000). *J. Biol. Chem.* **275**, 22487–22494.

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All procedures accord with current UK legislation.

Local Ca^{2+} signals in mouse submandibular acinar cells activate the Cl^- and not the K^+ channel

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Polarised Ca^{2+} signals that originate at and spread from the apical pole have been shown to occur in acinar cells from lacrimal, parotid, submandibular and pancreatic glands. However, Ca^{2+} signals that are restricted to the apical pole of the cell have been previously demonstrated only in pancreatic acinar cells where the primary function of the Ca^{2+} signal is to regulate exocytosis. We have investigated whether local Ca^{2+} signals also occur in submandibular acinar cells where the primary function of the Ca^{2+} signal is to drive fluid and electrolyte secretion, a process that requires simultaneous activation of a basolateral K^+ channel and an apical Cl^- channel.

Acinar cells were isolated from the submandibular glands of humanely killed male CD-1 mice by collagenase digestion. For each experiment, cells were allowed to settle onto a poly-L-lysine-coated coverslip that formed the base of the perfusion chamber and placed on the stage of an inverted microscope. Patch-clamp whole-cell and microfluorimetry experiments were performed using cells loaded with fura-2 either by pre-incubation with fura-2 AM or via the patch pipette to measure simultaneously changes in $[Ca^{2+}]_i$ and K^+ and Cl^- channel activity.

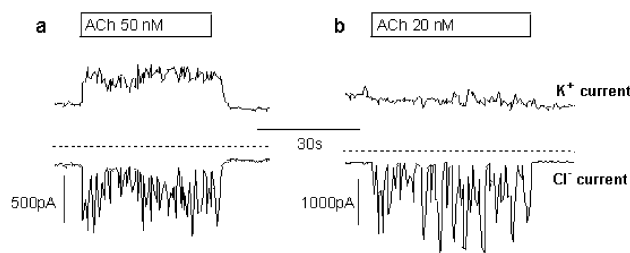


Figure 1. Activation of both the Cl^- and K^+ currents measured under voltage-clamp conditions in response to a

global Ca^{2+} signal (a) and activation of the Cl^- current alone by a Ca^{2+} signal localised to the apical pole (b).

We show that the response to an intermediate (20–500 nM) concentration of ACh occurred as a series of transient increases in $[\text{Ca}^{2+}]_i$ that originated at the apical pole of the cell and then spread as a Ca^{2+} wave to the basolateral pole. Each transient increase in $[\text{Ca}^{2+}]_i$ caused activation of first the Cl^- conductance and then the K^+ conductance (Fig. 1a), thus confirming the distribution of these channels to the apical and basolateral poles, respectively, and demonstrating that this type of response would easily support fluid secretion. The response to a low (5–20 nM) concentration of ACh also comprised a series of transient elevations of $[\text{Ca}^{2+}]_i$, although these Ca^{2+} signals did not spread from the apical pole of the cell. Furthermore, only the Cl^- and not the K^+ conductance responded to local elevation of $[\text{Ca}^{2+}]_i$ (Fig. 1b). This mode of activation might appear less effective in driving fluid secretion because the membrane depolarisation that results from Ca^{2+} activation of the Cl^- conductance is not counterbalanced by Ca^{2+} activation of the K^+ conductance. However, the effects of membrane depolarisation in these cells are self limiting because the K^+ conductance is voltage as well as Ca^{2+} activated, and therefore the K^+ conductance will increase even in the absence of Ca^{2+} activation.

Our data indicate that submandibular acinar cells are capable of local Ca^{2+} signalling and, by introducing a role for voltage activation of the K^+ conductance into the model, that these Ca^{2+} signals could support fluid secretion.

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All procedures accord with current UK legislation.

Effects of ageing and diabetes mellitus on intracellular free calcium concentration in isolated rat parotid acinar cells

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Ageing and diabetes mellitus (DM) have previously been shown to elicit marked changes in parotid gland salivary function, in terms of its ability to secrete α -amylase, and in its morphological macrostructure. Both ageing and DM are associated with decreased α -amylase output and infiltration of numerous lipid vacuoles in the parotid gland compared with age-matched controls (Anderson, 1987). Since cytosolic calcium ($[\text{Ca}^{2+}]_i$) plays a major role in the stimulus-secretion coupling process in most exocrine cells, then it is pertinent to investigate the homeostasis of this physiological divalent cation during ageing and DM compared with their respective controls. Diabetes mellitus was induced in adult male Wistar rats by a single intraperitoneal (i.p.) injection of streptozotocin (STZ, 60 mg (kg body wt) $^{-1}$; Sharma *et al.* 1985). Control animals were injected with a similar volume of citrate buffer. The animals were tested for DM 4 days after STZ injection and 2 months later when they were humanely killed for the experiment. Parotid glands from either age-matched control and diabetic rats or from two age groups of young (2–6 months) and aged (16 months) rats were isolated and subsequently dissociated into acinar cells using collagenase. Cells were loaded with 2 μM fura-2 AM for the measurement of $[\text{Ca}^{2+}]_i$ using fluorescence photometry (Pariente *et al.* 2000). All values are expressed as ratio units (F_{340}/F_{380}).

After 2 months of STZ treatment diabetic rats weighed significantly (Student's unpaired *t* test; $P < 0.001$) less (mean \pm S.E.M.; 232 ± 7.2 g, $n = 8$) compared with age-matched controls (381 ± 12.53 g, $n = 8$). Similarly, blood glucose level was much higher in diabetic rats (407.50 ± 35.90 mg dl $^{-1}$, $n = 8$) compared with control (81.20 ± 3.1 mg dl $^{-1}$, $n = 8$). Basal $[\text{Ca}^{2+}]_i$ in young (2–6 months) and aged (16 months) rats was 0.362 ± 0.008 ($n = 11$) and 0.411 ± 0.02 ($n = 10$), respectively. Stimulation of 16-month-old parotid acinar cells with acetylcholine (ACh; 1×10^{-5} M) resulted in a significant ($P < 0.05$) decrease (in both the peak and plateau phases) in $[\text{Ca}^{2+}]_i$ compared with acinar cells obtained from 2- to 6-month-old rats. Peak values of 1.272 ± 0.05 ($n = 11$) and 1.022 ± 0.02 ($n = 10$) were recorded from parotid acinar cells isolated from 2- to 6- and 16-month-old animals, respectively. DM produced no significant change in basal $[\text{Ca}^{2+}]_i$ with values of 0.360 ± 0.008 ($n = 17$) compared with age-matched control values of 0.363 ± 0.008 ($n = 11$). Similarly, DM evoked no significant change in the initial peak $[\text{Ca}^{2+}]_i$ with values of 1.210 ± 0.03 ($n = 17$) compared with age-matched control values of 1.272 ± 0.05 ($n = 11$). In contrast, DM had a significant ($P < 0.001$) effect on the plateau phase of $[\text{Ca}^{2+}]_i$ measured 202.93 ± 0.29 s ($n = 18$) after ACh application. Typically, the $[\text{Ca}^{2+}]_i$ was 0.730 ± 0.01 ($n = 18$) and 0.958 ± 0.02 ($n = 11$) in DM and age-matched control fura-2 AM-loaded acinar cells, respectively. The results indicate that both DM and ageing are associated with decreased levels of $[\text{Ca}^{2+}]_i$ in parotid acinar cells in response to ACh stimulation. Ageing induces alteration in Ca^{2+} release from intracellular stores, whereas DM is associated with a decrease in Ca^{2+} entry into the cell.

Anderson, L.C (1987). *J. Dent. Res.* **66**, 425–429.

Pariente, J.A. *et al.* (2000). *J. Memb. Biol.* **179**, 27–35.

Sharma, A.K. *et al.* (1985). *J. Neurol. Sci.* **67**, 285–297.

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Intracellular targeting of inositol trisphosphate receptors

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Inositol 1,4,5-trisphosphate (IP_3) receptors belong to a family of intracellular Ca^{2+} channels that mediate release of Ca^{2+} from intracellular stores and thereby generate increases in cytosolic $[\text{Ca}^{2+}]$ that are often spatially complex. IP_3 receptors are expressed largely within the membranes of the endoplasmic reticulum, but they are also found in the nuclear envelope, Golgi, secretory vesicles and perhaps in the plasma membrane. Targeting of IP_3 receptors to appropriate membranes is likely to be important in determining the patterns of Ca^{2+} release evoked by extracellular stimuli. A previous study in *Xenopus* oocytes suggested that the six transmembrane regions (TMR) together with the carboxyl terminal of the type 1 IP_3 receptor was sufficient to ensure its retention within the endoplasmic reticulum (Sayers *et al.* 1997). Recent work in mammalian cells further supports a role for TMRs in efficient targeting of IP_3 receptors to intracellular membranes (Galvan *et al.* 1999).

We use confocal imaging of mouse insulinoma cells (MIN6) and COS-7 cells to determine the localization of both the endoplasmic reticulum (using an antibody to an ER-resident protein, BiP) and expressed fragments of the type 1 IP_3 receptor tagged with green fluorescent protein (GFP). Results are from at

least six independent transfections for all constructs. We confirm that a protein containing all six TMR and the carboxyl terminal of the receptor is targeted to the ER. The targeting was unaffected by deletion of the carboxyl terminal. Fusion proteins that included only the first four TMR, the first two TMR or the last two TMR of the IP₃ receptor together with the linking regions were also targeted to the ER. We conclude that both TMR1–2 and TMR5–6 are independently capable of targeting the type 1 IP₃ receptor to the ER. Targeting of IP₃ receptors to the ER appears to be a redundant process mediated by non-sequence-specific properties of the TMRs and does not require the carboxyl terminal cytoplasmic tail.

Galvan, D.L. *et al.* (1999). *J. Biol. Chem.* **274**, 29483–29492.

Sayers, L.G. *et al.* (1997). *Biochem. J.* **323**, 273–280.

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Parathyroid hormone potentiates inositol trisphosphate-evoked Ca²⁺ signals by a cAMP-independent mechanism

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In human embryonic kidney (HEK) 293 cells stably expressing human type 1 parathyroid hormone (PTH) receptors, PTH (residues 1–34) potentiates the Ca²⁺ signals evoked by receptors that stimulate inositol 1,4,5-trisphosphate (IP₃) formation by a mechanism that is independent of protein kinase A (Short & Taylor, 2000). We previously proposed that PTH increases either the sensitivity of IP₃ receptors or the size of the Ca²⁺ pool available to IP₃. Here we attempt to discriminate between these possibilities.

Incubation of fura-2-loaded cells with a membrane-permeant analogue of IP₃ (IP₃-BM) stimulated a slow release of intracellular Ca²⁺ stores. The resulting Ca²⁺ signals were increased by addition of PTH, but only if it is added before IP₃-BM had completely emptied the intracellular Ca²⁺ stores. When added 5 min after addition of IP₃-BM, PTH increased the cytosolic [Ca²⁺] to 435 ± 46 nM (mean ± S.E.M., *n* = 3), but after 10 min it increased it to only 69 ± 24 nM (*n* = 5). The amount of Ca²⁺ remaining within intracellular stores was similar after prolonged stimulation with IP₃-BM alone (100 μM, 10 min) or after stimulation with carbachol (CCh) in combination with PTH. We conclude that PTH potentiates responses to IP₃ without increasing the size of the IP₃-sensitive Ca²⁺ store. Because carbachol alone stimulates formation of insufficient IP₃ to completely empty intracellular Ca²⁺ stores, PTH potentiates responses to even a maximal concentration of carbachol (Short & Taylor, 2000).

Intracellular cAMP levels increased from a basal level of 5 ± 1 to 736 ± 52 pmol well⁻¹ (*n* = 6) after a 45 s incubation with 10 nM PTH and to 1016 ± 187 pmol well⁻¹ (*n* = 6) after stimulation with 100 nM PTH. Incubation with an inhibitor of adenylyl cyclase (SQ 22536, 1 mM) reduced the amount of cAMP formed in response to 10 or 100 nM PTH by about 80%. In parallel experiments measuring the ability of PTH to potentiate carbachol-evoked ⁴⁵Ca²⁺ release from cells, SQ 22536 had no effect on responses to maximal or submaximal concentrations of PTH. We conclude that PTH sensitises IP₃ receptors to IP₃ without affecting the size of the IP₃-sensitive Ca²⁺ pool by a mechanism that is independent of cAMP.

Short, A.D. & Taylor, C.W. (2000). *J. Biol. Chem.* **275**, 1807–1813.

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The role of actin in antigen-stimulated inositol 1,4,5-trisphosphate receptor clustering in RBL-2H3 cells

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Cross-linking of IgE receptors (FcεRI) by multivalent antigens in basophils results in the activation of phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K). PLC γ activation leads to the production of inositol 1,4,5-trisphosphate (IP₃) and release of calcium from intracellular stores triggering exocytosis of secretory granules. Previous studies have shown that activation of the FcεRI leads to changes in IP₃ receptor (IP₃R) distribution in rat basophilic leukaemia cells (RBL-2H3), a mast cell line. At rest the IP₃R distribution is homogenous throughout the cell; upon activation the IP₃Rs cluster and form discrete spots of about 1 μm diameter (Wilson *et al.* 1998). We have examined the potential role of actin-myosin in this process.

In our experiments, RBL-2H3 cells were primed with anti-DNP-IgE (1 μg ml⁻¹) for 12–24 h and activated with the multivalent antigen DNP-BSA (1 μg ml⁻¹) for 1 h. We then localised IP₃R type II, calreticulin, BiP, actin and myosin II using GFP constructs and immunocytochemistry. All experiments were carried out at least twice and all observations consistently seen in at least three images.

After cell activation IP₃R type II receptors aggregate, as shown by Wilson *et al.* (1998). However, we also saw that these receptor aggregates appear to cluster preferentially towards one region of the cell, usually the granular region. Staining of F-actin with phalloidin, or actin-GFP, showed actin throughout the cell with a concentration in the subplasmalemmal region. We visualised the distribution of myosin IIA using an antibody and GFP construct (Wei & Adelstein, 2000). Myosin II was mainly found in the cell periphery. The concentrations of either actin or myosin II were not associated with the IP₃R aggregates. These data suggest that actin and myosin II are not the sole mediators of IP₃R distribution and indeed may not play any role. We then tested a role for actin using Latrunculin, an agent that depolymerises the actin cytoskeleton. We show that Latrunculin-B (50 μM, 1 h) dramatically reduced actin but actually enhanced IP₃R clustering. Under conditions of cell surface receptor stimulation, remodelling of the actin cytoskeleton is regulated by PI3K, which can be inhibited by wortmannin. Wortmannin (100 nM, 1 h) did not prevent the aggregation of the IP₃R but did prevent the IP₃Rs from concentrating in the granular region of the cell. We are conducting experiments to test for the selectivity of these effects on IP₃R distribution.

Wei, Q. & Adelstein, R.S. (2000). *Mol. Biol. Cell* **11**, 3617–3627.

Wilson, B.S. *et al.* (1998). *Mol. Biol. Cell* **9**, 1465–1478.

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Cross-talk between the SR and mitochondria during agonist-induced Ca^{2+} signalling in rat uterine smooth muscle cells

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Mitochondria have a well-established capacity to accumulate calcium ions entering the cytosol from the extracellular space or released from the sarco/endoplasmic reticulum (SR/ER) of many cell types including smooth muscle cells. The contribution of mitochondria to cytosolic calcium ($[\text{Ca}^{2+}]_i$) regulation has been observed during SR Ca^{2+} release in both visceral (McCarron & Muir, 1999) and vascular smooth muscle cells (Gurney *et al.* 2000). In the present study, we have investigated whether release of calcium from mitochondria can replenish the agonist-sensitive SR Ca^{2+} pool.

Cells were enzymatically isolated from the uteri of 19- to 21-day pregnant rats, humanely killed by cervical dislocation after CO_2 anaesthesia. Single cells were co-loaded with mag-fluo-4 AM for the SR Ca^{2+} ($[\text{Ca}^{2+}]_L$) measurement and fura-2 AM for $[\text{Ca}^{2+}]_i$ measurement (Shmigol *et al.* 2001). Individual mitochondria were visualised using Mito-Tracker Green or rhod-2.

Confocal microscopy of cells co-loaded with mag-fluo-4 and rhod-2 revealed that in uterine myocytes some of the mitochondria are localised close to the SR, which suggests a possible interaction between these two organelles during agonist-induced Ca^{2+} signalling. This was further investigated by simultaneous whole-cell measurements of $[\text{Ca}^{2+}]_L$ and $[\text{Ca}^{2+}]_i$. A decrease in $[\text{Ca}^{2+}]_L$ accompanied by a rise in $[\text{Ca}^{2+}]_i$ was observed during application of the purinergic agonist ATP. Repetitive applications of $100 \mu\text{M}$ ATP in the absence of extracellular Ca^{2+} led to the depletion of the SR. Calcium release from mitochondria by application of $10 \mu\text{M}$ CCCP in the presence of oligomycin elicited little or no change in $[\text{Ca}^{2+}]_i$ but produced a restoration of $[\text{Ca}^{2+}]_L$ to approximately 90% of its initial value ($n = 4$). Inhibition of the SR Ca^{2+} -ATPase with 200 nM thapsigargin prevented the increase in $[\text{Ca}^{2+}]_L$ during CCCP application ($n = 3$). In the presence of thapsigargin, CCCP application now caused a substantial rise in $[\text{Ca}^{2+}]_i$. These data are compatible with the idea that the SR takes up Ca^{2+} released from mitochondria.

When the agonist-sensitive SR Ca^{2+} pool was depleted by repetitive application of ATP in Ca^{2+} -free solution, Ca^{2+} released from mitochondria in the absence of extracellular Ca^{2+} led to a partial (approximately 50%, $n = 3$) restoration of the response to ATP, indicating close interaction between the SR and mitochondria.

In conclusion, our data suggest that during agonist-induced Ca^{2+} signalling, mitochondria accumulate some of the Ca^{2+} released from the SR and then, during the restoration phase, feed it back to the agonist-sensitive SR Ca^{2+} pool.

Gurney, A.M. *et al.* (2000). *Cell Calcium* 27, 339–351.

McCarron, J.G. & Muir, T.C. (1999). *J. Physiol.* 516, 149–161.

Shmigol, A.V. *et al.* (2001). *J. Physiol.* 531, 707–713.

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All procedures accord with current UK legislation.

InsP₃ and Ca^{2+} waves in guinea-pig colonic smooth muscle

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Ca^{2+} waves in smooth muscle, from sarcolemma agonist activity, play an important role in normal physiological function. Yet the mechanisms underlying wave propagation are unclear. One proposal is that a small catalytic Ca^{2+} release from the InsP₃-gated channel, on the sarcoplasmic reticulum, activates neighbouring ryanodine receptors (RyR) to generate a more robust Ca^{2+} release. Ca^{2+} diffuses from this latter site to activate nearby RyR (Ca^{2+} -induced Ca^{2+} release) and so the cycle of release and diffusion continues through the cell by repeated activation of RyR. However, experimental studies on the dual roles of InsP₃ and RyR in the generation of Ca^{2+} waves, in intact cells, have been hampered by the inability to evoke rapid and reproducible step changes in InsP₃ in subcellular regions. As a result, our understanding of the generation of waves in smooth muscle derives, predominantly, from studies using pharmacological approaches, which may lack specificity in intact cells. In the present study the initiation and propagation of Ca^{2+} waves, in single voltage-clamped smooth muscle cells, was examined using methods enabling elevations in InsP₃ to occur in subcellular regions in the intact cell. The cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) was imaged (with fluo-3) simultaneously at high temporal resolution (100 frames per second; 562 nm pixels at the cell, 160×160 pixel array).

From male guinea-pigs stunned by a blow to the head and killed by exsanguination, following the guidelines of the Animal (Scientific Procedures) Act, 1986, a segment of colon was removed and single smooth muscle cells prepared (McCarron & Muir, 1999). Depolarisation (-70 mV to 0 mV) increased $[\text{Ca}^{2+}]_c$ uniformly throughout the cell; the magnitudes and time courses were similar in all regions. The rate of decline of $[\text{Ca}^{2+}]_c$, after the depolarisation, though slower than the rise, was also similar throughout. In contrast, the $[\text{Ca}^{2+}]_c$ increase by sarcolemma agonists, which generate InsP₃, was not uniform but often appeared as a localised rise which then moved through the cell with approximately constant amplitude though with a variable velocity (a Ca^{2+} wave). The contribution of RyR to wave propagation was examined. Local increases in InsP₃ (in a $5 \mu\text{m}$ diameter region) were evoked by spot flash photolysis in single cells ($V_M -70 \text{ mV}$; mean bulk average $[\text{Ca}^{2+}]_c$ $99 \pm 21 \text{ nM}$, mean $\pm \text{S.E.M.}$, $n = 7$) and produced an increase in $[\text{Ca}^{2+}]_c$ of a magnitude similar to that evoked by the agonist, but did not generate a propagating Ca^{2+} wave; $[\text{Ca}^{2+}]_c$ declined in amplitude from the site of release. In the same cell, after depolarising to -20 mV , which elevated bulk average $[\text{Ca}^{2+}]_c$ ($276 \pm 32 \text{ nM}$, mean $\pm \text{S.E.M.}$, $n = 7$) and activated RyR, as evidenced by the occurrence of STOCs, local InsP₃ increases still failed to evoke a propagating Ca^{2+} wave. The failure of InsP₃ itself to evoke a Ca^{2+} wave could not be accounted for by a requirement for Ca^{2+} release at a specific initiating site, since focal release of InsP₃ at different $5 \mu\text{m}$ diameter locations throughout the cell evoked approximately equal increases in $[\text{Ca}^{2+}]_c$, which declined in amplitude from the release site. However, in the presence of a low concentration of a sarcolemma agonist that generated InsP₃ (which itself did not evoke an increase in $[\text{Ca}^{2+}]_c$) focal release of InsP₃ generated a propagating Ca^{2+} wave. PKC activation did not enable wave propagation since indolactam did not significantly alter the release evoked by InsP₃. On the other hand dialysing the cell with low concentrations of free InsP₃ permitted local increases in $[\text{Ca}^{2+}]_c$ from the release of caged InsP₃, to evoke a propagating Ca^{2+} wave. Thus low concentrations of InsP₃ may

increase the Ca^{2+} sensitivity of the InsP_3 receptor. These results suggest that a global subthreshold increase in InsP_3 throughout the cell, by agonists, may allow a propagating Ca^{2+} wave to occur by Ca^{2+} -induced Ca^{2+} release acting on the InsP_3 receptor rather than RyR.

McCarron, J.G. & Muir, T.C. (1999). *J. Physiol.* **516**, 149–161.

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Cyclic ADP ribose (cADPR) and Ca^{2+} release from the sarcoplasmic reticulum (SR) in guinea-pig colonic smooth muscle

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Although confirmed in sea urchin eggs, the ability of cADPR to release Ca^{2+} from mammalian smooth muscle SR is disputed. Even where cADPR is acknowledged to release Ca^{2+} from the SR, its precise mechanism of action is unresolved. In particular, the contribution of the FK506 binding protein 12.6 (FKBP12.6) in mediating the cADPR response is unclear. In the present study the ability of cADPR to modulate Ca^{2+} release from the SR was examined in a smooth muscle that lacked FKBP12.6 but contained functional RyR and IP_3R , which may modulate the physiological response of the tissue to excitatory agonists (McCarron *et al.* 2002). Male guinea-pigs were humanely killed by cervical dislocation followed by exsanguination according to the requirements of the Animal (Scientific Procedures) Act, 1986. Data presented are means \pm s.e.m. A Student's paired *t* test was used to test for significant difference ($P < 0.05$). The absence of FKBP12.6 in colonic smooth muscle and the protein's presence in brain (both guinea-pig) were confirmed by Western blots. In single guinea-pig colonic myocytes voltage-clamped in the whole-cell configuration and in which the cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) was measured using fluo-3, photolysed IP_3 (25 μM) and caffeine (10 mM; by hydrostatic application) each significantly ($P < 0.01$) increased $[\text{Ca}^{2+}]_c$ ($\Delta F/F_0 = 1.3 \pm 0.2$ and 1.3 ± 0.1 units, respectively, $n = 12$). However, photolysed cADPR (50 or 500 μM) increased neither bulk average $[\text{Ca}^{2+}]_c$ ($\Delta F/F_0 = 0.01 \pm 0.01$ units, $n = 11$) nor local subsarcolemma Ca^{2+} (the frequency and amplitude of spontaneous transient outward currents were unaltered). Nor did cADPR increase Ca^{2+} transients evoked by depolarisation (-70 to $+10$ mV, $n = 6$), even though low concentrations of caffeine (~ 500 μM) were effective in this respect (depolarisation-evoked response increased to $174 \pm 40\%$, $n = 10$, $P < 0.01$, control value was 100%). These results suggest that cADPR did not enable Ca^{2+} -induced Ca^{2+} release. Furthermore, cADPR did not increase the Ca^{2+} signal evoked by caffeine in concentrations that produced submaximal responses. In contrast to its failure to alter $[\text{Ca}^{2+}]_c$ in smooth muscle, flash photolysis of caged cADPR increased $[\text{Ca}^{2+}]_c$ significantly ($P < 0.05$) in sea urchin eggs ($\Delta F/F_0 = 3.1 \pm 0.9$ units, $n = 10$). Together these results suggest that in mammalian smooth muscle lacking FKBP12.6 cADPR does not regulate Ca^{2+} release.

McCarron, J.G. *et al.* (2002). *J. Cell Sci.* (in the Press).

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All procedures accord with current UK legislation.

InsP_3 -induced Ca^{2+} release in rat sensory neurones: imaging of intra-ER Ca^{2+} transients

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The existence of functional InsP_3 -induced Ca^{2+} release in neurones is firmly established. Several studies have demonstrated that metabotropic agonists trigger Ca^{2+} release in various types of nerve cells (see Verkhratsky & Petersen, 1998, for review). The InsP_3 -driven Ca^{2+} release was also observed in Purkinje neurones in brain slices in response to synaptic stimulation (Rose & Konnerth, 2001) suggesting its functional importance. Yet beside its proven existence, the dynamics of intra-ER Ca^{2+} concentration changes in response to InsP_3 remain unknown.

Free calcium concentration within the ER lumen ($[\text{Ca}^{2+}]_L$) was monitored in cultured neurones isolated from dorsal root ganglia obtained from neonatal (1–3 days old) Sprague-Dawley rats, killed according to UK legislation. The cells were loaded with Ca^{2+} probe by incubation with the membrane-permeable form of mag-fura-2 (5 mM for 20 min at 37°C), so that the probe was trapped within both intracellular organelles and the cytoplasm. To remove the cytoplasmic portion of the dye the cellular membrane was permeabilised by brief (7–10 s) application of saponin (0.001%) in 'intracellular' solution (mM: KCl 140, ATP 3, MgCl_2 2, CaCl_2 0.4, EGTA 1, Hepes/KOH 20; pH 7.4, free Ca^{2+} concentration 100 nM). Fluorescence images were captured using an Olympus IX70 inverted microscope ($\times 40$ UV objective) equipped with a charge-coupled device cooled intensified camera (Pentamax Gene IV, Roper Scientific, UK). The specimen was alternately illuminated at 340 and 380 nm by a monochromator (Polychrom IV, TILL Photonics, Germany) at a cycle frequency of 1–5 Hz. Control over the experiment, image storage and off-line analysis was performed by use of MetaFluor/MetaMorph software (Universal Imaging Corporation, USA) running on a Windows 98 workstation.

Treatment with saponin triggered a rapid decrease in fluorescence signal at both excitation wavelengths, which was associated with a progressive increase in 340/380 nm ratio. The resting $[\text{Ca}^{2+}]_L$ varied between 200 and 500 μM . Brief (15–30 s) application of InsP_3 in concentrations between 1 and 10 μM triggered decrease in the $[\text{Ca}^{2+}]_L$, which recovered after the removal of the drug. Stimulation of ryanodine receptors by short (5 s) administration of 20 mM caffeine triggered a much faster transient fall in $[\text{Ca}^{2+}]_L$ to 70–90 μM . Prolonged incubation with 5–10 μM InsP_3 led to a progressive slow depletion of $[\text{Ca}^{2+}]_L$ which stabilised at the same level. Application of caffeine immediately after prolonged incubation with InsP_3 did not produce an additional decrease in $[\text{Ca}^{2+}]_L$. Similarly if the stores were depleted by prolonged application of caffeine, InsP_3 was not able to trigger further Ca^{2+} release. We conclude that Ca^{2+} -induced Ca^{2+} release (Solovyova *et al.* 2002) and InsP_3 -induced Ca^{2+} release mechanisms share a common ER Ca^{2+} store in DRG neurones.

Rose, C.R. & Konnerth, A. (2001). *Neuron* **31**, 519–522.

Solovyova, N., Veselovsky, N., Toescu, E.C. & Verkhratsky, A. (2002). *EMBO J.* **21**, 622–630.

Verkhratsky, A. & Petersen, O.H. (1998). *Cell Calcium* **24**, 333–343.

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Demonstration of integrator unit and data acquisition software for laser-scanning microscopy

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Laser-scanning confocal and 2-photon laser sources are used in biophysical research to quantify fluorescence in optical sections of biological preparations. Current commercial systems suffer from two major technical limitations: (i) simultaneous acquisition of multiple channels of analog data cannot be easily stored and accessed as one data file, (ii) images acquired in line scan mode cannot be recorded indefinitely, the limit is usually set by a maximum file size (e.g. 30 000 lines). To overcome these limitations a fast integrator circuit was developed that used pixel clock and laser scan signals in conjunction with the photomultiplier (PMT) output from a confocal microscope (BioRad Radiance 2000). The current circuit can integrate the PMT signal over cycle periods as short as 1 ms. Using the accompanying sweep timing signal, these data can be acquired by a separate A/D converter (National Instruments PCI-MIO-16E1) to recreate the *x-y* or line scan image on separated data acquisition software. Software written by one of the authors (J.D.) can acquire PMT signals and associated analog signals (e.g. current and voltage) display the signals and stream the data to hard disk. The data file can be stored as binary or 16-bit tif format. This configuration allows continuous acquisition of image data and associated high bandwidth analog data with a file size that is only limited by the capacity of the computer hard disk. Programs for off-line examination and data extraction are also available. A commercial version of the integrator unit with higher specifications and additional features is currently under trial (M.T. & J.G.).

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Involvement of SH-groups in the regulation of PMCA and SERCA functional activity

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The changes of cellular Ca^{2+} homeostasis via modulation of the Ca^{2+} -transporting systems could be the origin of a number of functional disorders in organisms. Functioning of membrane

transporting systems is regulated through the amino acid residues in active centres of their molecules. SH-groups are the most sensitive part of the membrane Ca^{2+} -transporting molecules and are identified as an important part of the functional domains of Ca^{2+} -ATPases and $\text{Na}^{+}\text{--}\text{Ca}^{2+}$ exchanger. Because the types and properties of SH-groups of Ca^{2+} , Mg^{2+} -ATPases of secretory cell membrane are still unknown, the aim of our work was to identify and describe functional SH-groups in SERCA and PMCA molecules. To reveal the functional importance of SH-groups, their properties and type, we used *p*-chloromercuribenzoate (PCMB). The study was performed using post-nucleus, post-mitochondria microsome fractions obtained from isolated secretory cells of rat submandibular salivary gland by a series of consequent centrifugations. Using specific inhibitors (ouabain and thapsigargin) and specific marker tests (digitonin treatment) allowed us to identify the microsome fraction as a mixture of plasma membrane and endoplasmic reticulum vesicles, most of which are inside-out. Specific activities of Ca^{2+} , Mg^{2+} -ATPases in the microsome fraction was measured using the Fiske-Subarrow method. We show that the membrane fraction is rich with active Ca^{2+} , Mg^{2+} -ATPase molecules. The maximal initial rate of ATP hydrolysis is $0.29 \pm 0.06 \mu\text{M P}_i \text{ min}^{-1} (1 \text{ mg protein})^{-1}$. The activity of the thapsigargin-insensitive fraction of total Ca^{2+} , Mg^{2+} -ATPase (PMCA) was $0.11 \pm 0.01 \mu\text{M P}_i \text{ min}^{-1} (1 \text{ mg protein})^{-1}$ and thapsigargin-sensitive (SERCA) $0.4 \pm 0.01 \mu\text{M P}_i \text{ min}^{-1} (1 \text{ mg protein})^{-1}$; data are expressed as means \pm S.E.M., $n = 8$ in all experiments.

We have shown that PCMB (1–1000 μM) has an inhibitory effect on the Ca^{2+} , Mg^{2+} -ATPases. Its apparent inhibitory constant I_{50} for PMCA and SERCA are 245 ± 47 and $52 \pm 6 \mu\text{M}$, respectively. At concentrations higher than 400 μM , PMCA and SERCA were completely blocked. Such data allowed us to speculate about the availability of functionally important SH-groups in the catalytic centre of PMCA and SERCA. In order to understand the mechanism of Ca^{2+} , Mg^{2+} -ATPase inhibition by PCMB, we used dithiothreitol (DTT), a powerful chelator of SH-groups. Incubation of vesicles with DTT caused increase of PMCA and SERCA activities (by 22 ± 8 and $48 \pm 12\%$) allowing us to suppose that DTT could facilitate ATP access to the catalytic centre of molecule. The most prominent restoring effect of DTT on SERCA and PMCA inhibited by PCMB was 45 ± 8 and $32 \pm 11\%$, respectively. That is why we suppose that the functioning of PMCA is predominantly regulated by masked SH-groups. Summarizing all data obtained we can conclude that PMCA and SERCA have high reactive SH-groups, which comprise an important part of the catalytic domain of their molecules and regulate their functioning.

All procedures accord with current local guidelines.

Mitochondrial role in Ca^{2+} homeostasis of rat arterial smooth muscle cells

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Mitochondrial Ca^{2+} uptake increasingly appears to be important in Ca^{2+} homeostasis of many cell types. Here, we examined the effect of a mitochondria Ca^{2+} uptake inhibitor on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) using rat arterial smooth muscle cells. Single cells were dissociated from femoral arteries of rats humanely killed according to a Schedule 1 protocol. $[\text{Ca}^{2+}]_i$ was measured using fura-2 from whole-cell voltage-clamped cells.

Membrane potential was raised from -70 to -50 to -30 mV, yielding a concomitant increase in $[\text{Ca}^{2+}]_i$. When a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, $1\text{ }\mu\text{M}$), was applied whilst membrane potential was maintained at -30 mV, an increase in $[\text{Ca}^{2+}]_i$ was noted in all cells tested. In some cells, the increase in $[\text{Ca}^{2+}]_i$ occurred in two phases. The initial, smaller increase in $[\text{Ca}^{2+}]_i$ was followed by a larger elevation in $[\text{Ca}^{2+}]_i$. The average maximum increase in $[\text{Ca}^{2+}]_i$ caused by CCCP was $280 \pm 180\text{ nM}$ (mean \pm S.E.M., $n = 6$). Next, the effect of CCCP on Ca^{2+} transients caused by Ca^{2+} release was examined. Caffeine (20 mM) was applied repeatedly using a U-tube superfusion system (Evans & Kennedy, 1994) to cells voltage-clamped at -70 mV. When paired half-decay times ($\tau_{1/2}$, time required to reduced increase in $[\text{Ca}^{2+}]_i$ by 50 %) were measured in the control cells, no significant difference was noted ($7.2 \pm 2.0\text{ s}$ and $8.0 \pm 1.7\text{ s}$, $n = 6$, paired *t* test). In a separate set of experiments, caffeine transients were triggered, and upon the restoration of resting $[\text{Ca}^{2+}]_i$, CCCP was applied. This treatment evoked a transient increase in $[\text{Ca}^{2+}]_i$ with the average peak increase of $500 \pm 317\text{ nM}$ ($n = 5$). In the continued presence of CCCP, caffeine applications were repeated producing prolonged Ca^{2+} transients. $\tau_{1/2}$ after CCCP application was $33.2 \pm 7.5\text{ s}$, significantly different from that before CCCP ($3.4 \pm 0.2\text{ s}$, paired *t* test, $n = 5$, $P < 0.05$). The effect of CCCP was partially reversible with the mean $\tau_{1/2}$ of $9 \pm 1.2\text{ s}$ after wash-out of CCCP. CCCP is thought to inhibit mitochondria Ca^{2+} uptake by depolarization of the mitochondrial membrane. When mitochondria membrane potential was reported with Rhodamine 123, CCCP application increased fluorescence intensity, suggesting mitochondrial depolarization. Similar results were obtained with application of diazoxide. On the other hand, Rhodamine 123 signal was slightly reduced by application of oligomycin, suggesting mitochondria membrane hyperpolarization.

Our results suggest that mitochondrial Ca^{2+} uptake is important when $[\text{Ca}^{2+}]_i$ is raised by sustained membrane depolarization or Ca^{2+} release.

Evans, R.J. & Kennedy, C. (1994). *Br. J. Pharmacol.* **113**, 853–860.

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All procedures accord with current UK legislation.

Aromatic amino acids fail to alter neomycin- or extracellular Ca^{2+} -induced ERK 1,2 activity in calcium receptor-expressing HEK cells

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The extracellular calcium-sensing receptor (CaR) plays an important role in whole body calcium homeostasis. Agonists for CaR include extracellular calcium (Ca_o^{2+}) and the aminoglycoside antibiotic neomycin and stimulation of CaR results in Ca_i^{2+} mobilisation and phosphorylation/activation of extracellular-signal regulated kinase (ERK). Conigrave *et al.* (2000) have reported that in CaR-transfected HEK-293 cells (CaR-HEK), aromatic L-amino acids act as allosteric activators of the receptor. That is, in the presence of partially activating concentrations of CaR agonist, cotreatment with an aromatic amino acid such as L-Phe or L-Trp increases agonist-induced Ca_i^{2+} mobilisation.

However, the L-amino acids were only investigated for their effects on Ca_i^{2+} mobilisation. Thus in the current study we investigated whether L-Phe or L-Trp can increase CaR agonist-induced ERK activity in HEK cells stably transfected with CaR (a kind gift from NPS Pharmaceuticals, UT, USA). ERK 1,2 phosphorylation was quantified by immunoblotting using a phospho-specific anti-ERK 1,2 polyclonal antibody. All experiments were performed at 37°C for 5 min in an experimental buffer containing 0.5 mM Ca^{2+} (unless otherwise stated). Data are from a minimum of three independent experiments.

A partially activating concentration of neomycin ($30\text{ }\mu\text{M}$) stimulated ERK 1,2 phosphorylation 3.7-fold (± 1.5 , S.E.M.) above control, an effect which was submaximal since $100\text{ }\mu\text{M}$ neomycin induced a 38.8-fold (± 14.1) stimulation of ERK. However, when cotreated with $30\text{ }\mu\text{M}$ neomycin, L-Phe (10 mM) did not increase the stimulatory effect of neomycin on its own (Neo and L-Phe, $+1.7 \pm 0.4$ -fold). This lack of effect of L-Phe was not agonist specific, since when 2.5 mM Ca_o^{2+} was employed, L-Phe failed to increase the Ca_o^{2+} -induced response (Ca_o^{2+} , $+10.5 \pm 5.2$ -fold; Ca_o^{2+} and L-Phe, $+8.4 \pm 3.2$ -fold). Again, the response to 2.5 mM Ca_o^{2+} was not maximal since 5 mM Ca_o^{2+} caused a 130-fold (± 45.1) stimulation of ERK. Next, we tested whether the lack of effect was amino acid specific. However, L-Trp (10 mM) also failed to increase $30\text{ }\mu\text{M}$ neomycin-induced ERK activation (Neo, $+7.6 \pm 4.7$ -fold; Neo and L-Trp, $+5.8 \pm 1.8$ -fold) or 2.5 mM Ca_o^{2+} -induced ERK activation (Ca_o^{2+} , 4.4 ± 2.2 -fold; Ca_o^{2+} and L-Trp 7.6 ± 3.4 -fold). Again, increasing the CaR agonist concentration in the absence of L-Trp caused substantial elevations in ERK activity ($100\text{ }\mu\text{M}$ neomycin, $+249.6 \pm 171$ -fold; 5 mM Ca_o^{2+} , $+68 \pm 25.2$ -fold). In all experiments, the addition of L-Phe or L-Trp (10 mM) alone had no effect on basal ERK phosphorylation.

Thus the aromatic amino acids L-Phe and L-Trp do not stimulate the CaR to induce ERK activation. If aromatic amino acids are allosteric activators of CaR, so eliciting Ca_i^{2+} mobilisation, then the cell must somehow differentiate between amino acid and 'classic' agonist-induced stimuli to effect differential intracellular signalling. It will be necessary to investigate the reported effects of L-amino acids on CaR using cells that endogenously express the receptor and by measuring a variety of intracellular signals and physiological responses.

Conigrave, A.D. *et al.* (2000). *Proc. Natl Acad. Sci. USA* **97**, 4814–4819.

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Chronic exposure to cAMP upregulates T-type Ca^{2+} channels and TTX-insensitive Na^+ channels in cultured rat chromaffin cells

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We have studied the long-term effects of the membrane-soluble cAMP analogue (8-CPT-cAMP) on the properties of voltage-gated Ca^{2+} channels expressed in cultured rat chromaffin cells from humanely killed rats. Whole-cell Ca^{2+} currents were measured in bath solutions containing 10 mM Ca^{2+} plus 300 nM TTX to block voltage-gated Na^+ channels. In most experiments, cells were held at -80 mV holding potential (V_h). In these conditions, the inward Ca^{2+} currents measured in cells of 3–8 days in culture had similar voltage-dependent

characteristics. The currents started activating at about -30 mV, reached maximal amplitude at $+15$ mV and reversed around $+75$ mV. The I/V characteristics showed a single negative peak at $+15$ mV and maximal voltage dependence at -12 mV. Ca^{2+} currents were fully blocked by $100 \mu\text{M}$ Cd^{2+} and were not affected by replacement of Na^+ with TRIS $^+$ or by adding $50 \mu\text{M}$ Ni^{2+} . This suggests a dominance of high-threshold Ca^{2+} currents and little or no contribution of both low-threshold (T-type) and Na^+ TTX-insensitive currents in control conditions. Nifedipine ($3 \mu\text{M}$) blocked $62 \pm 1.8\%$ (mean \pm S.E.M., $n = 33$ cells) of the total current at 0 mV ($V_h = -40$ mV), suggesting a large contribution of L-channels to the high-threshold Ca^{2+} currents (Hernández-Guijo *et al.* 1999).

Addition of 8-CPT-cAMP ($200 \mu\text{M}$ for 1–4 days to cells in culture) caused the appearance of a new inward current component, which started activating at ~ -50 mV and was quickly inactivating. The time course of inactivation varied from cell to cell. It was either fast ($\tau_f = 3 \pm 0.6$ ms at -20 mV, $n = 5$), slow ($\tau_s = 21 \pm 1.1$ ms, $n = 5$) or a mixture of the two. The amplitude of this component increased with the day of culture and with the duration of 8-CPT-cAMP exposure. In some cells, after 4 days exposure, the contribution was comparable or even larger than the high-threshold Ca^{2+} currents available in normal conditions. Pharmacological dissection revealed the existence of two distinct components: (1) a fast inactivating TTX-insensitive Na^+ current, which disappeared when external Na^+ was replaced with TRIS $^+$ and remained unaltered in the presence of $50 \mu\text{M}$ Ni^{2+} or when external Ca^{2+} was lowered to 0.5 mM and, (2) a T-type low-threshold Ca^{2+} current which persisted when TRIS $^+$ replaced external Na^+ . This component was blocked by $50 \mu\text{M}$ Ni^{2+} and preserved with $30 \mu\text{M}$ Cd^{2+} . A conditioning pulse of 80 ms to -30 mV largely inactivated both transient currents. 8-CPT-cAMP had no action on the amplitude and voltage dependence of high-threshold Ca^{2+} currents available in control conditions. L-type channels, which contributed to 62% of the currents in control conditions, were found not to be significantly different after 2 days of 8-CPT-cAMP incubation ($61 \pm 2.3\%$, $P > 0.05$, paired t test, $n = 30$). It is concluded that 8-CPT-cAMP selectively stimulates the functional expression of ion channels (Ca^{2+} and Na^+), which lower the threshold of firing rate in rat chromaffin cells.

Hernández-Guijo, J.M. *et al.* (1999). *Eur. J. Neurosci.* **11**, 3574–3584.

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Unitary properties of human pancreatic α_{1D} (Cav1.3) L-channel isoform expressed in HEK 293 cells

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We transiently transfected the full length human pancreatic α_{1D} subunit, containing exon 8A in repeat I (Koschak *et al.* 2001), together with the $\alpha_2\delta$ and β_3 subunits in HEK 293 cells and analysed the elementary properties of the corresponding single-channel currents.

Single α_{1D} channel activity could be well resolved in cell-attached patches using 100 mM BaCl_2 and $5 \mu\text{M}$ Bay K 8644. Under these

conditions, α_{1D} channel activity was already evident at relatively low voltages (-30 mV). Step depolarizations of 200 ms to 0 mV from -80 mV revealed uniform single channel activity, characterised by brief openings (2.5 ± 0.29 ms, mean \pm S.E.M., $n = 25$ patches) rarely alternated by long lasting ones (> 10 ms). Open probability (P_o), calculated by excluding the first and last closure of the channel and null sweeps (Carabelli *et al.* 2001), was 0.025 ± 0.005 ($n = 4$) at -30 mV and steeply increased with voltage to reach maximal activation at $+20$ mV (P_o 0.2 ± 0.03 , $n = 12$), with half-maximal P_o at -14.2 mV ($V_{1/2}$). The mean open time increased from 1.2 ± 0.15 ms (-30 mV, $n = 4$) to 3.2 ± 0.2 ms ($+10$ mV, $n = 12$), while mean shut time decreased from 25.5 ± 2.7 ms ($n = 4$) to 12.8 ± 1.1 ms ($n = 12$). Unitary conductance was 17.7 ± 1.4 pS ($n = 3-29$). The steep voltage dependence of α_{1D} channel gating was also evident from the time course of averaged currents. Time to peak of mean currents was 45 ms at -30 mV and decreased to 7 ms at $+10$ mV ($\tau_{act} < 1.5$ ms). Mean amplitude progressively increased with voltage, reaching a maximal value at -10 mV. Averaged currents showed variable degrees of inactivation during pulse duration from -10 to $+10$ mV.

These findings are in line with whole-cell current recordings of the same α_{1D} subunit expressed in tsA-2001 cells (Koschak *et al.* 2001), characterised by low-threshold of activation ($V_{1/2} = -17.5$ mV in 20 mM Ba), fast activation ($\tau_{act} < 1$ ms at 0 mV) and weak inactivation during pulses of 200 ms. Compared with the L-channel of bovine chromaffin cells, the P_o appears significantly lower ($P_{o,max}$ 0.2 versus 0.56 ± 0.05 , $n = 14$, $P < 0.01$, paired t test) and shifted by $20-25$ mV toward negative voltages. The shift reduces to $3-4$ mV when comparing the voltage dependence of P_o with the L-channel of rat insulin secreting RINm5F cells (Magnelli *et al.* 1996) and mouse pancreatic β -cells (Smith *et al.* 1993), suggesting a different distribution of α_{1D} subunits among different neurosecretory cells.

Carabelli, V. *et al.* (2001). *J. Physiol.* **532**, 73–90.

Koschak, A. *et al.* (2001). *J. Biol. Chem.* **276**, 22100–22106.

Magnelli, V. *et al.* (1996). *Pflügers Arch.* **431**, 341–352.

Smith, P.A. *et al.* (1993). *J. Gen. Physiol.* **101**, 767–797.

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The role of Ca^{2+} in the regulation of IEG expression in pancreatic acinar cells

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Ca^{2+} is an important intracellular messenger that regulates a number of processes including gene expression. The aim of this study was to investigate the role of Ca^{2+} in the regulation of immediate early genes (IEG). Expression of IEG rapidly activated by a variety of extracellular stimuli is an important part of the transcriptional intracellular machinery. IEG protein products effectively modulate the expression of 'late' genes.

Experiments were performed on acutely isolated pancreatic acinar cells from mice. Total RNA was reverse transcribed to cDNA and amplified by 30 cycles of PCR with the primers for c-fos, c-myc, c-jun genes and b-actin (internal control). RT-PCR reaction products were subjected to electrophoresis and relative changes in IEG expression were expressed as a percentage.

Elevated extracellular calcium ($[Ca^{2+}]_o$) levels were alone able to activate expression of IEGs: 1 or 10 mM of Ca^{2+} resulted in 23 ± 4.0 and 44 ± 8.1 % increase for c-fos; 21 ± 6.4 and 28 ± 8.4 % for c-myc; and 15 ± 3.4 and 32 ± 5.7 % for c-jun, respectively, compared with those at 0 Ca^{2+} + 200 μ M EGTA; data are expressed as means \pm S.E.M., $n = 6$ in all experiments. Stimulation with calcium-mobilising agonists for 30 min induced much higher levels of expression. A supramaximal concentration of CCK (10 nM) evoked a pronounced $[Ca^{2+}]_o$ -dependent rise in the expression level: the IEG cDNA content was lowest in Ca^{2+} -free (200 μ M EGTA) solution, increased at the physiological level of 1 mM $[Ca^{2+}]_o$ and maximal at 10 mM $[Ca^{2+}]_o$. Quantitatively it yielded an increase of: 102 ± 22 and 163 ± 15 % for c-fos; c-myc 73 ± 13 and 106 ± 24 %; c-jun 49 ± 8 and 59 ± 9 % at 1 and 10 mM of extracellular Ca^{2+} , respectively. We suggest that extracellular calcium, through a Ca^{2+} influx mechanism, plays an important role in rapid induction of IEG expression.

We have found that the increase in c-fos expression induced by 10 μ M acetylcholine (ACh) was also $[Ca^{2+}]_o$ dependent although smaller than for CCK. ACh-induced expression of c-fos in 1 and 10 mM $[Ca^{2+}]_o$ increased by 36 ± 8.1 and 62 ± 14.5 %, respectively. The addition of atropine to the 10 mM $[Ca^{2+}]_o$ containing solution did not cause any detectable changes in the c-fos expression, providing further evidence that the effect of the ACh-induced increase of c-fos expression at $[Ca^{2+}]_o = 10$ mM is mostly due to increased transmembrane Ca^{2+} influx. Finally, we have shown that incubation of cells for 30 min with 1 μ M thapsigargin in 0 Ca^{2+} + 200 μ M EGTA solution did not significantly change IEG expression. Incubation of acini in solutions with 1 or 10 mM Ca and 1 μ M thapsigargin caused elevation of c-fos expression by 59 ± 8 and 70 ± 7 %, respectively. We conclude that extracellular calcium through a Ca^{2+} influx mechanism plays an important role in the rapid induction of IEG expression.

All procedures accord with current UK legislation.

A possible role of acetylcholine secretion in the generation of spontaneous $[Ca^{2+}]_i$ oscillations in developing murine primary muscle cells

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In developing murine primary skeletal muscle cells spontaneous electrical activity is associated with $[Ca^{2+}]_i$ transients and twitches. We have already observed that such spontaneous $[Ca^{2+}]_i$ oscillations were modulated by α -bungarotoxin. Moreover, patch-clamp experiments showed spontaneous openings (i.e. in the absence of exogenous acetylcholine) of the acetylcholine receptor (AChR) channel in oscillating cells. Thus we suggested a role for AChR channel activity in the generation of the spontaneous $[Ca^{2+}]_i$ oscillation in developing murine primary skeletal muscle cells. To further understand this phenomenon, experiments have been performed on murine primary skeletal muscle cells (i28) isolated from hindlimb muscles of a mouse killed by cervical dislocation. Then, i28 cells were differentiated *in vitro* and loaded with the dye fura-2. The videoimaging technique was used to measure the $[Ca^{2+}]_i$ oscillations.

In the first series of experiments we investigated the effect of agents capable of enhancing the probability of the AChR channel

openings. In particular, nicotine was applied at submaximal concentrations (50–200 nM). In i28 oscillating cells, the addition of the agonist invariably caused a sudden increase in the frequency of spontaneous $[Ca^{2+}]_i$ oscillations. In addition, nicotine was also able to trigger the oscillatory activity when administered during the silent periods between two consecutive bursts of spontaneous $[Ca^{2+}]_i$ oscillations ($n = 20$). We also considered the possibility that developing i28 cells could release acetylcholine, activating their own AChRs (Hamann *et al.* 1995). Thus the effect of acetylcholinesterase on spontaneous $[Ca^{2+}]_i$ oscillations was investigated. When the enzyme (200 units ml^{-1}) was administered, the oscillatory activity stopped in 45 % of the cells observed ($n = 22$). In contrast, the acetylcholinesterase inhibitor edrophonium (500 nM) caused an increase in the frequency of spontaneous $[Ca^{2+}]_i$ transients (6/8). Moreover, preliminary immunocytochemistry experiments demonstrated the presence of choline acetyltransferase immunoreactivity in developing i28 cells.

To conclude, our results confirmed the involvement of AChR channels in the generation of spontaneous $[Ca^{2+}]_i$ oscillations. Interestingly, the secretion of acetylcholine by differentiating i28 cells may sustain the activity of the AChR channels and consequently the $[Ca^{2+}]_i$ oscillations.

Hamann, M. *et al.* (1995). *J. Physiol.* **489**, 791–803.

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Differing roles for JNK in the modulation of osmosensitive taurine efflux in C6 glioma and HeLa cells

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Taurine efflux through the volume-sensitive organic osmolyte/anion channel (VSOAC) is activated by hypotonic shock. The signalling pathways that link membrane stretch or the reduction in intracellular osmolarity to activation of taurine efflux remain unclear. Tyrosine phosphorylation is implicated in this process since the tyrosine kinase inhibitor tyrphostin a23 reduces osmosensitive taurine efflux in astrocytes (Mongin *et al.* 1999). Significantly, members of the mitogen-activated protein kinase (MAPK) family are coactivated with taurine efflux by hyposmolarity. To determine whether MAPKs are involved in regulating taurine efflux in the glioma cell line C6, inhibitors of the three key MAPKs were tested: the extracellular-signal regulated kinase (ERK) inhibitor PD 98059, the p38 inhibitor SB 202190 and the c-Jun NH_2 -terminal kinase (JNK) inhibitors dicumarol (Krause *et al.* 2001) and SP 600125. JNK inhibitors were also tested on the epithelial cell line HeLa to determine whether JNK was implicated in other cell lines. ^{14}C -taurine efflux was measured using methods previously described (Belsey *et al.* 2002). Since taurine efflux can be inhibited by blocking the pore of VSOAC or by inhibiting its activation pathway, the test compounds were either pre-incubated or directly applied to HeLa and C6 cells.

At 100 μ M, tyrphostin a23 inhibited taurine efflux in HeLa after pre-incubation and when applied directly by 25.0 ± 1.6 and 32.2 ± 10.0 % (\pm S.E.M.; $n = 3$ for all data), respectively. It also inhibited taurine efflux in C6 cells after pre-incubation and when

applied directly by 25.4 ± 4.9 and 37.2 ± 1.7 %, respectively. Taurine efflux is primarily activated by a decrease in osmolarity rather than membrane distension (Deleuze *et al.* 2000), unlike ERK and p38, which are primarily activated by membrane distension. Consistent with this notion, we found only a minimal effect on taurine efflux when both ERK and p38 were inhibited in C6 glioma. JNK is hypotonically activated, although little is known about its involvement in modulating taurine efflux. At $100 \mu\text{M}$, dicumarol inhibited taurine efflux in C6 after pre-incubation and when applied directly by 72.6 ± 4.3 and 80.3 ± 2.3 %, respectively. It also blocked taurine efflux in HeLa by 90.3 ± 3.5 %, although it had only a minimal effect following pre-incubation. The more specific JNK inhibitor SP 600125 had minimal effect in HeLa when pre-incubated or when directly applied, supporting the notion that JNK plays no role in taurine efflux in this cell line and indicating that any blockade effect by dicuramol is not related to its effect on JNK. However, pre-incubating SP 600125 in C6 produced 50.7 ± 4.0 % block at $100 \mu\text{M}$. Taken together, these results suggest that JNK plays a role in the osmosensitive taurine efflux pathway but this involvement is cell-type specific, since JNK only appears to play a role in C6, not HeLa.

Belsey, M.J. *et al.* (2002). *Br. J. Pharmacol.* **135**, 1P.

Deleuze, C. *et al.* (2000). *J. Physiol.* **523**, 291–299.

Krause, D. *et al.* (2001). *J. Biol. Chem.* **276**, 19244–19252.

Mongin, A.A. *et al.* (1999). *Am. J. Physiol.* **276**, 1226–1230.

Role of PI3K γ in β -adrenergic stimulation of cardiac contractility and L-type calcium current

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In cardiac myocytes, voltage-gated L-type calcium channels play a crucial role in excitation and contraction coupling. The two main receptor systems involved in the regulation of contractility are the muscarinic and the β -adrenergic, which exert opposite effects on the calcium current (I_{Ca}) through the activation of G-proteins. Recent reports have demonstrated that the different subtypes of β -adrenergic receptors are coupled to both G_i and/or G_s ; β_2 -AR can activate G_i and G_s proteins, whereas β_1 -AR couple only to G_s (Zhu *et al.* 2001). The modulation of I_{Ca} is mainly due to hormones and mediators acting through the activation of different protein kinases; among them, phosphoinositide 3-kinases (PI3Ks) have been recently discovered to play an important role in the transduction of the intracellular signalling mediated by G-protein coupled and tyrosine kinase receptors (Macrez *et al.* 2001; Steinberg, 2001). A signalling pathway from PI3K to the serine/threonine protein kinase Akt/PKB is implicated in some cellular responses; in particular, it has been shown that in adult mouse cardiac myocytes, β_2 -ARs coupled to G_i signalling, activate PI3K-Akt pathway through the $G\beta\gamma$ subunit and are directly implicated in the survival effect (Naga Prasad *et al.* 2000; Zhu *et al.* 2001).

In our study, we compared the effect of the β -adrenergic agonist, Isoproterenol (Iso), on wild-type and PI3K knock-out mice, looking at the modulation of contractility in atrial and papillary muscles and of I_{Ca} in isolated ventricular cells (Gallo *et al.* 2001). Animals were killed according to the procedures of the National guidelines.

The amplitude of contractility evoked by Iso in wild-type atrial muscle was significantly different, compared with the knock-out animals. The maximal Iso ($1 \mu\text{M}$) effect was 7.8 ± 0.7 -fold with respect to the control values in $n = 5$ experiments in the wild-type mice (all data are expressed as means \pm S.E.M.). The positive inotropic effect was significantly reduced in the knock-out mice (4.5 ± 0.6 ; $n = 6$, t test**). Surprisingly, in the papillary muscle the inotropic effect seemed rather to be higher in the PI3K-deficient mice (6.3 ± 2.9 ; $n = 5$), than the wild-type (3.4 ± 0.5 ; $n = 6$). The role of PI3K γ in the modulation of I_{Ca} by Iso was studied in adult cardiac myocytes, enzymatically dissociated from wild-type and knock-out animals. In 13 cells from wild-type mice, the percentage of stimulation of Iso above the control values was 30.2 ± 2.0 %, and, similarly to papillary muscle, the Iso effect was higher in the knock-out cells (86.2 ± 6.5 %, $n = 5$, t test**). Taken together, these data support the idea that, in cardiac muscle, PI3K γ has a physiological role in the regulation of I_{Ca} and contractility. The differential effects of Iso observed in atrial and ventricular preparations suggest that in ventricular cells, the intracellular kinase-mediated signal, which modulates I_{Ca} and inotropism, should be linked to a G_i β -ARs-coupled subtype. The opposite inotropic response of atrial and ventricular tissues to Iso might be explained on the basis of different β -ARs distribution within the heart.

Gallo, M.P. *et al.* (2001). *Pflügers Arch.* **441**, 621–628.

Macrez, N. *et al.* (2001). *Circ. Res.* **89**, 692–699.

Naga Prasad, S.V. *et al.* (2000). *JCB* **275**, 4693–4698.

Steinberg, S.F. (2001). *Circ. Res.* **89**, 641–644.

Zhu, W.Z. *et al.* (2001). *Proc. Natl Acad. Sci. USA* **98**, 1607–1612.

All procedures accord with current National guidelines.

Analysis of the non-genomic action of progesterone in human spermatozoa, at the single cell level

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Micromolar concentrations of the steroid hormone progesterone induce calcium influx and acrosome reaction (AR) in human sperm. Calcium influx occurs as an initial transient, lasting up to 4 min, followed by a sustained phase. The study aimed to analyse the effects of progesterone concentration on the calcium transient and subsequent AR.

Highly motile spermatozoa from human donors were harvested using direct swim-up with sEBSS media (0.3 % BSA), and were incubated for 6 h at 6 million cells ml^{-1} . For single cell analysis, sperm were labelled with Oregon Green Bapta AM, adhered to coverslips in a perfusion chamber and imaged at 25°C . For fluorimetric studies sperm were labelled with fura-2 AM and imaged at 37°C ; a $K_d = 224 \text{ nM}$ was assumed.

Progesterone sensitivity was measured across a series of concentrations ranging from 0.3 nM to $30 \mu\text{M}$. AR was induced at a concentration of 3 nM ($P < 0.025$, $n = 9$, paired t test) and all higher concentrations. The amplitude of the initial calcium transient was analysed using fluorimetry. A dose-response curve showed that a maximum $[\text{Ca}^{2+}]_i$ was stimulated with $0.3 \mu\text{M}$ progesterone ($730 \pm 156 \text{ nM}$, S.E.M. $n = 8$); however, this was not significantly different from doses of 30 nM, $3 \mu\text{M}$ and $30 \mu\text{M}$.

($P > 0.05$, paired t test). Stimulation by 3 nM progesterone gave a $[Ca^{2+}]_i$ transient of 70 ± 11 nM ($n = 7$), which was significantly lower than stimulation with 30 nM ($P = 0.04$, $n = 5$, paired t test). A slight but significant maintained elevation of resting $[Ca^{2+}]_i$ (20 ± 3 nM, $P = 0.01$, $n = 4$, paired t test) was seen when cells were stimulated with 0.3 nM progesterone.

Aitken *et al.* (1996) reported that after treatment with 5 μ M progesterone sperm became refractory to a second progesterone stimulus for 30–60 min. Populations of cells were stimulated sequentially with 3 nM, 30 nM and then 3 μ M progesterone with a 7 min delay between additions, and were shown to re-evoked at each concentration in the fluorimeter. Single-cell analysis was then used to assess the number of cells responding to each progesterone concentration in the series. Analysis of 1209 cells revealed that 68% were showing significant calcium transients at all three concentrations, indicating re-evocation of the progesterone transient $[Ca^{2+}]_i$ pathway within individual cells. However, this re-evocation of the response does not occur if the initial concentration used is 0.3 μ M or greater.

We conclude that progesterone exerts its full effect at doses much lower than those previously used and at these doses cells can respond to repeated stimuli.

Aitken, R.J. *et al.* (1996). *Endocrinology* 137, 3999–4009.

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.

a secondary increase in R_f in normal cells to 3.12 ± 0.87 ($n = 3$, $P < 0.04$) and 8.05 ± 1.71 ($n = 3$, $P < 0.001$), respectively, so that the final R_f was no less than that seen in PE. As exposure to AA induces a strong contraction, the secondary rise in R_f could be an artifact of a delayed contraction. However, fluorescence ratio imaging showed that in 11/18 cells the secondary rise in R_f preceded the contraction.

AA can also be metabolised by a monooxygenase (MOX). Thus the effect of COX or LOX inhibition on $[Ca^{2+}]_i$ could be explained by metabolites of the COX and LOX pathways interacting to inhibit the secondary $[Ca^{2+}]_i$ increase, or an increase in the effective [AA] potentiating Ca^{2+} mobilisation either directly or indirectly via a MOX metabolite. The potentiated AA response seen in normal cells after indomethacin pretreatment (4.55 ± 1.4 , $n = 3$) was inhibited by also pretreating with the MOX inhibitors metyrapone (50 μ M: $26 \pm 6\%$, $n = 3$; $P < 0.001$) and isoniazid (200 μ M: $19 \pm 7\%$, $n = 3$; $P < 0.001$). We conclude that in pre-eclampsia the activities of COX or LOX are reduced or that of MOX increased, diverting more AA through the MOX pathway to cause a secondary $[Ca^{2+}]_i$ rise in response to AA.

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

Altered arachidonic acid metabolism and Ca^{2+} mobilisation in pre-eclamptic human umbilical artery smooth muscle cells

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Pre-eclampsia (PE) is a disease of pregnancy with maternal hypertension as one of its defining symptoms. The aetiology of PE may involve a disturbance of arachidonic acid (AA) metabolism reflected by altered levels of circulating AA and its metabolites that are known to affect Ca^{2+} mobilisation and will cross the placental barrier. We have investigated the effect of AA on $[Ca^{2+}]_i$ in human umbilical artery smooth muscle cells (HUASMC) isolated and cultured from cords obtained with ethical approval and informed consent. We measured the fluorescence ratio (R_f) of fura-2 at 340/380 nm excitation in single or small groups of 3–5 cells as a measure of cytosolic $[Ca^{2+}]$.

Both normal and PE HUASMC responded to 50 μ M AA with an increase in R_f that was larger in PE (2.99 ± 0.47 , $n = 19$) than normal (1.44 ± 0.28 , $n = 16$) (means \pm S.E.M., n = number of cords, $P < 0.009$, Student's unpaired t test). The response in PE cells was also qualitatively different, often showing a secondary increase in R_f that was delayed by up to 600 s. The increased response in PE cells could be the result of altered AA metabolism via the cyclo-oxygenase (COX) or lipoxygenase (LOX) pathways. To test this we used indomethacin and NDGA (10 μ M, 20 min pre-incubation) as COX and LOX inhibitors, respectively. Neither significantly affected the response of PE cells to AA (2.97 ± 0.93 , $n = 8$; 3.80 ± 1.33 , $n = 8$; $P > 0.4$), but both induced