C6

Characterisation of Ca^{2+} currents in human prostate smooth muscle cells

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Benign prostatic hyperplasia (BPH) is a stromal disease that subsequently affects epithelial growth. Growth factors activate downstream intracellular pathways, but the linkage to cellular proliferation is unclear. In many cells growth factors raise intracellular Ca^{2+} (de Laat *et al.* 1999) and Ca^{2+} channel activity correlates with cell proliferation. T-type channels are prominent during developmental cell growth, and activity is modulated by growth hormone or conditions leading to cellular hypertrophy and proliferation (Guo *et al.* 1998), but the channels are less evident in differentiated cells. Ca^{2+} channels have not been characterised in human prostate smooth muscle, and the aim was to carry out an analysis prior to an investigation of the cellular functions of growth factors.

Prostate samples were obtained, with Ethical Committee approval, from patients undergoing either transurethral resection of the prostate (TURP) or radical prostatectomy. They were stored in Ca^{2+}-free Hepes-Tyrode solution. Isolated cells were prepared by collagenase-based digestion and, in general, this was easier with prostatectomy samples as retrieved tissue was less damaged. Experiments were carried out in HCO_3^-/CO_2-buffered Tyrode solution at 37 °C. Whole-cell recordings used patch-electrodes, and whole-cell capacitance was measured on membrane rupture to scale membrane currents to unit capacitance. Cs^+-based electrode solutions, to block outward currents, were used to record inward currents, and K^+-based solutions to record resting potentials. Data are means ± S.D., and differences between data sets ($P < 0.05$) were tested using Student's unpaired *t* test.

The resting potential was −63 ± 11 mV ($n = 8$) and action potentials were elicited with Cs^+-filled electrodes; maximum upstroke velocity was about 0.8 V s^{-1}. Inward current was dependent on superfusate Ca^{2+} with peak current at 0 mV. Current density (holding potential, V_h, (−100 mV) was significantly less in cells from TURP chips (72 ± 6 pF) compared to prostatectomy (71 ± 5 pF) samples (1.9 ± 0.27 *vs.* 3.3 ± 0.40 pA pF^{-1}, $n = 14$ and 24, respectively), otherwise there were no differences between the two groups. The current could be divided into two components (L-type and T-type) as determined by the dependence on V_h −100 or −40 mV) or their sensitivity to 30 μM verapamil and 20 μM $NiCl_2$. Separate experiments showed that these were maximal concentrations of blockers. Half-maximum activation was −7.2 ± 1.0 mV for L-type and −36.1 ± 2.0 mV for T-type current ($n = 15$). The L-type current was the larger component. At their respective maximum voltages L-type current density was 1.56 ± 0.47 pA pF^{-1} (+10 mV) and T-type current was 0.83 ± 0.16 pA pF^{-1} (−20 mV).

This is the first study to characterise systematically inward current in human prostate smooth muscle. Mean peak net inward current is about 60 % of that in detrusor (Sui *et al.* 2001) and has two components that have properties of L-type and T-type channels. Of interest is the Ni^{2+} dependency of the T-type component as there are several isoforms of the α-subunit. The α_{1G} subtype has a low affinity for Ni^{2+} compared to the α_{1H} subtype (Lee *et al.* 1999). In detrusor the low affinity subtype is present (Sui *et al.* 2001) but in prostate the low [$NiCl_2$] (20 μM) used for block suggests the α_{1H} subtype. Thus any manipulation of prostate channel activity could have selective effects over detrusor.

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

C7

The cellular properties of human sub-urothelial myofibroblasts

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The mechanism whereby the urinary bladder senses fullness is unclear. ATP is released from the serosal surface when the bladder is stretched (Ferguson *et al.* 1997). Furthermore, $P2X_3$ receptor-deficient knock-out mice exhibit urinary retention and their absence from sub-urothelial sensory afferents may underlie reduced sensation of bladder fullness (Cockayne *et al.* 2000). Recently a sub-urothelial myofibroblast layer has been described with cells that make close appositions to nerves (Wiseman *et al.* 2003) and are connected to each other through gap junctions (Sui *et al.* 2002). We have studied the electrophysiological properties of these cells and their response to ATP to determine if they could play a role in the sensory process.

Human bladder wall samples were obtained with Ethical Committee approval from patients undergoing either cystectomy or bladder augmentation. The urothelium was dissected from the underlying detrusor and digested at 37 °C with a collagenase-based medium. The tissue was partially disrupted into round urothelial cells and ovoid or spindle-shaped cells with or without dendrite-like structures. Experiments were performed at 37 °C in a HCO_3^-/CO_2-buffered superfusate. Electrophysiological recordings were made with patch-type electrodes filled with a KCl/EGTA-based solution. Intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) was measured with the fluorochrome Fura-2. Data are presented as means ± S.D.

Cell capacitance was 27 ± 16 pF ($n = 53$) and membrane potential was −63 ± 13 mV ($n = 16$). However the membrane potential showed either spikes or small fluctuations. Membrane resistance was 8.5–9.0 × 10^4 Ω cm^2 from resting potential changes to small depolarising or hyperpolarising currents. Under voltage clamp, on depolarisation from −100 mV sustained outward currents were preceded in some cells by a small, transient inward current. Inward current was attenuated in Ca^{2+}-free superfusate and peaked at about −10 mV (25 ± 10 pA). Outward current showed outward-going rectification with spontaneous transient components, exhibited a reversal potential at about −80 mV and was greatly attenuated by 30 mM tetraethylammonium chloride. ATP at 30 μM increased $[Ca^{2+}]_i$ from 90 ± 60 nM to a peak of 832 ± 500 nM (18 transients, 4 cells). ATP was also applied to several epithelial cells, but no Ca^{2+} transients were observed. ATP at 100 μM generated, after a delay, transient inward currents when the membrane was clamped at −60 mV. The maximum peak inward current was 23 ± 17 pA ($n = 6$).

It is possible to isolate cells from the urothelial layer of the bladder, distinct from epithelial cells: they are not contaminant smooth muscle cells from the underlying detrusor but represent a distinct urothelial/suburothelial cell population, previously identified as myofibroblasts. The cells had many characteristics

of excitable cells and responded to ATP so that they could form an electrical network to distribute an electrical signal over a reasonable area upon focal depolarisation. These properties are consistent with the hypothesis that these cells form an intermediate stage in the modality of bladder sensation and could act as a variable gain integrating stage.

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C8

Colocalization and expression of β_2-adrenergic receptor and large conductance calcium-activated potassium channel in human myometrium

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β_2-Agonists have been shown to modulate K^+ currents and stimulate the activity of large conductance calcium-activated potassium (BK_{Ca}) channels in myometrium (Anwer *et al.* 1992; Hamada *et al.* 1994). However, it is not known whether the mechanism of this activation involves a direct or indirect interaction between the β_2-adrenergic receptor (AR) and BK_{Ca} channels. The aim of this study was to investigate the nature of this association using confocal immunofluorescence and co-immunoprecipitation studies.

This study was approved by the Southern Derbyshire Ethics Committee and written informed consent was obtained from all tissue donors. Myometrial biopsies were obtained from singleton term pregnant women (gestational age >37 weeks) undergoing either (1) elective Caesarean section before the onset of labour ($n = 6$) or (2) emergency Caesarean section after spontaneous labour (cervical dilatation > 3 cm; $n = 6$). Tissue was divided into two parts. One half for confocal microscopy was processed to yield cytospins. Thus, isolated myometrial cells were obtained following enzymatic dispersal, and fixed in 2% (w/v) paraformaldehyde for double staining immunofluorescence with the avidin–biotin complex technique. The remaining tissue was stimulated with 1 mM ritodrine (a β_2-sympathomimetic) for immunoprecipitation with either β_2-AR or BK_{Ca} channel antibody. Proteins were resolved by 10% SDS–PAGE then blotted with either β_2-AR or BK_{Ca} antibody as appropriate.

Confocal microscopic visualization demonstrated the colocalization of β_2-AR and BK_{Ca} channel proteins mainly at the plasma membrane of human myometrium from both labouring and non-labouring women. Co-immunoprecipitation experiments revealed that β_2-AR antibody was able to immunoprecipitate BK_{Ca} channel protein and that BK_{Ca} antibody was able to immunoprecipitate β_2-AR protein in both groups of women.

This study demonstrated that β_2-AR and BK_{Ca} channel are colocalized in the human myometrium. They are also apparently linked by a direct protein–protein interaction. This close association suggests the synergistic role of these two membrane proteins in myometrial relaxation. Further studies are needed to examine the functional correlation between the β_2-AR and BK_{Ca} channel in pregnant human myometrium particularly at the onset of labour.

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C9

Role of P1-receptors in the contractile function of guinea-pig and human detrusor smooth muscle

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Nerve-mediated contractions of detrusor smooth muscle are mediated through the release of two neurotransmitters, acetylcholine and ATP (Burnstock *et al.* 1978). These are rapidly broken down in the synapse by specific extracellular enzymes. The breakdown product of ATP, adenosine, reduces contractions in other smooth muscle to reduce contractures (Lynch & Huddart 1991). Adenosine may modulate contraction either by acting on the muscle or the motor-nerve ending and is believed to act through P1-receptors that are divided into four subtypes (A1, A2a, A2b, A3). The site and subtypes of these receptors in detrusor smooth muscle are unclear and this study has addressed these questions.

Detrusor strips were obtained from guinea-pig bladders or human biopsy samples, the latter obtained with local ethical committee permission and informed patient consent. Guinea-pigs were humanely killed. Strips were superfused at 37 °C in HCO_3^-/CO_2-Tyrode solution (pH 7.35). Contractions were evoked either by field-stimulation of embedded motor nerves (3 s train of 0.1 ms pulses, 1–40 Hz, abolished by 1 mM TTX) or direct activation of the muscle with the acetylcholine analogue carbachol (10 mM). All chemicals except ZM241385 were obtained from Sigma UK. Data are expressed as means ± S.D. (n), where n is the number of experiments. Student's unpaired t test was used to test for significance between data sets ($P < 0.05$). $pEC_{50} = -\log_{10}EC_{50}$.

Adenosine (1 mM), the non-selective P1-agonist 5'-(*N*-ethylcarboxamido)adenosine (NECA) (10 mM) and the A1-selective agonist N^6-cyclopentyladenosine (CPA) (10 mM) reduced nerve-mediated contractions in guinea-pig tissue. (63 ± 11% (4), 61 ± 11% (6), 71 ± 11% (10) of control, respectively, 8 Hz stimulation). The reduction by CPA was significantly less than that induced by adenosine or NECA. Concentrations were maximal and were determined for each agent previously (pEC_{50} values were 3.88 ± 0.83 (4), 6.44 ± 0.29 (6) and 7.01 ± 0.86 (4), respectively). The A2a- and A2b-antagonists ZM241385 (100 nM) and alloxazine (1 mM) had no significant effect. Carbachol contractures were not significantly altered by any P1-agonist. There was a frequency-dependent action of P1-agonists, with greater attenuation at low stimulation frequencies. Nerve-mediated contractions with human detrusor strips from stable and unstable bladders were not reduced by any of the above P1-agonists. Carbachol contractures were also unaffected. Force–frequency relationships in the presence of α,β-methylene-ATP (10 mM) reduced contractions to a greater proportion at lower stimulation frequencies.

We interpret these data to signify that adenosine and analogues with A1-activity exert a presynaptic action on guinea-pig detrusor preparations, but there is little effect postsynaptically. In human there was little effect by the P1-receptor agonists pre- and postsynaptically. P1-receptor agonists are more effective at reducing contractions at lower stimulation frequencies that coincide with a proportionally higher release of ATP.

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C10

Prostaglandin $F_{2\alpha}$-induced contraction and increased intracellular calcium in small pulmonary arteries of the rat

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The mechanisms by which prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induces increases in $[Ca^{2+}]_i$ and activates smooth muscle contraction are unclear. We have studied the effects of $PGF_{2\alpha}$ on contraction and $[Ca^{2+}]_i$ in the rat pulmonary artery using fura PE-3 AM-loaded isolated intrapulmonary arteries.

Rats were humanely killed by cervical dislocation.

Low concentrations of $PGF_{2\alpha}$ (10–100 nM) caused a transient increase in $[Ca^{2+}]_i$ followed by a sustained plateau but did not induce contraction. Further increases in $[PGF_{2\alpha}]$ (3–30 μM) caused a dose-dependent increase in tension but no further increase in $[Ca^{2+}]_i$. In nominally Ca^{2+}-free solution, the transient component of the Ca^{2+} response was still present but the $[Ca^{2+}]_i$ plateau was abolished. Even without further elevation in $[Ca^{2+}]_i$, tension still increased in a dose-dependent manner. Tension dose–response curves show that V_{max} in Ca^{2+}-free physiological salt solution (PSS) was 70 % of that in Ca^{2+}-containing PSS, i.e significantly smaller ($P < 0.05$, Student's paired t test). However, EC_{50} values were similar: 3.4 ± 0.7 μM ($n = 7$, mean ± S.E.M.) in Ca^{2+}-containing PSS and 5.1 ± 0.8 μM ($n = 7$) in Ca^{2+}-free PSS. The $[Ca^{2+}]_i$ transient and plateau could be reproduced with the selective $PGF_{2\alpha}$ receptor (FP) receptor agonist fluprostenol. However, no increase in tension was caused by fluprostenol even at 10 μM. The $[Ca^{2+}]_i$ transient and plateau evoked by both fluprostenol and $PGF_{2\alpha}$ could be inhibited with the selective FP receptor antagonist AL-8810, or with thapsigargin. In contrast the thromboxane A_2 analogue U-46619 caused a sustained dose-dependent increase in $[Ca^{2+}]_i$ and tension. The selective thromboxane A_2 receptor (TP) antagonist SQ29584 completely blocks increases in $[Ca^{2+}]_i$ and tension caused by U-46619, and abolished the rise in tension, but not the $[Ca^{2+}]_i$ response, induced by $PGF_{2\alpha}$.

These results suggest that $PGF_{2\alpha}$ elevates $[Ca^{2+}]_i$ via FP receptor-induced Ca^{2+} release from intracellular stores with subsequent capacitative calcium entry. However, this is not sufficient to cause contraction, which requires simultaneous activation of TP receptors.

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PC9

Sphingosylphosphorylcholine-mediated vasoconstriction of rat small pulmonary arteries

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Bioactive sphingolipids and their metabolites such as sphingosylphosphorylcholine (SPC) are believed to play an important role in regulating various cellular processes, primarily via receptors of the EDG family. Recent evidence has suggested a role for sphingolipids in the control of vascular tone, but the signalling pathways involved may differ significantly depending on the vascular bed, with calcium entry, release from stores, and activation of RhoA and Rho kinase having variable degrees of importance (Coussin *et al.* 2002; Shirao *et al.* 2002). The action of sphingolipids has not been investigated in the pulmonary circulation, where there is evidence that Rho kinase may be of particular importance. We therefore examined the effect of SPC on small (300–500 μm i.d.) intrapulmonary arteries (IPA) of the rat.

Rats were humanely killed by cervical dislocation. IPA were mounted on a small vessel myograph. In some cases intracellular calcium was estimated simultaneously using Fura-PE3. Data are given as means ± S.E.M. and were tested for significance using ANOVA.

SPC (3–100 μM) caused a relatively slowly developing, concentration-dependent vasoconstriction in IPA, which reached a plateau after ~30 min. The maximum tension was 111 ± 15 % of the response to 80 mM KCl ($n = 7$), with an EC_{50} of 18 ± 6 μM. Removal of the endothelium or application of 100 μM L-NAME caused ~20 % increase in tension without altering the EC_{50} ($n = 4$ and 7). Simultaneous recording of tension and intracellular calcium showed that the development of tension was associated with a rise in intracellular calcium, although tension continued to increase after calcium reached a steady state. Removal of calcium from the bathing solution shifted the concentration–response curve to the right (EC_{50}: 97 ± 43 μM, $n = 6$, $P < 0.05$), without having a significant effect on maximum tension (135 ± 39 %, $n = 6$). Once tension had reached steady state following application of 10 μM SPC, application of the L-type calcium channel blocker diltiazem (10 μM) had a relatively minor effect on tension and intracellular calcium. However, the Rho kinase inhibitor Y27632 (10 μM) caused partial relaxation of ~35 % ($n = 5$).

We conclude that SPC-induced constriction of rat IPA is mediated through both calcium entry, primarily via a voltage-independent pathway, and Rho kinase-dependent calcium sensitisation, but that different receptors may be responsible for these two actions.

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