Temperature-induced contraction in human radial artery smooth muscle

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In coronary artery bypass grafting (CABG), vasospasm of arterial conduits is a significant clinical problem. Unlike the internal mammary artery, the radial artery (RA) is stored in theatre before grafting. Storage induces temperature shocks upon the RA, both following the initial isolation and upon the return of the graft to body temperature. We have studied the effect of temperature changes in native and cultured human RA smooth muscle.

Sections of RA were obtained surplus to CABG, with ethical committee approval. Rings of RA, pretensioned at 30 mN for 1 h, were then relaxed to 10 mN for a further 30 min. RA smooth muscle cells (hRASMCs) were cultured from explants as described previously (Conant *et al.* 2002) and loaded with fura-2. Data were collected at 37 °C, unless indicated, from at least three separate patients or batches of cells and are presented as means ± S.E.M.

In arterial rings the addition of media at 22 °C to the sample chamber resulted in a transient increase in tension from a basal value of 10.2 ± 0.5 mN to a peak value of 14.6 ± 0.6 mN ($n = 29$). Glyceryl trinitrate (GTN; 0.5 mg ml^{-1}; 2.2 mM) or 0.5 mg ml^{-1} (1.5 mM) papaverine prevented this response. In hRASMCs the rapid addition of media at 22 °C led to a transient increase in $[Ca^{2+}]_c$, in 53 % of the cells tested, from a resting value of 221 ± 50 nM ($n = 100$), to a peak of 315 ± 14 nM. The presence of 1 mM EGTA did not affect the response in either arterial rings or hRASMCs.

In arterial rings gradual cooling to 22 °C led to a slow drop in tension from 15.0 ± 0.6 mN ($n = 25$) to 11.8 ± 0.5 mN. Subsequent rewarming led to an increase in tension to 26.8 ± 1.6 mN, beyond that of the initial baseline, which was reduced in the presence of GTN, papaverine or EGTA. hRASMCs slowly cooled to 22 °C and then rewarmed to 37 °C showed a rapid and sustained increase in $[Ca^{2+}]_c$ from 103 ± 9 to 194 ± 19 nM ($n = 144$) accompanied by spontaneous $[Ca^{2+}]_c$ oscillations in 23 ± 10 % of the cells. The increase in basal calcium was unaffected by GTN or EGTA but calcium oscillations were.

In conclusion the human RA is sensitive to a rapid drop in media temperature. Whilst slower cooling reduces basal tension, subsequent rewarming leads to an increase in tension beyond initial values. These responses are mirrored by changes in $[Ca^{2+}]_c$ in cultured hRASMCs. The effects of temperature should be considered during the preparation of the RA for CABG.

Conant, A.R. *et al.* (2002). *J. Cardiovasc. Pharmacol.* **39**, 130–141.

All procedures accord with current local guidelines.

Modulation of contractility in phenoxybenzamine-treated human radial arteries prepared for coronary artery bypass surgery

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The use of the radial artery (RA) in coronary artery bypass surgery has increased over the last few years. This is due to its superior patency compared with saphenous vein grafts. However, the RA is at increased risk of spasm compared with the internal mammary artery. This can have an adverse clinical outcome due to myocardial hypoperfusion. Recently Taggart *et al.* (2000) described the use of phenoxybenzamine (PhB) to prevent spasm in the RA. PhB binds irreversibly to the α_1 adrenoceptor, the dominant adrenoceptor in the RA (He & Yang, 1998) and treatment with PhB renders the graft insensitive to catecholamines present in the circulation post-operatively (Taggart *et al.* 2000). However, circulating levels of other vasoconstrictors also rise in the post-operative period (Downing & Edmunds, 1992), which means that additional strategies may be required to prevent spasm.

Sections of RA, treated with 1 mg ml^{-1} (1.6 mM) PhB, were obtained surplus to surgery with ethical approval. Rings of RA, pretensioned at 30 mN for 1 h, were then relaxed to 10 mN for a further 30 min, before the addition of agonists. We then went on to evaluate the effect of 0.5 mg ml^{-1} (2.2 mM) glyceryl trinitrate (GTN), papaverine (0.5 mg ml^{-1}; 1.5 mM) and 10 μM diltiazem against these responses. Responses were compared using Student's unpaired t test ($P < 0.05$). Data are presented as means ± S.E.M.

Sections of RA treated in theatre with papaverine, not PhB, responded to noradrenaline with an EC_{50} of 1.8 ± 0.6 mM and a maximal tension of 34.0 ± 1.9 mN ($n = 10$). PhB-treated arterial sections were insensitive to a 2 or 20 μM application of noradrenaline ($n = 35$). PhB-treated RA did respond to 100 nM vasopressin (26.7 ± 2.1 mN; $n = 11$), endothelin-1 (23.0 ± 1.7 mN; $n = 12$) and angiotensin II (27.9 ± 2.0 mN; $n = 21$). In addition, PhB-treated RA also responded to isomolar KCl in a concentration-dependent manner, giving responses at 60 mM of 25.7 ± 1.4 mN ($n = 31$). Acute addition of papaverine and GTN effectively antagonised responses elicited by all of the vasoconstrictors tested. Diltiazem reduced responses to KCl but was much less effective against endothelin-1 and had no effect against angiotensin II or vasopressin.

We conclude that receptors other than the adrenoceptors may play an important role in the aetiology of spasm in the RA and that GTN or papaverine may be useful supplements to PhB.

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Effects of organ culture on contractility and phospholipase C isoform expression of rat mesenteric arteries

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The phospholipase C (PLC) family of enzymes consists of a number of isoforms that are differentially regulated and expressed. This differential regulation may be representative of differential cellular function. PLCγ is highly expressed in fetal and carcinoma tissue, suggesting a role in cell division. In contrast, PLCδ is present in large amounts in differentiated vascular smooth muscle and has been proposed to be involved in cytoskeletal organisation (Lymn & Hughes, 2000).

The aim of these studies was to examine the relationship between contractility and PLC isoform expression in small vessels maintained in organ culture.

Mesenteric small arteries from male Wistar rats, which were humanely killed, were mounted on steel wires and cultured in serum-free medium (NCTC) or 10% dialysed fetal calf serum (dS) with a cutoff of 12–14 kDa, for up to 4 days. After culture, arteries were mounted at a normalised resting tension in an unpressurised wire myograph. Isometric contraction was assessed in response to 144 mM potassium solution (K), noradrenaline (NA, 5 μM), 5-hydroxytryptamine (5-HT; 10 μM) and NA + K. Contractile responses were calculated as effective active pressure (EAP) based on Laplace's law. Protein expression of vessels was determined by Western blotting with specific antibodies followed by densitometric analysis. Data are expressed as % change in expression with respect to fresh vessels.

Organ culture resulted in a progressive decrease in contraction to all stimuli.

Table 1. Effect of organ culture on responses to agonists

	Day 0	Day 4	
		NCTC	dS
K	21 ± 1	9 ± 2*	10 ± 2*
NA	18 ± 2	10 ± 2*	10 ± 2*
5-HT	17 ± 2	11 ± 2*	11 ± 2*
NA+K	25 ± 2	13 ± 2*	12 ± 2*

Data are EAP (kPa). All data are expressed as means ± S.E.M. and analysed by ANOVA, $^*P \leqslant 0.05$.

Preliminary data suggest that while there is no significant change in the expression of SM α-actin ($n = 8$) compared with control vessels following organ culture, calponin expression is significantly reduced (NCTC −34 ± 7%*, dS −46 ± 12%*, $n = 7$). Expression of PLCγ_1 was not significantly affected by organ culture, although PLCδ_1 expression was significantly downregulated in NCTC (80 ± 12%* of control values, $n = 8$).

Organ culture reduced contractile responses to a range of agonists, possibly as a consequence of smooth muscle dedifferentiation. This loss of contractility in vessels incubated in NCTC was not due to changes in the contractile proteins but was associated with a significant reduction in PLCδ_1 expression.

Lymn, J.S. & Hughes, A.D. (2000). *NIPS* **15**, 41–45.

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The effects of intra- and extravascular pressure on rat coronary artery diameter

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Myogenic tone is an important autoregulatory mechanism ensuring that blood flow is kept constant despite alterations in perfusion pressure. Its existence has been demonstrated *in vitro* using isolated pressurized small arteries where increases in transmural pressure (TmP) have been obtained by increasing intravascular pressure (IvP). *In vivo*, however, coronary vessels are subject to compressive forces of the surrounding contractile cardiac muscle. Transmural pressure will thus be determined by both intra- and extravascular pressure (EvP) changes. It is unclear whether IvP and EvP have similar effects on active arterial diameter. This study describes a new technique that allows the effect of variations in both intra- and extravascular pressures on isolated arterial diameter to be investigated.

Wistar rats were humanely killed by cervical dislocation and septal coronary arteries (200–250 μm diameter) were dissected out. Each artery was mounted on a modified pressure myograph where they were constantly superfused with physiological salt solution (pH 7.4, 37 °C, 95% air and 5% CO_2). A lid was secured over the myograph chamber, which allowed EvP to be altered (via a 95% air and 5% CO_2 source). The internal diameter of vessels was determined using a video dimension analyser.

At an IvP of 60 mmHg, and thus an outward TmP of 60 mmHg, coronary arteries (mean internal diameter of 223 ± 9 μm, $n = 5$) developed myogenic tone (124 ± 21.39 μm). Elevation of EvP to 40 mmHg, which effectively reduced the outward TmP to 20 mmHg, reduced the diameter of all vessels by 25 ± 3.9%. Subsequent elevation of IvP to 100 mmHg, at a maintained EvP of 40 mmHg, to return TmP to 60 mmHg increased arterial diameter to 99.6 ± 1.9% of that observed at the original TmP of 60 mmHg (due to IvP of 60 mmHg and EvP of 0 mmHg).

The effect of different IvPs on arterial diameter was also assessed when EvP was 0 mmHg, $n = 8$ (IvP range = 20–60 mmHg) and when EvP was maintained at 40 mmHg, $n = 4$ (IvP range = 60–100 mmHg), such that similar TmPs were obtained. Arterial diameters were similar at each TmP regardless of the relative contribution of IvP and EvP to it (0.77 ± 0.13 and 0.87 ± 0.09 for 20 mmHg; 0.88 ± 0.09 and 0.95 ± 0.07 for 40 mmHg and 0.94 ± 0.05 and 0.97 ± 0.04; data are means ± S.E.M.) for a TmP of 60 mmHg observed at an EvP of 40 and 0 mmHg, respectively (Student's unpaired t test, $P > 0.05$) (data normalised to diameter at IvP of 60 and EvP of 0 mmHg for each vessel).

Thus we demonstrate for the first time that changes in both intra- and extravascular pressure may modify coronary artery diameter. Furthermore we have shown that it is the outward transmural pressure that is important in determining arterial diameter, which may be determined by a combination of intra- and extravascular pressures.

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Sphingosine 1-phosphate (S1P)-induced intracellular signalling pathways involved in vasoconstriction

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S1P, a lipid released from activated platelets, influences physiological processes via activation of the endothelial differentiation gene (EDG) family of G protein-coupled receptors. The potential role of S1P in vasoconstriction has not been examined. The aim of this study was to determine the S1P-induced intracellular signalling events in rat aorta (Ao) and cerebral artery (CA) that may contribute to vasoconstriction. Freshly dispersed CA and Ao smooth muscle cells were isolated by enzymatic digestion from humanely killed rats and loaded with fura-2. Fluorescence Ca^{2+} imaging was carried out using a CCD camera. RhoA activation was assessed using a GST-rhotekin 'pull-down' assay which binds to GTP bound (i.e. active) RhoA. Following isolation of GST-rhotekin, proteins were separated using SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with an anti-RhoA antibody. Results are means ± S.E.M.

Transient intracellular Ca^{2+} increases were induced by 1 μM S1P in dispersed cells from CA (mean peak amplitude, 0.45 ± 0.01 ratio units, $n = 109$). Stimulation of Ao cells with S1P produced only a small Ca^{2+} increase (maximum 30 μM S1P, 0.13 ± 0.03 ratio units, $n = 32$). In both smooth muscle types, 100 nM ET-1 evoked similar transient Ca^{2+} increases. Pre-incubation with either thapsigargin or the phospholipase C inhibitor, U73122, almost completely abolished the S1P-induced Ca^{2+} increases evoked by 1 μM S1P in CA cells. In CA rings, S1P stimulation resulted in a sustained rise in tension and was inhibited by addition of 10 μM Y27632, a rho-kinase inhibitor. Peak S1P-induced contraction was 35 ± 5% ($n = 5$) of the ET-1-induced contraction. In Ao rings no change in tension was observed following incubation with S1P. RhoA was activated by S1P in a time course compatible with a role in the S1P-induced contraction of CA. Following 5 min stimulation with S1P, the proportion of GTP-RhoA compared with total RhoA protein increased 4.2 ± 0.6-fold ($n = 3$) in isolated CA. S1P did not activate RhoA in isolated Ao. Immunoblots of homogenates from CA and Ao stained with specific antibodies revealed that EDG-1 receptor expression was similar in Ao and CA. EDG-3 receptor expression was 4.4 ± 0.2-fold ($n = 4$) greater and EDG-5 receptor expression 4.2 ± 0.8-fold ($n = 4$) greater in CA than in Ao.

In conclusion, S1P can activate intracellular signalling pathways compatible with its potential role as a vasoconstrictor. These pathways are not activated in all smooth muscle types, which may correlate with the differential expression of EDG receptor subtypes.

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Intracellular resistance and connexin expression in stable and unstable human bladder smooth muscle

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Unstable bladder contractions may contribute to the symptoms of the overactive bladder and it is debated if they originate in the smooth muscle or from neuronal influences. A myogenic cause implies fundamental changes to detrusor smooth muscle and one possible factor is protrusion junctions between cells (Elbadawi *et al.* 1993), leading to speculation that enhanced intercellular coupling co-ordinates electrical activity and generates greater spontaneous activity. However, only gap junctions mediate electrical coupling and there is sparse evidence for them in detrusor. This study measured the intracellular resistivity of human detrusor from normal and unstable bladders and how this related to the presence of gap junction proteins.

Bladder biopsies were obtained at open surgery, placed in Ca-free Hepes buffer and used immediately. Patients had urodynamically defined stable or overactive bladders. Local Ethical Committee approval and patient consent were obtained for each specimen. Intracellular resistivity, and in particular junctional impedance was measured from preparation impedance (20 Hz–300 kHz; Fry *et al.* 1999). Immunoconfocal detection of Cx43, Cx40 and Cx45 used established monoclonal or polyclonal antibodies (Coppen *et al.* 1998). Data are medians (25%, 75% interquartiles); differences between data sets used Mann-Whitney tests.

Intracellular resistivity, R_i, after correction for extracellular resistance was 817 (572, 1173; $n = 15$) Ω cm, and 1426 (1264, 1787; $n = 10$) Ω cm in samples from stable and unstable bladders and was significantly greater ($P < 0.05$) in the latter group. R_i values were divided into cytoplasmic (R_c) and junctional (R_j) resistivities. R_c values were not significantly different in the two groups (294 (253, 411) *vs.* 374 (306, 412) Ω cm, $P > 0.05$), whereas R_j values were significantly greater in the unstable group (511 (368, 778) *vs.* 1052 (895, 1368) Ω cm, $P = 0.01$). Punctate connexin43 labelling was intense in a suburothelial band of cells, but unequivocal detrusor labelling was not observed in the muscle layer. Connexin40 labelling was prominent in endothelial cells of larger arterioles but not elsewhere. Connexin45 labelling showed as small, sharply defined areas specifically in the detrusor and localized to boundaries between smooth muscle cells. Northern blots of the separated mucosal and detrusor layers mirrored that of the corresponding confocal microscopy images. Mean connexin45 transcript levels were reduced to 65% in the unstable compared with the stable bladders ($P < 0.05$).

Connexin45 is expressed in detrusor smooth muscle. The decrease in connexin45 transcript quantity, coupled with a raised junctional resistivity implies that intercellular coupling is reduced in the unstable bladder. How this contributes to aberrant contractile function remains to be determined.

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The role of mitochondria in the control of Ca signalling in rat ureteric smooth muscle

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Mitochondria can act as a Ca^{2+} uptake compartment due to the large electrochemical gradient in favour of Ca^{2+} accumulation. Although a role for mitochondria in the control of Ca signalling under physiological conditions had been largely dismissed, recent data indicate that in some types of smooth muscle cells, mitochondria may play an important role, by affecting the spatio-temporal aspects (McGeown *et al.* 1996; Drummond & Tuft, 1999; McCarron & Muir, 1999). In the present work, therefore, we have examined the role of mitochondria in control of Ca^{2+} signalling in rat ureter smooth muscle cells.

Single cells were produced by enzymatic digestion, following humane killing of the rats. The cells were loaded with the Ca-sensitive indicator fura-2, voltage-clamped and the mitochondrial Ca^{2+} uptake inhibitor-carbonyl cyanide *m*-chlorophenyhydrazone (CCCP), with and without oligomycin B – a blocker of the mitochondrial ATP synthase, applied.

CCCP in voltage-clamped rat ureteric cells produced a significant ($P < 0.05$, *t* test), elevation of the resting baseline level of intracellular Ca^{2+} ($n = 27$). This elevation was associated with the generation of inward current. There was also a significant decrease in the rate of restoration of the Ca^{2+} transients induced by depolarising voltage steps or application of 10 μM carbachol, by 1.42 ± 0.25 ($n = 27$) and 1.53 ± 0.34 times ($n = 4$), respectively.

Clearly alteration of mitochondrial Ca^{2+} homeostasis has profound effects on cytosolic Ca^{2+} handling and excitability, in these cells. Since 3 mM ATP and 11 mM Hepes were present in the pipette solution an inhibition of ATP production or decrease in pH seem to be unlikely mechanisms to contribute to the effect of CCCP on Ca^{2+} signalling in rat ureteric cells. Interaction between the mitochondrial and SR Ca^{2+} store are suggested by preliminary data showing that the frequency of Ca^{2+} puffs and STOCs were transiently increased in the presence of CCCP. These data suggest that mitochondria can modulate the characteristics of both elementary and global Ca^{2+} events in rat ureteric cells.

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Effect of bladder outflow obstruction on the carbachol-induced $[Ca^{2+}]_i$ rise in a fetal sheep model

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Posterior urethral valves (PUV) result in congenital bladder outflow obstruction exclusively affecting boys (Woolf & Thiruchelvam, 2001). As a consequence of this prenatal obstruction, these boys often have persistent postnatal bladder dysfunction in later life, with resultant end-stage renal failure and delayed achievement of urinary continence. To understand the pathophysiology of PUV, this study examined the role of intracellular Ca^{2+} ($[Ca^{2+}]_i$) regulation in altered contractility using a fetal lamb model with induced *in utero* bladder outflow obstruction.

Partial bladder outflow obstruction was induced in male fetal sheep by placement of an omega-shaped urethral ring and urachal ligation midway through gestation, at 75 days (full-term 150 days) under 2–3 % halothane anaesthesia in a NO/O_2 mixture. Animals were killed 30 days after surgery (105 days) with I.V. sodium pentobarbitone. Procedures accorded to UK legislation. Sham-operated control fetuses underwent urethral and urachal exposure only. Detrusor smooth muscle was obtained from the mid-region of bladders after removal of mucosa and serosa. Isometric twitch tension was measured from small muscle strips (less than 1 mm in diameter) and $[Ca^{2+}]_i$ measured in single dissociated myocytes loaded with the fluorescent indicator fura-2 (Wu & Fry, 1998). Contraction and $[Ca^{2+}]_i$ rise were elicited by carbachol, an analogue of the functional neurotransmitter acetylcholine. Data were expressed as means ± S.E.M. and Student's unpaired *t* test used to test the statistical significance between data sets.

In multicellular preparations, the force of contraction in response to carbachol (10 μM) was significantly reduced in obstructed fetal bladders (obstructed: 2.4 ± 0.5 mN mg^{-1}, $n = 4$; sham-operated control: 4.7 ± 0.2 mN mg^{-1}, $n = 3$; $P < 0.05$). In isolated detrusor cells, 10 μM carbachol elicited a $[Ca^{2+}]_i$ transient from a resting value of 118 ± 8 nM with an net increase of 629 ± 86 nM ($n = 16$), whilst in cells from obstructed bladders, the net rise was only 414 ± 59 nM from a resting value of 144 ± 2 nM ($n = 20$, $P < 0.05$). Further experiments determined the concentration dependence of the carbachol-induced $[Ca^{2+}]_i$ rise over a range from 0.03 to 100 μM. The minimal effective concentration was around 0.1 μM and the maximal effect around 10 μM. An EC_{50} of 0.8 ± 0.2 μM ($n = 6$) was obtained from the dose–response relationship for the control group, whilst the relationship was shifted to the right in the obstruction group (EC_{50} 2.4 ± 0.5 μM, $n = 6$; $P < 0.05$).

These results demonstrate that bladder outflow obstruction during fetal development results in a reduced muscarinic receptor efficacy and sensitivity in detrusor myocytes. The impaired Ca^{2+} regulation coupled to the receptor activation in these cells may in part account for the reduced bladder contractility found after *in utero* bladder obstruction.

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T-type calcium currents in freshly dispersed smooth muscle cells isolated from human proximal urethra

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Hollywood *et al.* (2001) described the presence of a nickel-sensitive inward current in human proximal urethral smooth muscle, which had properties typical of T-currents found in other tissues. Recently, Koh *et al.* (2001) described a novel, voltage-sensitive inward current in murine colon that had kinetics and nickel sensitivity similar to T-currents but was blocked by external Ba^{2+} and almost abolished in 5 mM Na^+. The aim of the present study was to determine whether the currents in human bladder neck smooth muscle cells possessed characteristics more typical of T-type calcium current or the voltage-dependent non-specific cation current described by Koh *et al.* (2001).

Smooth muscle cells were isolated from biopsy samples taken from the proximal 1 cm of the urethra in seven male and two female patients (age range 20–68 years) who gave written, informed consent. This study was approved by the Queen's University Ethical Committee. Cells were perfused with Hanks' solution containing 1.8 mM Ca^{2+} at 37 °C and studied using the amphotericin B perforated-patch technique with Cs^+-rich pipette solution. When cells were held at −100 mV and depolarised in 10 mV steps for 500 ms, inward currents were evoked at potentials positive to −70 mV. The current–voltage (*I–V*) relationship consisted of two peaks: one at ~−40 mV and the other at 0 mV. In the presence of 300 nM nifedipine, the current at 0 mV was reduced from −156 ± 37 to −38 ± 13 pA (mean ± S.E.M., $n = 6$, $P < 0.05$, paired *t* test) whereas the current at −40 mV was little affected (−47 ± 13 to −41 ± 11 pA, $n = 6$, $P = 0.2$). The nifedipine-insensitive current at −40 mV peaked within 15 ± 2 ms ($n = 4$) and inactivated at −50, −40 and −30 mV with time constants of 31 ± 4, 22 ± 3 and 16 ± 2 ms, respectively ($n = 7$).

The current at −30 mV was unaffected by TTX and not significantly reduced in 5 mM Na^+ (−85 ± 4 to −66 ± 10 pA, $P = 0.14$, $n = 5$), suggesting that Na^+ ions made very little contribution. When Ca^{2+} was replaced with Ba^{2+}, the current at −30 mV was increased from −80 ± 16 to −91 ± 15 pA ($P = 0.34$, $n = 5$). Replacement of Ca^{2+}-containing Hanks' solution with 5 mM EGTA, Mg^{2+}-substituted Hanks' solution, reduced the peak current at −40 mV from −67 ± 12 to −33 ± 9 pA ($P < 0.01$, $n = 6$). When *I–V* relationships were constructed before and after Ca^{2+} removal, currents activated at potentials positive to −70 mV, peaked at ~−10 mV and inactivated at negative potentials. When Mg^{2+} was removed from the calcium-free solution, the current was dramatically enhanced, suggesting that it represented the flow of monovalent cations through T-type calcium channels.

In conclusion, these results suggest that the negatively activating inward current in human bladder neck myocytes resembles T-type calcium current rather than a voltage-sensitive non-selective cation conductance.

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Effects of noradrenaline on transient BK current in freshly dispersed smooth muscle cells from the rabbit urethra

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The urethra is thought to play an important role in maintaining urinary continence by generating sufficient tone to prevent leakage of urine from the bladder. This tone is partly myogenic in nature, but can be augmented by adrenergic nerves acting on post-junctional α_1-receptors. In the present study we have examined the effects of exogenous noradrenaline on K^+ currents in smooth muscle cells isolated from the rabbit urethra. Rabbits were killed with pentobarbitone (I.V.) and their urethras removed.

Smooth muscle cells were isolated by enzymatic dispersal and studied with the amphotericin B perforated patch technique at 37 °C using K^+-rich pipette solutions. When cells were held at −60 mV and depolarized to 0 mV for 500 ms, a small transient inward current was evoked and this was followed by a larger transient outward current. The transient outward current was blocked by penitrem A (100 nM), suggesting that the outward current was carried through large conductance calcium-activated K^+ (BK) channels described previously in these cells (Hollywood *et al.* 1999). Application of noradrenaline (10 μM) decreased the amplitude of the transient BK current from 860 ± 126 to 315 ± 60 pA (mean ± S.E.M.) and increased the amplitude of the inward current from −29 ± 21 to −48 ± 20 pA ($n = 16$, $P < 0.05$, paired *t* test). In the presence of the α_1 adrenoceptor antagonist prazosin (1 μM), NA failed to inhibit the BK current (574 ± 61 pA before NA compared with 563 ± 55 pA during NA, $n = 5$), suggesting that its effects were mediated through α_1-adrenoceptors. When calcium release from IP_3-dependent stores was blocked with 100 μM 2APB (Maruyama *et al.* 1997), NA failed to depress the BK current ($n = 8$, $P > 0.5$), suggesting that its effects were mediated via release of calcium from an IP_3-sensitive store.

To investigate the effect of NA on evoked action potentials, cells were studied under current clamp and brief injections of depolarizing current applied. Membrane potential was maintained at ~−60 mV by the injection of a steady backgound current. In six cells, action potentials were elicited that had a mean overshoot of 2.5 ± 3.4 mV and a duration of 42 ± 1.3 ms. In the presence of NA, the overshoot increased to 17 ± 4 mV and the duration increased to 65 ± 7 ms ($n = 6$, $P < 0.05$). These results suggest that in the presence of noradrenaline the action potential is broadened, allowing a greater influx of calcium. This may contribute to the increase in tension observed in the urethra in response to exogenous NA.

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Dual modulation of calcium-activated chloride channels by niflumic acid in rabbit coronary myocytes

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Ca^{2+}-activated Cl^- channels (Cl_{Ca}) play an important role in the regulation of resting membrane potential of vascular smooth muscle cells during agonist-mediated tone. Niflumic acid (NFA) is recognized as the most potent inhibitor of Cl_{Ca}. However, when Cl_{Ca} in rabbit pulmonary artery myocytes was evoked by a sustained level of intracellular calcium, NFA both inhibits and stimulates the current (Piper *et al.* 2002). We examined this phenomenon further by studying the effect of NFA on $I_{Cl(Ca)}$ evoked in coronary artery myocytes by dialysis with K^+-free pipette solutions containing 500 nM Ca^{2+} using the whole-cell patch-clamp technique. Coronary myocytes were freshly isolated from New Zeland White rabbits that were killed by an anaesthetic overdose (pentobarbital 1 mg kg^{-1}) via the ear vein. Cells were prepared as described previously (Greenwood *et al.* 2001). All pooled data are expressed as means ± S.E.M. Application of 100 μM NFA inhibited $I_{Cl(Ca)}$, which was followed by a marked increase in current level above control values after washout (mean increase of instantaneous, late and tail currents was 47 ± 13, 80 ± 28 and 50 ± 16 %, respectively, $n = 4$). The activation kinetics of enhanced $I_{Cl(Ca)}$ during a depolarizing step increased (at +90 mV, $\tau = 0.4 \pm 0.1$ and 0.2 ± 0.02 s before and after washout of NFA, respectively; $P < 0.05$, Student's paired t test, $n = 4$) while the mono-exponential decay of the tail current at −80 mV became biexponential upon washout of the drug ($\tau = 44 \pm 4$ ms in control, $\tau_{fast} = 19 \pm 2$ ms and $\tau_{slow} = 176 \pm 29$ ms after washout of NFA; $P < 0.05$, ANOVA, $n = 4$). We next sought to determine the concentration-dependence of enhanced $I_{Cl(Ca)}$ after washout of NFA. Cells were stepped to +60 mV for 30 s (HP = −50 mV) and exposed to a given concentration of NFA with a fast flow perfusion system for 2 s. Whilst the relative increase of $I_{Cl(Ca)}$ following washout of NFA was independent of drug concentration, the time to achieve 50 % of the maximal increase of current was concentration dependent (almost instantaneous, 641 ± 169 ms and 1421 ± 400 ms following a 2 s exposure to 10 μM, 100 μM or 1 mM NFA, respectively; $P < 0.05$, Student's paired t test, $n = 4$). Moreover, increased $I_{Cl(Ca)}$ could be blocked as effectively and as quickly as the control current by a second exposure to 100 μM NFa after a 500 ms washout. This indicates that both control and stimulated $I_{Cl(Ca)}$ currents can be inhibited by NFA. We propose the existence of at least two binding sites on smooth muscle Cl_{Ca}: a high-affinity inhibitory site, which is hiding a low-affinity site revealed upon washout of the drug.

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All procedures accord with current local guidelines.

Two cell types in the media of rabbit portal vein

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The rabbit portal vein discharges spontaneous action potentials accompanied by contractions when the whole vein, or a portion of it, is bathed in physiological salt solution. This spontaneous activity is myogenic and seems to be due to the presence of multiple pacemakers in the wall of the portal vein (Sutter, 1990). Enzymatic dispersion of the rabbit portal vein revealed two types of cells: more numerous smooth muscle cells (SMCs) were mixed with unusual cells morphologically resembling closely the interstitial cells of Cajal, which were shown to be specialised pacemaking cells in the gut (Sanders *et al.* 1999). Their presence in blood vessels has not been previously reported and we were intrigued to investigate their features, functions and location in the portal vein.

A 20 mm section of portal vein was removed from male New Zealand rabbits (2–3 kg) killed by an overdose of pentobarbitone. Experiments were performed at room temperature on segments of the opened vein after removal of fat and the adventitial layer, or on freshly isolated single cells within a few hours of enzyme and mechanical dispersion. Single cells were studied with standard whole-cell and amphotericine B perforated patch methods or imaged after loading with the fluorescent Ca^{2+}-sensitive indicator fluo-4 AM. ***c-kit***-labelled living tissue was obtained as described elsewhere (Hirst & Edwards, 2001): portal vein segments were incubated with ACK-2 rat monoclonal antibodies (from Gibco BRL or from Santa Cruz) and then with Alexa Fluor 633 conjugated anti-rat antibodies (Molecular Probes). In controls, primary antibodies were omitted from the first incubation solution. All single-cell or whole tissue imaging was performed using a laser scanning confocal microscope.

In contrast to relaxed SMCs, which have a more constant shape (90–150 μm in length with a mean of 121 ± 23 μm and about 8–10 μm in width with a mean of 8.8 ± 1.1 μm; ± S.D., $n = 47$), interstitial cells varied somewhat in their morphology (from 40 to 300 μm in length with a mean of 147 ± 51 μm and from 4 to 12 μm in width with a mean of 7.2 ± 2.0 μm; ± S.D., $n = 98$), but they always had a variable number (from several to several tens) of numerous, branching, extremely thin (often less than 1 μm wide) processes which could be up to several tens of micrometres in length. Interstitial and SMCs had no differences in electrophysiological characteristics. Under voltage clamp both cell types revealed well-developed voltage-gated nifedipine-sensitive L-type Ca^{2+} current but no Na^+ current. After loading with fluo-4 AM SMCs, and less commonly, interstitial cells, displayed various spontaneous calcium events including Ca^{2+} sparks and Ca^{2+} waves. Ca^{2+} waves were followed by contraction of the SMCs, but not interstitial cells. The latter usually had brighter fluorescence than SMCs but appeared darker under phase contrast. Application of noradrenaline (1–10 μM), caffeine (1–10 mM) or high K^+ solution (60 mM) evoked a rise of $[Ca^{2+}]_i$ in both cell types and a contraction of SMCs, but no change in the shape of interstitial cells was observed. Some interstitial cells showed spontaneous $[Ca^{2+}]_i$ transients (lasting about 2 s) propagating from the cell body to the end of the processes. Surviving contacts between the processes of interstitial cells and SMCs could be found after the cell isolation. Portal vein tissue showed cells stained with Methylene Blue and ***c-kit***-positive cells in the media about 7–30 μm from the endothelium.

It is possible that these interstitial cells could be responsible, as in gut muscles, for driving its spontaneous activity.

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All procedures accord with current UK legislation.

Contractile responses in the absence of extracellular calcium provide insights of reticular calcium compartments in vascular smooth muscle

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Studying the influence of calcium-free medium and of reticular calcium levels upon contractility in isolated aorta, we previously established the depletion protocol. The stores sensitive to α-adrenergic stimulation partially overlap those mobilized by caffeine. We examined this aspect and the influence of extracellular magnesium in de-endothelialised rat aorta rings in isometric conditions (animals were killed by decapitation). Phenylephrine effects were observed in calcium-free medium (1 mM EDTA), when given after 30, 60 or 90 min incubation. We then examined the influence of caffeine (1 mM), ryanodine (0.01 mM) and various concentrations of magnesium (0 to 10 mM) upon the mentioned store-dependent contractions (results are % of the submaximal response in the same ring; mean ± S.E.M.; $n = 6$). The biphasic contraction in calcium-free medium is based upon two reticular compartments. The transient caffeine-sensitive response proved to be ryanodine sensitive; the store is exhausted after calcium-free incubation with EDTA for 60 min, as also shown by others. The second component, caffeine-insensitive, is abolished at 90 min incubation as above, though not affected in the first 60 min ($P < 0.01$; Student's unpaired t test). Potentiation of the mentioned contractions by low magnesium was confirmed, while increased magnesium lowered the ratio between the transient and the sustained response. It has been suggested that reticular compartments communicate at rest. We suspect that depletion of the caffeine-sensitive one leads to redistribution, favouring depletion of the other. Functional tests in calcium-free medium are still useful, including the study of reticular compartments. Magnesium effects upon smooth muscle contractility are well characterized, yet this study provides particular evidence regarding the influence of extracellular magnesium upon the depletion of reticular calcium compartments in standardized conditions.

All procedures accord with current local guidelines.

The role of the sarcoplasmic reticulum (SR) in developing uterine smooth muscle

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In smooth muscle cells, the SR is an important regulator of cytosolic Ca^{2+}, membrane excitability, and a source of Ca^{2+} for contraction. In some smooth muscles, important developmental differences have been found in the size and function of the SR (Hillemeier *et al.* 1991; Nakanishi *et al.* 1997). There are, however, no comparative data on the myometrium. The aim of this study is therefore to investigate the role of neonatal uterine SR, to further our understanding of the control of uterine activity.

Simultaneous measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and force were made on strips of uterus taken from 2-day and 10-day-old neonatal and non-pregnant adult rats, after humane killing. The presence of an SR Ca^{2+} store in the neonate was determined by using (i) 20 μM cyclopiazonic acid (CPA), a specific inhibitor of the SR Ca^{2+} pump, and (ii) 100 μM carbachol, in the presence and absence of extracellular Ca^{2+}. Data were normalised to high-K^+ (150 mM)-induced contractions, and are expressed as means ± S.E.M.; significance was tested using Student's unpaired t test, with significance taken at $P < 0.05$; n is number of animals.

The neonatal uterus was spontaneously active, producing Ca^{2+} transients followed by phasic contractions at a frequency of 4.5 ± 0.7 per 10 min (37°C). Emptying the SR with CPA significantly increased this frequency and basal $[Ca^{2+}]_i$ and force to 174 ± 4 and 241 ± 12 % ($n = 4$), respectively. Carbachol induced significantly greater maximal $[Ca^{2+}]_i$ and force in neonate compared with adult myometrium (140 ± 4 *vs.* 113 ± 10 % $[Ca^{2+}]_i$ and 182 ± 19 *vs.* 120 ± 7 % force; $n = 6$, respectively). There was also marked force production after $[Ca^{2+}]_i$ had returned to basal levels in the neonates (9.7 ± 1.8 *vs.* 1.4 ± 0.5 min in the adult). Oxytocin (100 nM) produced little or no effect on the neonatal uterus but greatly potentiated force and $[Ca^{2+}]_i$ in the adults. In zero Ca^{2+} solutions, carbachol-induced SR Ca^{2+} release increased myometrial $[Ca^{2+}]_i$ by 122 ± 5 % in 2-day neonates, 77 ± 3 % in 10-day neonates and 44 ± 5 % in adult rats compared with high-K^+ controls ($n = 5$).

These data clearly show that spontaneous force and Ca^{2+} transients and a functional SR Ca^{2+} store exist in neonatal rat uterus. As with adult uterus, this SR Ca^{2+} seems to limit spontaneous contraction, suggesting a negative feedback mechanism. Significantly more SR Ca^{2+} is released in neonatal rat myometrium than adult myometrium and the younger the rat, the more this release is. The neonatal uterus has a greater response to carbachol than adult uterus possibly due to (i) the relatively large size of the functional SR store in the neonate and/or (ii) increased carbachol-induced sensitisation of the contractile apparatus in the neonate.

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Effects of intracellular and extracellular pH change on Ca^{2+} signalling and force in pregnant myometrium

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Uterine activity in labour can produce acidosis. In turn, intracellular pH change can greatly alter the spontaneous contractile ability of human myometrium, although the mechanisms involved are poorly understood. We have therefore determined the effects of both intra- and extracellular pH change on contractile activity and intracellular $[Ca^{2+}]$ during spontaneous, oxytocin-induced and high K^+ depolarization-induced stimulation of longitudinal strips from human myometrium.

Human non-labouring myometrial tissue was obtained with ethical permission and informed consent, from women undergoing elective Caesarean section at term (37–42 weeks gestation). Strips of longitudinal muscle were incubated overnight with 7 μM indo-1 AM for $[Ca^{2+}]_i$ measurements. These were then attached to a force transducer and tissue superfused continuously with physiological saline at 35–37 °C. Changes in pH_i were produced by isosmotic replacement (40 mM) of NaCl with sodium butyrate or NH_4Cl. Changes in pH_o were produced by addition of NaOH or HCl. Oxytocin (10 nM) and high-K^+ at 40 mM were used. EGTA (1 mM) was added to solutions of zero calcium. Statistical analysis was performed on paired data using Student's *t* test (95 % confidence); *n* is the number of samples.

Our data show that both intracellular and extracellular acidification significantly reduce or even abolish phasic activity whether arising spontaneously or in the presence of oxytocin ($n = 7$). Furthermore, these contractile changes can be accounted for by the changes in $[Ca^{2+}]$. Alkalinization produced the opposite effects, i.e. phasic contractions and Ca^{2+} transients increased ($n = 7$). However, baseline or maintained tension changes could not be accounted for by changes in $[Ca^{2+}]_i$. Thus intracellular acidification reduced baseline tension in both spontaneous and oxytocin-stimulated preparations and reduced maintained tension in depolarised preparations, but increased $[Ca^{2+}]_i$ in all cases. Application of weak acid, in calcium-free solution, both with ($n = 4$) and without oxytocin ($n = 7$) led to a rise in $[Ca^{2+}]_i$ but an immediate loss of tension in all cases.

We suggest that the effects on phasic activity are due to inhibition of Ca^{2+} entry via surface membrane channels and during periods of maintained tension (as produced with application of high-K^+ solution) pH-sensitive Ca^{2+} release from the SR occurs but is not sufficient to overcome the inhibitory effects at the myofilaments. We conclude that alterations of both intra- and extracellular pH significantly affect Ca^{2+} signalling and force production in the human myometrium and may therefore contribute to dysfunction in labour.

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All procedures accord with current local guidelines.

Intracellular stores buffer nerve-evoked Ca^{2+} transients in smooth muscle cells during purinergic transmission in mouse vas deferens

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We have recently demonstrated that field (nerve) stimulation of the mouse vas deferens evokes focal, highly intermittent, α,β-methylene ATP-sensitive $[Ca^{2+}]$ transients in smooth muscle. These focal $[Ca^{2+}]$ transients arise adjacent to nerve terminal varicosities and can be used to detect simultaneously the highly intermittent release of packets of ATP from each varicosity along a nerve terminal branch. These focal transients have been termed neuroeffector Ca^{2+} transients (NCTs; Brain *et al.* 2002). In the present study, agents that modify Ca^{2+}-induced Ca^{2+} release (CICR) or the sequestration of Ca^{2+} by intracellular stores, have been used to investigate mechanisms that might influence the amplitude or time course of NCTs. Mice (Balb/C) were humanely killed according to UK legislation. The Ca^{2+} indicator Oregon Green 488 BAPTA-1 10 kDa dextran was applied to the cut end of the isolated vas deferens for 8–10 h to load nerve terminals orthogradely (Brain & Bennett, 1997) and to fill a population of smooth muscle cells (Brain *et al.* 2002). Smooth muscle cell labelling could also be achieved by exposing the vas deferens to 10 μM Oregon Green 488 BAPTA-1 AM for 2 h at 36 °C. Preparations were examined with a Leica inverted confocal microscope; images were recorded at a frequency of 4 Hz when nerves were field stimulated with trains of stimuli at 2 Hz (0.6 ms pulse width; 10 V amplitude). The amplitude and time course of recovery of NCTs were measured from junctions that demonstrated a relatively high probability of evoking NCTs per field stimulus (0.05–0.1).

The amplitude of NCTs was not affected ($P > 0.05$, Student's paired two-tailed *t* test) by either the endoplasmic reticulum Ca^{2+} ATPase blocker cyclopiazonic acid (CPA; 10 μM; 3 ± 12 %; number of junctions, $n_j = 11$; number of preparations, $n_p = 3$; mean ± S.E.M.) or thapsigargin (1 μM; -20 ± 10 %; $n_j = 9$, $n_p = 4$). However, CPA significantly slowed the return of Ca^{2+} to its resting concentration: control time constant of 200 ± 13 ms; time constant in CPA of 600 ± 100 ms ($P < 0.05$). Thapsigargin slowed the time constant of recovery only slightly (by 12 ± 6 %; $P < 0.05$). Caffeine (3 mM) greatly increased the frequency of spontaneous focal $[Ca^{2+}]$ transients within the smooth muscle cell (by more than 15-fold; $n_p = 4$). These caffeine-induced Ca^{2+} transients did not preferentially occur at the same locations as evoked NCTs and may be due to the release of Ca^{2+} from intracellular stores. Hence there is no detectable contribution of CICR to NCTs, but CPA- and thapsigargin-sensitive Ca^{2+} ATPase, presumably located on the endoplasmic reticulum, are responsible for sequestering at least some of the Ca^{2+} that enters through P2X receptors. This sequestration buffers the focal Ca^{2+} transients and presumably contributes to the filling of intracellular Ca^{2+} stores.

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All procedures accord with current UK legislation.

Involvement of imidazolinic receptors in the inhibitory action of agmatine upon smooth muscle tonic but not phasic contraction

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Membrane imidazoline receptors (I-1) are present in smooth muscles. We used the endogenous I-1 agonist agmatine (1 μM to 10 mM), and the specific antagonist efaroxan (0.1 mM), aorta as a typical tonic muscle and uterus as a phasic one. Aorta rings from male adult Wistar rats and uterine horn fragments from virgin female rats were studied in isometric conditions, in oxygenated saline solution (bicarbonate buffer, pH 7.2–7.4), at 37 °C (animals were killed by decapitation; all experiments conformed to local/national guidelines). Submaximal contractions were induced in the aorta by 10^{-5} M phenylephrine and in the uterus by 10^{-5} M carbachol; results are expressed as % active tension (mean ± S.E.M.; $n = 6$). Agmatine does not affect aortic relaxation induced by 0.01 M carbachol. It partially relaxes (maximum 58.6 ± 8.3 % at 10 mM agmatine; IC_{50} ~213 μM) aorta rings precontracted by phenylephrine; there was similar inhibition with agmatine pretreatment; both effects are abolished by efaroxan; contractile force initially (1 min) developed is not significantly influenced by agmatine. Agmatine does not alter the baseline tone of uterine smooth muscle, but inhibits carbachol-induced contraction and reduces mean active force to 62.8 ± 4.9 % (maximal at 10 mM agmatine; IC_{50} ~460 μM) based upon frequency reduction (maximum 67.4 ± 4.6 %; IC_{50} ~423 μM) without significant changes in the amplitude of transient contractions, not altered by efaroxan. In the aorta agmatine inhibits the maintenance of contractile force acting on I-1 receptors endothelium independently. In the uterus agmatine inhibits the phasic contraction by an I-1-independent mechanism apparently absent in the aorta.

All procedures accord with current national guidelines.

Intracellular acidification makes a partial contribution to the inhibitory effect of metabolic inhibition on spontaneous Ca release in rat ventricular myocytes

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When cardiac cells are overloaded with calcium, spontaneous release of calcium ions from the sarcoplasmic reticulum (SR) produces propagating calcium waves. In previous work we have shown that the frequency of occurrence of these waves is decreased by metabolic inhibition and that this is accompanied by an increase of SR Ca^{2+} content (Overend *et al.* 2001). These effects were attributed to a decrease in the open probability of the SR Ca^{2+} release channel or ryanodine receptor (RyR).

The aim of the present work was to investigate the mechanism by which metabolic inhibition decreases RyR open probability. Specifically, we have investigated the contribution of changes of intracellular pH as intracellular acidification has been shown to decrease both the frequency of Ca^{2+} sparks (Balnave & Vaughan-Jones, 2000) and the amplitude of systolic Ca^{2+} release from the SR (Choi *et al.* 2000). Experiments were performed on isolated rat ventricular myocytes. The occurrence of Ca^{2+} waves was determined from cell length while simultaneously measuring intracellular pH with the indicator carboxy SNARF.

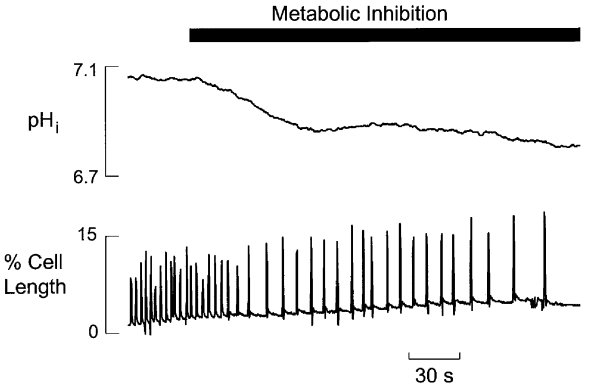

Figure 1. Metabolic inhibition (2 mM CN, zero glucose) inhibits spontaneous waves of contraction and causes intracellular pH to fall.

As shown in Fig. 1, the decrease of frequency of Ca^{2+} waves was associated with an intracellular acidosis. In five out of eight cells this was associated with an abolition of spontaneous Ca^{2+} release and, in the remainder with a decreased frequency. In order to see whether the acidosis accounts for the decrease of frequency, we have compared the effects of metabolic inhibition with those produced by intracellular acidification (at constant external pH) with butyrate. The results showed that although an equivalent acidosis to that produced by metabolic inhibition did decrease the frequency of waves, the effect was quantitatively less. Complete inhibition of waves was never observed. We conclude that, although intracellular acidification contributes to the effects of metabolic inhibition, something else (perhaps a decrease of ATP concentration) is also important.

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All procedures accord with current UK legislation.

The positive lusitropic effect of n-3 polyunsaturated fatty acids is inhibited by H89 in isolated rat ventricular myocytes

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n-3 polyunsaturated fatty acids (PUFAs) produce anti-arrhythmic effects by reducing surface membrane excitability and inhibition of spontaneous release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Kang *et al.* 1995; Negretti *et al.* 2000). These effects may be direct actions of PUFAs on ion channels in surface and SR membranes. Alternatively PUFAs act as second messengers and are precursors of biologically active molecules, e.g. prostaglandins and thromboxanes. As second messengers n-3 PUFAs lead to activation and inhibition of various kinases that might be involved in control of contraction in cardiac muscle. Here we report lusitropic effects in cardiac myocytes consistent with activation of protein kinase A (PKA).

Figure 1A shows the effect of the n-3 PUFA eicosapentaenoic acid (EPA, 10 μM) on field-stimulated contractions. In EPA contraction peaks sooner and relaxation is faster. In Fig. 1B the experiment is repeated in the presence of the PKA inhibitor H89 (20 μM). Both effects of EPA are absent. Due to the biphasic nature of the contractions, as a measure of the lusitropic effect we have calculated the area under the contractions after normalising the amplitude. Under control conditions EPA reduces the area of the contraction to 64.7 ± 2.7 % (mean ± S.E.M., $n = 6$, $P < 0.0001$, paired t test) of control, in the presence of H89 EPA has no effect on the area under the normalised contraction (103.5 ± 3.1 %; $P > 0.35$).

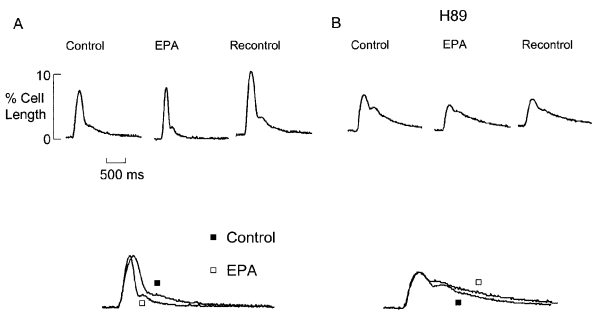

Figure 1. EPA (10 μM) causes a positive lusitropic effect that is inhibited by H89 (20 μM). The recontrol solution contained 1 mg ml^{-1} BSA to help remove EPA.

These results suggest that the lusitropic effects of EPA are due to activation of PKA. Although n-3 PUFAs are known to inhibit PKA (Mirnikjoo *et al.* 2001), they also inhibit phosphodiesterase so cAMP levels rise (Picq *et al.* 1996). This may overcome the inhibition of PKA, resulting in increased phosphorylation levels of e.g. phospholamban, leading to more rapid sequestration of Ca^{2+} by the SR following systolic release.

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Identification of the major ionic currents in single cells isolated from the rat ureter

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The rat ureter has a distinctive action potential, with an initial fast, single spike, followed by a temperature-sensitive plateau phase (Burdyga & Wray, 2002). Ca^{2+}-induced Ca^{2+} release is absent, unlike in guinea-pig ureter, although agonists can increase [Ca^{2+}] (Burdyga *et al.* 1995). As the ionic currents in rat ureter have not been characterised, we determined them in order to better understand the above processes.

Wistar rats (250–350 g) were killed by cervical dislocation and their ureters removed. Single cells were isolated by enzymatic digestion and held at −80 mV using the whole-cell patch-clamp technique. Cells were depolarised in either caesium or potassium pipette solution to reveal inward or outward currents, respectively. All experiments were carried out at room temperature and all values represent means ± S.E.M.; n is the number of cells.

Stepping to depolarizing potentials produced a voltage-dependent slow non-inactivating current. The peak amplitude of this current was 1323 ± 180 pA ($n = 14$) and it was inhibited by 1 mM tetraethylammonium (TEA; 35 ± 7 % of the control; $n = 4$). At a concentration of 10 mM TEA, 20 % of the current was still present ($n = 2$). In some cells a voltage-sensitive fast transient outward current similar to the A-current was seen. This current had a peak amplitude of 424 ± 60 pA, and was inhibited by 1 mM 4-aminopyridine (49 ± 4 % of control; $n = 3$). A calcium-dependent inward tail current was observed which was abolished by cadmium chloride. When caesium was present in the pipette, depolarisation produced voltage-dependent inward currents (peak 61 ± 8 pA; $n = 14$) which had a bell-shaped current–voltage relationship, were inhibited by 1 mM cadmium chloride and 1 μM nifedipine, and were enhanced by 1 mM Bay K 8644 (232 ± 43 % of the control; $n = 14$).

These results suggest that several K currents exist in these cells, a TEA-sensitive calcium-activated K current, a TEA-insensitive delayed rectifier and a fast transient outward current that was inhibited by 4-aminopyridine. The bell-shaped current–voltage relationship and the ability of cadmium chloride and nifedipine to abolish the inward current suggests that this current is via L-type voltage-operated calcium channels. The calcium-dependent inward tail current may be due to a calcium-activated chloride current. Further experiments using more specific inhibitors and elevated temperature are in progress to clarify these findings.

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All procedures accord with current UK legislation.

Identification and characterization of calcium-activated chloride currents in uterine smooth muscle cells

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Calcium-activated chloride currents ($I_{Cl\text{-}Ca}$) have been observed in several types of smooth muscle (Arnaudeau *et al.* 1994). These currents may be important in the generation of spontaneous electrical activity, which has obvious implications in pregnancy and parturition where the control of electrical activity is necessary. In uterine smooth muscle cells these currents can be activated by calcium release from the sarcoplasmic reticulum through the use of oxytocin. As L-type current (I_{Ca}) is the major source of activator Ca^{2+} in uterine cells, the aim of our work was to characterise the $I_{Cl\text{-}Ca}$ and determine the role of L-type I_{Ca} in its activation.

Pregnant female Wistar rats (18–21 days gestation) were killed humanely and cells obtained from longitudinal myometrium via enzyme isolation. Whole-cell membrane currents were recorded using the conventional patch-clamp technique. Cells were perfused with Krebs solution and the pipettes contained 140 mM KCl or CsCl, 8 mM NaCl, 4 mM MgATP, 10 mM Hepes and 5 μM

EGTA. Cells were maintained at a holding potential of −60 mV before application of depolarising voltage pulses.

With KCl pipette solution an initial inward Ca^{2+} current followed by a large outward K^+ current was observed with depolarising voltage pulses. A tail current was observed upon repolarisation. In the majority of cells this tail current was a result of the outward K^+ current deactivation. In approximately 30 % of the cells (28 out of 90), a long-lasting inward tail current was observed. CsCl pipette solution was used to remove interfering K^+ currents and enable the characterisation of this inward tail current. Its reversal potential was 4 ± 6 mV, $n = 4$, i.e. very close to the expected Cl^- reversal potential under our conditions. Using Ba^{2+} as the charge carrier, or niflumic acid (an inhibitor of chloride channels) resulted in the loss of the inward tail current. The current–voltage relationship of the peak tail current closely followed the current–voltage relationship of the peak I_{Ca} but the rate of its decay was much faster compared with the decay of $[Ca^{2+}]$ ($t = 39 \pm 2.7$ *vs.* 920 ± 46 ms, $n = 7$, respectively, $P < 0.001$, paired t test). Bay K, a Ca^{2+} channel agonist, increased peak I_{Ca} and peak tail current. There was a strong correlation between the amplitudes of the peak I_{Ca} and inward tail current ($r = 0.92 \pm 0.012$, $n = 66$, $P < 0.0001$). The results expressed are the mean ± S.E.M.

Our data suggest a calcium-activated chloride current is present in a subpopulation of uterine myocytes. Ca^{2+} entering the cell through L-type channels can activate this current. These currents will contribute to the excitability of the uterus and possibly to pacemaker potentials.

Arnaudeau, S. *et al.* (1994). *Am. J. Obstet. Gynecol.* **171**, 491–501.

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The effect of metabolic inhibition on uterine contractions and intracellular calcium

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Uterine dystocia is characterised by weak, unco-ordinated contractions and a prolongation of labour; it is the commonest cause of Caesarean sections (CS). As hypoxia may be one of the causes of dystocia, we investigate in this study the effects of metabolic inhibition on contractions of human myometrium, with and without agonists.

Human myometrial biopsies were obtained with informed consent and ethical committee approval, from non-labouring women at term undergoing elective CS. Longitudinal muscle strips were dissected, loaded with indo-1 for Ca^{2+} measurement and attached to a pressure transducer. Tissues were superfused with physiological saline, pH 7.4 at 35 °C, and cyanide (2 mM) applied.

Spontaneous contractions of the myometrium, and their associated intracellular Ca^{2+} transients were rapidly (5 min) abolished by cyanide ($n = 7$); basal Ca^{2+}, however, was elevated. A significant ($P < 0.05$, paired t test) reduction in force production also occurred when agonists were used to stimulate the myometrium (oxytocin, 100 nM, $n = 9$, or carbachol, 100 μM, $n = 7$), although basal Ca^{2+} was markedly elevated. In all cases Ca^{2+} quickly returned to control levels and contractile activity recovered within 5–15 min upon removal of cyanide.

These data indicate that metabolic inhibition and hence hypoxia rapidly and profoundly reduces the ability of the uterus to contract, even when agonists are applied. As such these data support a role for hypoxia in dystocic labours. The mechanism of the contractile dysfunction is not entirely explained by changes in $[Ca^{2+}]$, but also point to altered sensitivity of the contractile proteins to Ca^{2+}, possibly as a result of acidosis. The cause of the persistent elevation of basal Ca^{2+} is not clear, but could be due to impaired mitochondrial Ca^{2+} handling or Ca^{2+} efflux mechanisms.

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All procedures accord with current local guidelines.

Extraction of cholesterol abolishes phasic contraction of rat and guinea-pig ureter

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The sarcolemma of smooth muscle cells is divided into spatially distinct microcompartments of sphingomyelin- and cholesterol-enriched rafts and glycerophospholipid-containing non-raft regions (Babiychuk & Draeger, 2000). Since the association of various signalling molecules with particular lipid micro-compartments is essential for their physiological functioning (Simmons & Toomre, 2000), knowledge of the mechanisms regulating membrane compartmentalization is of paramount importance. In the present study we have investigated in smooth muscle, whether extraction of membrane cholesterol, (1) causes destabilization of membrane rafts and (2) has functional consequences, and the possible mechanisms involved.

Thin-layer chromatography, Western blotting, purification of porcine stomach smooth muscle microsomes and their extraction with non-ionic detergent were performed as described previously (Babiychuk & Draeger, 2000). Ureters were excised from rats or guinea-pigs humanely killed by cervical dislocation after CO_2 anaesthesia, muscle strips dissected and tension recorded. Cholesterol was extracted from the ureteric strips by perfusion with Krebs solution containing 2 % methyl-β-cyclodextrin.

In the smooth muscle microscomes, methyl-β-cyclodextrin resulted in preferential solubilization of cholesterol, leaving other lipid constituents of the sarcolemma unaffected. However, such extraction resulted in destabilization of membrane rafts as judged from the loss of their resistance to non-ionic detergents ($n = 3$). Cholesterol extraction from rat ($n = 8$) or guinea-pig ($n = 3$) ureter resulted in the abolition of mechanical activity in response to electrical stimulation. This activity was restored in the presence of 5 mM TEA, an inhibitor of potassium channels ($n = 6$). The phasic but not tonic contractions of ureter caused by application of 10 μM carbachol were also completely inhibited ($n = 3$). Extraction of cholesterol had no effect on the amplitude of mechanical responses to depolarisation with 120 mM KCl ($n = 12$).

In conclusion, our data suggest that extraction of membrane cholesterol results in destabilization of membrane rafts. As a consequence of this there is a specific inhibition of the phasic contractions of rat and guinea-pig ureter, possibly due to a stimulation of K^+ channels. This in turn suggests a selective effect of rafts on K^+, but not Ca^{2+}, channels, in the smooth muscle membrane.

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Na^+–K^+-ATPase isoforms in pregnant and non-pregnant rat uterus

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Sodium pump function depends on the isoform composition of Na^+–K^+-ATPase, as isoforms show differential sensitivity to ions and inhibitors in a species- and tissue-dependant manner (Mobasheri *et al.* 2000). The control of uterine contraction is dependent on the sodium pump maintaining ionic gradients across the cell membrane and hence excitability. In order to better understand its role we have investigated the cellular distribution of Na^+–K^+-ATPase α and β isoforms and the effect of ouabain in the rat uterus.

Female pregnant and non-pregnant Wistar rats were humanely killed by cervical dislocation under CO_2 anaesthesia. Uterine horns were removed and transverse sections cut and mounted in OCT blocks. Cryostat sections (10 μm thick) were mounted on coated slides and fixed for 10 min in ice-cold methanol before blocking in 10 % normal goat serum in PBS. Expression of a panel of Na^+–K^+-ATPase isoforms was compared by indirect immunofluoresence using antibodies to the α and β subunit isoforms and goat anti-mouse or goat anti-rabbit IgG conjugated to FITC, respectively. Spontaneous contraction of strips of myometrial smooth muscle was measured in the presence or absence of oxytocin or in combination with the Na^+–K^+-ATPase inhibitor ouabain.

Abundant immunostaining of the α_1 and α_2 isoforms was observed in the basolateral membranes of uterine epithelia and in the myometrium. β_1 Subunit expression was positively correlated with areas shown to stain positively for the α_1 isoform. Diffuse α_2 but dense α_3 staining was visible throughout the endometrium of both pregnant and non-pregnant tissue. Relatively diffuse β_2 and β_3 staining was observed in all regions except for uterine glands, which produced higher levels of immunostaining for β_3. Application of 10 nM oxytocin increased force in non-pregnant tissue ($n = 3$). When oxytocin was applied following ouabain incubation (10 μM for 45 min), significantly more force was produced ($P < 0.05$, paired t test).

These results suggest that uterine Na^+–K^+-ATPase consists of α_1, α_2 and α_3 and β_1, β_2 and β_3 isoforms but the isoform combinations differ in the distinct anatomical regions of the uterus. The data with ouabain demonstrates the functional significance of the sodium pump in the myometrium, and suggests that elevation of intracellular Na^+ leads to Ca^{2+} entry via Na^+–Ca^{2+} exchange and hence augmentation of force.

Mobasheri, A. *et al.* (2000). *Biosci. Rep*. **20**, 51–91.

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Platelet-derived growth factor (PDGF)-induced activation of proliferative pathways in rat portal vein: comparison of neonatal and fully developed smooth muscle

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Proliferation of vascular smooth muscle cells occurs during blood vessel development and neointimal formation. This is regulated by activation of proliferative signalling pathways involving transcription factors, such as nuclear factor-κB (NF-κB) and cAMP response element-binding protein (CREB), as well as the mitogen-activated protein kinases, extracellular signal-regulated kinase1/2 (ERK1/2). The activation of these pathways by growth factors, such as PDGF, in developing compared with fully developed vascular smooth muscle has not been investigated. The aim of this study was to examine the PDGF-induced activation of NF-κB, CREB and ERK1/2 in neonatal rat portal vein (PV), previously characterised as a developing smooth muscle phenotype (Tasker *et al.* 1999). Freshly isolated PVs from 2- to 4-day-old and 6-week-old Sprague-Dawley rats killed by cervical dislocation were stimulated with PDGF-BB for 0, 15, 30 and 60 min. Tissues were immediately homogenised, proteins separated using SDS polyacryalamide gel electrophoresis and subjected to immunoblotting with specific antibodies. Freshly isolated PV strips were also used for contraction studies using a force transducer.

CREB activation (as measured by phosphorylation of CREB) peaked at 30 min after stimulation with PDGF-BB (50 ng ml^{-1}) (increased 280 % compared with unstimulated control, $n = 4$) in the neonatal PV and decreased to control levels by 60 min. PV from adult rats was activated by PDGF-BB to a lesser extent and peaked much earlier (increased 170 % by 15 min, $n = 4$). In contrast, PDGF-induced NF-κB activation (as measured by degradation of inhibitory factor-κBα (I-κBα)) revealed a different time course in developing, compared with fully developed, PV. NF-κB was maximally activated at 30 min (30 % decrease in I-κBα, $n = 3$), whereas neonatal PV did not induce NF-κB activation at any time points examined ($n = 3$). ERK1/2 were maximally activated by PDGF-BB after 15 min in both the neonate and adult PV; however, activation was sustained at 60 min in the adult, whereas it returned to control levels in the neonate. PDGF-BB-induced contractility was significantly greater in adult PV strips compared with neonate (32.3 ± 9.1 % compared with 1.3 ± 0.3 %, respectively, of maximal K^+ response, $n = 5$, mean ± S.E.M., $P < 0.05$, Student's unpaired t test).

In conclusion, these findings suggest a diverse role for activation of transcription factors and ERK1/2 in developing vascular smooth muscle compared with fully developed smooth muscle. This may have an important role in the regulation of proliferation.

Tasker, P.N. *et al.* (1999). *Circ. Res*. **84**, 536–542.

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A possible role for tissue transglutaminase in regulating PLCδ_1 in human vascular smooth muscle cells (HVSMC)

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Phospholipase C (PLC) links many extracellular signals to intracellular responses via the hydrolysis of phosphoinositide 4,5-biphosphate (PIP2). Although much is known about the regulation of the PLCβ and PLCγ isoforms, the mechanism of regulation of PLCδ_1 remains obscure. PLCδ_1 is the most predominant isoform in differentiated, contractile HVSMC and has been proposed to be involved in cytoskeletal organisation (Lymn & Hughes, 2000). Previous studies in our group have suggested that PLCδ_1 activity is dually regulated by GTP proteins with Rho A acting as the negative modulator (Hodson *et al.* 1998). Tissue transglutaminase (TGII/Gh) is a dual-functional enzyme, which can act either as a transglutaminase involved in protein modification and cross-linking or as a GTPase. TGII/Gh has previously been demonstrated to play a role in PLCδ_1 activation in rat liver (Feng *et al.* 1996).

The aim of this study is to determine the level of TGII/Gh expression and cellular localisation in relation to PLCδ_1 expression and redifferentiation of HVSMC.

HVSMC, derived from saphenous vein by a routine explant technique, were cultured in Dulbecco's modified Eagle's medium containing 15% fetal calf serum. The use of this tissue conformed to local ethics commitee guidelines. In order to induce a measure of redifferentiation HVSMC were serum deprived for 7 days. Expression of smooth muscle α-actin (SM α-actin), PLCδ_1 and TGII/Gh were determined by SDS-PAGE and Western blotting. Cellular location was determined using confocal microscopy. Protein expression in randomly cycling cells was taken to be 100% and all the data were expressed as means ± S.E.M. with respect to this. Statistical analysis was performed using Student's paired *t* test and a *P* value < 0.05 was considered as significant. Serum deprivation of HVSMC resulted in a significant increase in α-actin, PLCδ_1 and Rho A protein expression, while TGII/Gh expression was significantly decreased.

Table 1. Protein expression in HVSMC

SM α-actin	203 ± 19*
PLCδ_1	247 ± 167*
TGII/Gh	83 ± 7*
Rho A	200 ± 46*

*$P < 0.05$.

Serum deprivation resulted in α-actin becoming more structured with fibres running the length of the cell. PLCδ_1 also took on a more structured appearance. Intriguingly, PLCδ_1 and TGII/Gh were expressed in different locations in randomly cycling cells, but were co-localised in serum-deprived cells.

In conclusion, serum deprivation modulates both PLCδ_1 and TGII/Gh protein expression and induces a cellular association of these enzymes. These data suggest that TGII/Gh may play a role in determining not only PLCδ_1 but also contraction.

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The role of RhoA/ROCK pathway in the control of rat pulmonary artery and aorta contraction

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In the lung two important and specific mechanisms of local blood flow control are observed: hypoxic vasoconstriction and the resistance to the vasodilator effect of acidosis. We have recently reported that the resistance of pulmonary vessels to the vasodilator effect of acidosis is a property observed in isolated, endothelium-denuded vessels. This suggests that there are differences between the excitation–contraction coupling mechanisms in pulmonary and systemic vessels.

In smooth muscle, the level of myosin light chain (MLC_{20}) phosphorylation, which is the primary determinant of contraction, is regulated not only by fluctuation in cytoplasmic calcium, but also by other Ca^{2+}-independent mechanisms (so-called Ca^{2+}-sensitization mechanisms). It is now well recognised that the RhoA/Rho-associated kinase (ROCK) pathway plays a major role in Ca^{2+} sensitization through inhibition of myosin light chain phosphatase. The contribution of the RhoA/ROCK pathway to contraction differs in different muscles.

We hypothesised that in pulmonary vascular smooth muscle, the RhoA/ROCK pathway mediates a greater proportion of tension development than in systemic circulation.

We have studied the effect of two specific ROCK inhibitors Y27632 and HA1077 on tension development induced by agonists in both pulmonary artery (PA) and aorta endothelium-denuded rings. Vessels were submaximally (EC_{70}) contracted with the α-agonist phenylephrine (PE) or the thromboxane analogue U46619. Adding Y27632 (10^{-8} to 3×10^{-6} M) induces a concentration-dependent relaxation. The maximal relaxation induced by Y27632 was 90 ± 5 *vs.* 70 ± 8% ($n = 6$, $P < 0.05$, mean ± S.E.M., Student's *t* test), respectively, in PA and aorta contracted with PE. The calculated IC_{50} values for relaxation of PE-induced contraction reveals a greater potency of Y27632 in PA compared with aorta (0.38 ± 0.06 and 2.17 ± 0.24 μM, respectively, $n = 6$, $P < 0.05$). Similar results were obtained with the HA-1077. In the presence of the voltage-gated Ca^{2+} channel inhibitor nifedipine (Nif, 10 μM) and the Ca^{2+} store-depleting agent thapsigargin (TSG, 2 μM), PE-induced contraction remained unchanged in PA (90 ± 9% of the control, $n = 6$, $P > 0.05$) but was lowered in aorta (80 ± 2% of the control, $n = 6$, $P < 0.05$). The Nif/TSG-resistant component of the PE-induced contraction was also inhibited by Y27632 in a concentration-dependent manner, and the concentration–response curve to Y27632 was similar to that under control conditions. Similar results were obtained for U46619-induced tension development, whereas Y27632 did not alter KCl-induced contraction in either PA or aorta.

In freshly isolated smooth muscle cells from PA and aorta, loaded with fluo-3/AM, the PE (10 μM)-induced Ca^{2+} rise was unaffected by Y27632. Moreover, in the presence of Nif/TSG, PE did not induce Ca^{2+} changes.

The RhoA level expression, assessed by Western blotting, was similar in both PA and aorta.

These results suggest that Y27632 relaxes agonist-induced contraction in both PA and aorta rings independently of any

changes in Ca^{2+}. The greater relaxation induced by Y27632 and the greater potency in PA, suggest that the RhoA/ROCK pathway plays a greater role in agonist-induced tension development in this vessel, independently of any change in the RhoA level expression.

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The negative inotropic effects of halothane on contractile properties in the streptozotocin-induced diabetic rat heart

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Defective cardiac function is a frequent complication of human diabetes and is also a feature of experimentally induced diabetes (Yu *et al.* 1994; Tamada *et al.* 1998). In the normal heart volatile anaesthetics such as halothane have been shown to exert a potent negative inotropic effect (Housmans & Murat, 1988). In this study we have investigated the effects of halothane on the mechanism of contraction in ventricular myocytes from streptozotocin (STZ)-induced diabetic rats compared with age-matched controls. STZ (60 mg kg^{-1}) was administered intraperitoneally (I.P.) to male Wistar rats (250–300 g). Animals were killed humanely and ventricular myocytes were isolated by a combination of enzymatic and mechanical dispersal techniques and contraction was measured via a video edge detection system (Howarth & Levi, 1998). Cells were superfused with a normal Tyrode (NT) solution containing 1 mM Ca^{2+}. Following a train of steady-state contractions, myocytes were rapidly superfused with halothane (0.6 mM) for 1 min.

At 8–12 weeks after STZ treatment blood glucose levels in diabetic (mean ± S.E.M., 378.3 ± 17.6 mg dl^{-1}, $n = 9$) animals were significantly higher ($P < 0.01$; independent samples t test) compared with controls (88.3 ± 3.1 mg dl^{-1}, $n = 9$). Other characteristics of diabetic animals included significantly ($P < 0.05$; independent samples t test) reduced body weight and heart weight. The time to peak of contraction (t_{pk}) of myocyte shortening was significantly ($P < 0.01$; independent samples t test) prolonged in STZ myocytes (137.2 ± 4.1 ms, $n = 32$) *versus* control (105.5 ± 2.0 ms, $n = 31$), and was significantly ($P < 0.01$; paired t test) reduced when comparing pre- and post-application of halothane in both control (94.6 ± 2.4 ms, $n = 31$) and STZ myocytes (119.3 ± 3.4 ms, $n = 32$). Halothane significantly ($P < 0.01$; independent samples t test) reduced amplitude of contraction in both control (from 100 to 65.9 ± 2.7 %, $n = 31$) and STZ-induced (from 100 to 40.9 ± 3.2 %, $n = 32$) myocytes. This response was significantly ($P < 0.01$; independent samples t test) greater in STZ-induced myocytes compared with control. The time from the peak of contraction to half-decay was not significantly different between control and STZ cells in the presence or absence of halothane, but was significantly ($P < 0.01$; paired t test) decreased in control (46.5 ± 2.1 ms, $n = 31$ *vs.* 42.5 ± 2.0 ms, $n = 31$) and STZ-induced (48.6 ± 1.8 ms, $n = 32$ *vs.* 45.1 ± 1.9 ms, $n = 32$) myocytes comparing pre- and post-application of halothane, respectively. These results demonstrate that halothane evokes a markedly pronounced negative inotropic effect in the STZ-induced diabetic heart compared with the normal heart.