PC1

A cyclic GMP-dependent calcium-activated chloride channel in smooth muscle tissues: properties, distribution and identity

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We have recently described a unique cGMP-activated Ca^2 -activated chloride current ($I_{Cl(cGMP, Ca)}$) in smooth muscle cells (SMCs) (Matchkov *et al.* 2004) with properties distinct from the classical Ca^2 -activated chloride current ($I_{Cl(Ca)}$). In contrast to $I_{Cl(Ca)}$, the new current is activated by cGMP via PKG. In the following table the properties of the classical $I_{Cl(Ca)}$ and the novel $I_{Cl(cGMP, Ca)}$ are compared.

Interestingly, both currents were found to co-exist in smooth muscle cells, where they can be separated pharmacologically.

We characterised the distribution of the $I_{Cl(cGMP, Ca)}$ in vascular and non-vascular SMCs of different origin: aorta, pulmonary artery, tail artery, femoral artery, femoral vein, middle cerebral artery, renal artery, portal vein, superior mesenteric artery, mesenteric small artery and colon and compared distribution to that of classical $I_{Cl(Ca)}$. The conventional patch-clamp technique was used. Rats were humanely killed, tissues were excised, and fresh SMCs were enzymatically isolated.

We found that $I_{Cl(Ca)}$ and $I_{Cl(cGMP,Ca)}$ co-exist throughout the vascular tree, with the exception of the pulmonary circulation where only $I_{Cl(Ca)}$ was found. $I_{Cl(cGMP, Ca)}$ was dominant in cerebral artery and femoral vein, and was larger than $I_{Cl(Ca)}$ in aorta, renal, femoral, mesenteric small and superior mesenteric arteries. $I_{Cl(Ca)}$ was pronounced in portal vein and tail artery. $I_{Cl(cGMP, Ca)}$ was also present in SMCs of colon (Matchkov *et al.* 2005).

The present data point to the possibility that $I_{Cl(cGMP,Ca)}$ may play a significant role in the regulation of vascular and potentially non-vascular smooth muscle. Selective inhibition of $I_{Cl(cGMP, Ca)}$ inhibits vasomotion, demonstrating the essential role of this channel in synchronising cellular activity (Peng *et al.* 2001). This channel has all required properties to be a link between two major signalling pathways: calcium and NO/cGMP.

Table 1. Comparison of the properties of the classical $I_{Cl(Ca)}$ and the novel $I_{Cl(cGMP, Ca)}$

	The classical $I_{Cl(Ca)}$	$I_{Cl(cGMP,Ca)}$
$[Ca^{2+}]_i$ EC_{50}	>250 nM	~ 74 nM
$[cGMP]_i$ EC_{50}	No effect	~ 3-6 μM
Halide permeability	$I^- > Br^- > Cl^-$	$Br^- > I^- > Cl^-$ or $Cl^- > I^-$
Voltage- and time-dependence	At $[Ca^{2+}]_i$ below 1 μM	Independent
Niflumic acid IC_{50}	2-20 μM	No effect at 100 μM
DIDS IC_{50}	15-250 μM	No effect at 200 μM
Zn^{2+} IC_{50}	Not effective	~ 2-6 μM
SCN^- conductance	$SCN^- > Cl^-$	$Cl^- > SCN^-$ (blockade)

Matchkov VV, Aalkjaer C & Nilsson H (2004). *J Gen Physiol* 123, 121-134.

Matchkov VV, Aalkjaer C & Nilsson H (2005). *Pflugers Arch* (in press).

Peng H, Matchkov V, Ivarsen A, Aalkjaer C & Nilsson H (2001). *Circ Res* 88, 810-815.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC2

Ionized non-fatal whole-body irradiation inhibits large conductance Ca^{2+}-dependent K^+ channels in endothelial cells of rat coronary artery

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The endothelial lining is a very important system playing a crucial role in vascular tone regulation and is thought to be responsible for vasospasm development. Although it is known (Soloviev et al. 2003) that radiation selectively inhibits the EDRF/NO-dependent component of acetylcholine-induced vascular relaxation, while EDHF is able to maintain endothelium-dependent relaxation at a reduced level, the underlying mechanisms of this phenomenon remain unknown. The goal of this study was to evaluate the influence of single dose, whole-body γ-irradiation (6 Gy) on large-conductance Ca^{2+}-dependent potassium channels (BK_{Ca} channels) in primary cultured rat coronary artery endothelial cells using the whole-cell configuration of the patch-clamp technique. During irradiation, Wistar rats (250 g b.w.) were restrained in a plastic box, and the radiation beam was focused on the animal's chest. There was no change in housing, standard food or drinking water following irradiation. The animals were closely observed for unwanted effects and there were no visible signs of discomfort or illness. On the 9th and 30th days post-irradiation the hearts were taken from the animals anesthetized with ketamine/xylazine (1 ml/kg b.w.) to obtain the coronary artery endothelial cells.

The stimulation of control cells by increasingly depolarized voltage steps at potentials more positive than -40 mV elicited outward potassium currents that were followed by clearly expressed outward tail currents. The current-voltage relationships for peak and steady-state currents exhibited close to linear behaviour at potentials more negative then -40 mV and became non-linear at potentials above -40 mV. The reversal potential of the whole-cell current was -52±3 mV (n=8). A blocker of BK_{Ca} channels, paxillin (50 nM), abolished these currents consistent with the activation of large conductance BK_{Ca} channels. Irradiation significantly suppressed BK_{Ca} channels. Outward currents in irradiated cells isolated on the 9th day post-irradiation displayed two types - in one group of cells (20%) outward currents were suppressed significantly but not totally (type 1), while the remaining cells (80%) demonstrated total loss of outward currents (type 2). The reversal potentials for these whole-cell currents were -40±4 (n=7) and -10±2 mV (n=7), respectively. Paxillin was without effect on type 2 endothelial cells, and cells obtained on the 30th day post-irradiation, suggesting that BK_{Ca} channels were abolished. Irradiated cells displayed an almost linear I-V relationship indicating the absence of outwardly rectifying conductance. Increasing the extracellular potassium concentration from

5 to 10 mM decreased outward potassium currents induced by voltage ramp from -125 to +75 mV in both control and irradiated type 1 cells at potentials above -40 mV. A two-fold increase in potassium concentration led to a small increase in current at potentials lower than -75 mV, suggesting these cells expressed some inward rectifier channels.
The data obtained indicate that non-fatal, whole-body γ-irradiation suppresses large conductance, calcium-activated potassium channels, which control the driving force for Ca^{2+} entry and related NO synthesis in endotheliocytes. This may contribute to endothelium disorders following radiation impact.

Soloviev A, Tishkin S, Parshikov A, Ivanova I, Goncharov E & Gurney A (2003). Br J Pharmac 138, 837-844.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC3

Electrophysiological and pharmacological characterization of store-operated currents and capacitative Ca^{2+} entry in rat vascular smooth muscle cells

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Fura-2 fluorescence measurements of cytosolic free Ca^{2+} concentration have led to the hypothesis that depletion of intracellular Ca^{2+} stores in the A7r5 rat aortic smooth muscle cell line activates a Ca^{2+} entry mechanism known as capacitative or store-operated Ca^{2+} entry (CCE or SOCE, respectively). In a recent study (1), we described a store-operated current (I_{SOC}) in A7r5 cells, which was activated by passive depletion of Ca^{2+} stores with 1 μM thapsigargin or by active store depletion with 1-100 nM AVP. Because of different recording conditions, it has not been previously determined whether I_{SOC} corresponds to CCE measured using fura-2. Nor has the channel protein(s) responsible for this Ca^{2+} entry pathway been identified. In the present study, the pharmacological characteristics of I_{SOC}, including its inhibition by 2-aminoethoxydiphenylborane (2-APB), diethylstilbestrol, or micromolar Gd^{3+}, were found to parallel the effects of these drugs on CCE measured under identical external ionic conditions using fura-2 (Fig. 1). Rat mesenteric artery smooth muscle cells (MASMC) were prepared from arteries isolated from Sprague-Dawley rats anaesthetized with isolflurane (4% by inhalation); rats were then humanely killed. Thapsigargin-stimulated I_{SOC} in freshly isolated MASMC was similar to that recorded from A7r5 cells. Members of the TRP family of non-selective cation channels, TRPC1, TRPC4, and TRPC6 were detected by RT-PCR and Western blot in both A7r5 cells and MASMC. A stable A7r5 cell line expressing a small interfering RNA, which selectively reduced TRPC1 mRNA expression (by 43 ± 7% (mean ± S.E.M.), determined using real-time RT-PCR), exhibited significantly decreased thapsigargin-stimulated I_{SOC} (inward current measured at -100 mV (mean ± standard error): -0.21 ± 0.02 pA/pF (n=7), compared with -0.49 ± 0.05 pA/pF (n=8) measured in control cells; $p<0.001$, Student's t test), suggesting that this channel contributes to the store-operated pathway.

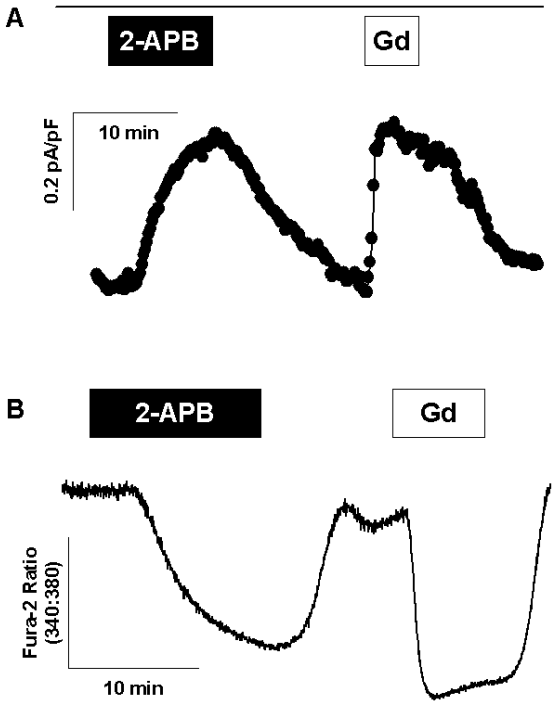

Figure 1. Similar pharmacological characteristics of I_{SOC} and CCE

A, I_{SOC} was pre-activated with 1 μ thapsigargin for 15 min. Time course of inward current recorded at -100 mV is presented. Treatment with 10 μM 2-APB for 10 min (black box) reversibly inhibits I_{SOC} to a similar extent as 100 μM $GdCl_3$ (white box, about 5 μM free Gd^{3+}) (representative of n=4). B, recording of fura-2 fluorescence from a population of A7r5 cells pre-treated with thapsigargin to deplete intracellular Ca^{2+} stores and activate capacitative Ca^{2+} entry (CCE). 2-APB and Gd^{3+} have similar inhibitory effects on CCE as on I_{SOC} (representative of 4 similar experiments). The same external solutions were used for I_{SOC} and fura-2 recordings.

Brueggemann LI, Markun DR, Barakat JA, Chen H & Byron KL (2005). *Biochem J* 388, 237-244.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC4

The effect of large conductance Ca^{2+}-activated K^+ channel modulators on Ca^{2+}-activated Cl^- currents

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The Ca^{2+}-activated Cl^- current (IClCa) has been characterised in a variety of smooth muscle preparations where activation causes membrane depolarisation and increased cellular excitability. Although this current is known to play a fundamental role in

smooth muscle excitation-contraction coupling the molecular identity of channel protein remains elusive. Various studies have shown that blockers of IClCa augment the large conductance Ca^{2+}-activated K^+ current (BKCa) suggesting that there may be some structural similarity between the two channels. Consequently we have investigated whether modulators of BKCa channels also affect IClCa in mouse portal vein smooth muscle cells. Mice were humanely killed. IClCa was measured in the whole cell configuration with an internal solution containing (mM): 106 CsCl, 20 TEA-Cl, 10 Hepes, 10 BAPTA, 0.42 $MgCl_2$, 3 Na_2ATP, 0.2 GTP(Na) and either 250 or 500nM free Ca^{2+}. The external solution contained 126 NaCl, 11 glucose, 10 Hepes, 1.2 $MgCl_2$ and 1.5 $CaCl_2$. Values are given as means ± S.E.M.

Application of the BKCa activators NS1619 (30 μM) and isopimaric acid (IpA, 3 μM) increased the late outward component of IClCa (termed I_{late}) by 159 ± 26% (n = 9) and 240 ± 16% (n = 5), respectively. Neither reagent affected the kinetics of the relaxation or the reversal potential of the current. NS1619 had no affect on the apparent Ca^{2+} sensitivity. The chloride channel blocker niflumic acid (NFA, 100 μM) alone both augmented and inhibited IClCa in a voltage-dependent manner as described previously (Piper *et al.* 2002) whereas the BKCa inhibitor Paxilline (Px, 1 μM) produced inhibition only, decreasing ilate by 66 ± 6% (n = 4). Whilst neither reagent alone completely inhibited IClCa, NFA and Px in combination completely blocked all components of the current and abolished the time-dependent relaxation (n = 5). The NS1619-enhanced current was partially inhibited by 100μM NFA (n = 3) and 1μM Px (n = 2), whilst a complete block of the current occurred when both reagents were combined. The IpA-enhanced current was partially inhibited by Px (n = 2), and transiently enhanced by NFA (n = 2). However, a combination of NFA and Px on the IpA-enhanced current again resulted in an almost complete block (n = 4).

These data show that agents known to interact with BKCa produce similar effects on IClCa and may provide important insights into the channel responsible for this conductance.

Piper AS *et al.* (2002). J Physiol **539**, 119-131.

S.S. is a BBSRC/GSK PhD student.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC5

Dihydropyridine-insensitive voltage-dependent calcium channels in terminal arterioles from rat mesentery

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The role of voltage-dependent calcium channels (VDCCs) for diameter regulation in rat mesenteric terminal arterioles (RMTA) has been studied using intra-vital microscopy. However, there is a need to develop preparations for studying vasomotor signals in isolated arterioles and in single cells isolated therefrom.

Arterioles were isolated from the mesentery of humanely killed rats using enzyme digestion and mechanical dispersion. These non-pressurized microvessels had one continuous layer of smooth muscle cells in a circular arrangement around the vessel lumen. The external diameter was 31±1.2 μm (mean±S.E.M.; n = 27) and the internal (lumenal) diameter was 17±1.3 μm (n = 7), thus classifying them as terminal arterioles (lumen <50 μm). Isolated arterioles were mounted on cover slips, loaded with the Ca^{2+} indicator Fura-PE3, and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated as F_{340}/F_{380}. Voltage-dependent Ca^{2+} influx was evaluated as % changes from baseline of the F_{340}/F_{380} during sustained depolarization using 75 mM bath KCl (hi-K) (Jensen et al, 2004).

The overall baseline F_{340}/F_{380} of 0.91±0.02 increased to 1.08±0.04 with hi-K solution (118.6±2.5 % increase relative to baseline, $P<0.001$, n = 27). Initially, we tested the effect of various modulators of L-type VDCCs. The dihydropyridine nitrendipine (10 μM), a specific L-type blocker, non-significantly changed the Ca^{2+} increase to hi-K from 128.5±6.2 % to 125.3±6.5 % relative to the baseline F_{340}/F_{380} (*N.S.*, n = 7). Bay-K8644 (1 μM), an L-type channel opener, did not change the resting $[Ca^{2+}]_i$, but induced a non-significant change of the Ca^{2+} increase to hi-K from 114.8±3.1 % to 120.6±3.3 % relative to baseline (*N.S.*, n = 5). The phenylalkylamine D-600 (2 μM) did not change the baseline ratio, yet it reduced the increase to hi-K from 113.3±2.8 % to 103.6±0.5 % relative to baseline (70±5 % inhibition, $P<0.05$, n = 4). $NiCl_2$ (500 μM), a fast and reversible inorganic blocker of T-type Ca^{2+} channels, reduced the baseline from 100 % to 97.5±0.4 % ($P<0.01$, n = 5), and reduced the Ca^{2+} increase to hi-K from 109.3±1.5 % to 102.1±0.1 % relative to baseline (79±2 % inhibition, $P=0.001$).

We conclude that this arteriolar preparation is suitable for measurements of $[Ca^{2+}]_i$ or whole-cell Ca^{2+} current following single cell dispersion. Although the presence of L-type VDCCs could not be excluded, the combined dihydropyridine insensitivity and Ni^{2+} sensitivity suggests that the voltage-dependent Ca^{2+} influx in isolated RMTA is carried by channels with a T-type pharmacology, thus confirming our previous investigations (Morita et al, 2002; Jensen et al, 2004). Of note is the effect of D-600 in RMTA, suggesting that its antihypertensive capacity may partially stem from inhibition of a T-type channel in the peripheral vasculature.

Jensen LJ et al (2004). Br J Pharmacol **142**, 709-718.

Morita H et al (2002). Br J Pharmacol **137**, 467-476.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC6

TRPC1 is involved in receptor-activated calcium entry in rat aortic smooth muscle cells

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In vascular smooth muscle, activation of plasma membrane ion channels is an important mechanism allowing for the increase in cytosolic calcium concentration and the development of contraction. In rat aorta, agonist-stimulated calcium entry is only partly inhibited by blockade of voltage-operated calcium chan-

nels (1), suggesting that another calcium entry pathway is activated. Canonical transient receptor potential (TRPC) proteins have been proposed as candidates for receptor- or store-operated calcium channels in smooth muscle cells (2). In order to determine the role of TRPC1 in the calcium signal evoked by agonists in rat aorta smooth muscle cells (VSMC), its expression was inhibited by transfecting the cells with small interfering RNA directed against TRPC1 (siRNA-TRPC1). Functional evaluation was performed by measuring the calcium signal by fluorescence microscopy in fura-2 loaded VSMC.

Rats were humanely killed; the aorta was quickly removed, cleaned from adherent tissue and endothelium was gently rubbed off. Primary culture of VSMC was performed by the explant technique. The gene expression of TRPC isoforms in VSMC was investigated by RT-PCR. mRNA encoding TRPC1, -3, -4, -5, -6 and -7, but not TRPC2 was detected in VSMC. The highest level of expression was found for TRPC1. In cells transfected with siRNA-TRPC1, TRPC1 mRNA expression was inhibited by 72 ± 3% (n=4). In contrast, transfection with nonsilencing control siRNA did not affect the level of expression of TRPC1 mRNA. Immunocytochemistry revealed that TRPC1 protein expression was markedly attenuated in siRNA-TRPC1 transfected VSMC but was not modified in cells transfected with nonsilencing siRNA. Measurement of calcium signal evoked by endothelin-1 in VSMC showed that, in the absence of external calcium, endothelin-1 induced a rapid but transient increase in cytosolic calcium, reflecting the release of intracellular calcium. Addition of calcium into the external solution in the presence of endothelin-1 evoked a sustained increase in cytosolic calcium, which was completely blocked by the ET-A receptor antagonist BQ-123 and by micromolar concentration of Gd^{3+}. In VSMC transfected with siRNA-TRPC1, calcium release evoked by endothelin-1 in calcium free solution was not modified, but the increase in cytosolic calcium observed after the addition of calcium into the external solution was markedly blunted. These results indicate that TRPC1 channel protein is involved in receptor-activated calcium entry in aortic VSMC.

Ghisdal P et al. (2003). J Physiol 551, 855-867.

Beech DJ et al. (2004). J Physiol 559, 685-706.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC7

Cloning and functional characterisation of a family of microbial inwardly rectifying (Kir) potassium channels

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The recent determination of 3D X-ray crystal structures for the prokaryotic inwardly-rectifying Kir channels KirBac1.1 and KirBac3.1 has provided a major advance in our understanding of the relationship between structure and function for this class of potassium channels. However, very little is known about the functional properties of these prokaryotic Kir channel homologs. In this study we have cloned KirBac genes from a diverse range of prokaryotic organisms and unicellular eukaryots. These 10 different KirBac sequences exhibit between 24% and 67% identity with KirBac1.1 at the amino acid level and show sequence diversity in structurally important regions. In order to assess their functional properties we have screened their ability to complement the growth of mutant E.coli strains deficient in K+ uptake (TK2420 and LB2003). Several of the KirBac gene products, including KirBac1.1 appear toxic to the sustained growth of E.coli and so appropriate host-independent expression vectors must be used. For this purpose we constructed our own expression vector based upon the Qiagen pQE-60. Complementation of growth was assessed using both standard and defined low K+ medium in culture and on plates. Some KirBac channels e.g. KirBac2.1 and KirBac6.1 appear to show highly efficient complementation of growth at <4mM external K+ concentrations, whilst others require >10mM K+. The KirBac channels also appear to exhibit differing sensitivities to inhibition by external barium and caesium. In particular growth of strains containing KirBac6.1 could be completely inhibited by 10 micromolar caesium chloride whilst other channels e.g. KirBac1.1 and KirBac2.1 require millimolar concentrations to achieve the same effect. These results demonstrate a clear range of functional diversity amongst this family of KirBac channels which, combined with the high resultion structural information now available, should provide an excellent resource for future structure-function and physiological studies.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC8

Extracellular matrix modulates capacitative calcium entry and TRP channel expression in human airway smooth muscle cells

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Extracellular matrix (ECM) modulates the phenotype of airway smooth muscle cells (ASM) (Hirst SJ, et al, 2000). Capacitative calcium entry (CCE), responsible for the control of relaxation and contraction of ASM is thought to be coded by TRP channel genes. Although studies have shown some links between ECM and CCE, direct effects of ECM on CCE in HASM are still not fully understood. The purpose of the present study was to elucidate the interplay between ECM and CCE in cultured human ASM (HASM) and underlying mechanisms with emphasis on TRPC genes.

HASM cells were isolated from resection tissue from patients undergoing pneumonectomy (ethical approval was obtained from the LREC of the City Hospital of Nottingham). Cells (passage 2 to 5) were transferred onto 96 well plates or glass cover slips pre-coated with Collagen -1 (Col-1), fibronectin (FN) or laminin (LN) (10μg/mL). Fluo-4 was used to measure intracellular Ca^{2+} on confluent monolayers and whole-cell patch clamp to study CCE currents induced by CPA (10 μM). Experiments

were performed in serum-free DMEM in the presence of cycloheximide (10 μM) to inhibit new protein synthesis. Taqman Real-time PCR to assess TRPC1, TRPC3, TRPC4 and TRPC6 gene expression was carried out on cDNA samples prepared from HASM cells growing on plates pre-coated with FN, Col-1 or LN. Data were analysed by ANOVA or unpaired t test as appropriate. CPA-induced Ca^{2+}release and CCE in cells growing on LN were increased by 25% (n=9, $p<0.01$) compared with responses in cells grown on Col-1 or FN. CPA-induced currents were nearly abolished by 1 mM concentrations of La^{3+}, Ni^{2+}, and Gd^{3+}, and decreased in a concentration-dependent manner by La^{3+} (0.01 to 100 μM). SKF93635 (up to 100 μM) was without any effect. Compared with control, peak SOCC currents at -100 mV were decreased by 66% (n=9, $p<0.01$), and 71% (n=10, $p<0.05$) respectively in FN, Col-1 treated cells while in LN treated cells peak SOCC current was increased by 67% (n=10, $p<0.05$). Compared with control, TRPC1 and TRPC3 gene expression were not significantly affected by Col-1, FN or LN treatment. TRPC4 was up-regulated by 31% and 70% in Col-1 and FN treated cells respectively while in LN treated cells TRPC4 was down-regulated by 25%. TRPC6 gene expression was up-regulated by 97% in FN-treated cells.

In the present study, we have shown that FN and Col-1 down-regulated CCE while exposure to LN protein produced a small up-regulation of CCE in cultured HASM. Matrix factors also regulated TRPC gene expression: in general an increase in TRPC6 expression was associated with a decrease in CCE. These results suggest that alterations in the matrix environment induce changes in calcium handling by HASM cells: this may account in part for altered responsiveness of asthmatic airways.

Hist SJ et al. (2000). Am J Respir Cell Mol Biol 23, 335-344.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC9

Differential sensitivity of smooth muscle, cardiac muscle and pancreatic β-cell K_{ATP} channels to adenine nucleotides

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ATP-sensitive K^+ (K_{ATP}) channels, composed of pore-forming Kir6.2 and regulatory SURx subunits, are critical for the physiological function of many tissues by acting as metabolic sensors. Metabolic regulation of K_{ATP} channel activity is achieved by changes in the intracellular concentrations of adenine nucleotides. Binding of ATP to Kir6.2 inhibits K_{ATP} channels, while interaction of Mg-nucleotides with SUR1 promotes channel opening. Recently, a mutation in Kir6.2 (F333I) that enhances the activatory effect of SUR1, has been reported (Tammaro et al. 2005). We used this mutation as a tool to analyse the activation properties of different SUR isoforms that contribute to the varied metabolic sensitivities of K_{ATP} channels containing SUR1, SUR2A and SUR2B subunits.

Macroscopic K_{ATP} currents were recorded from inside-out patches from *Xenopus laevis* oocytes 1-3 days after injection with Kir6.2-F333I and SURx mRNAs. The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 Hepes (pH 7.4) plus various ATP or ADP concentrations. The Mg-free internal (bath) solution contained (mM): 107 KCl, 1 K_2SO_4, 10 EGTA, 10 Hepes (pH 7.2). The Mg-containing solution consisted of Mg-free solution plus 2mM $MgCl_2$ and MgATP or MgADP (instead of ATP or ADP). Values are means±S.E.M.

In the absence of Mg^{2+}, only small differences were seen in the IC_{50} for ATP inhibition of SUR1 (211±28μM, n=6), SUR2A (128±9μM, n=4) and SUR2B (119±19μM, n=4) containing channels. However, in the presence of Mg^{2+}, SUR2A currents were blocked by ATP (IC_{50} = 541±81μM, n=5) whereas SUR1 or SUR2B currents were *activated* (by ~5 and ~1.5 fold in 1 mM MgATP, respectively). The stimulatory effect of MgATP on SUR1 channels was abolished by mutations that prevent MgATP binding/hydrolysis. Both SUR2A and SUR2B currents were activated by 1 mM MgADP: 5.5±1.0 (n=5) and 5.0±0.5 (n=5) fold, respectively. The fact that SUR2A exhibits activation by MgADP but not by MgATP suggests that ATP binding/hydrolysis may be less than that of SUR1 or SUR2B. The differences in nucleotide handling by the various SUR isoforms may help explain why K_{ATP} channels are active in pancreatic beta cells over a wide range of metabolic conditions while in smooth muscle or the heart they open only under hypoxia or metabolic inhibition.

Tammaro P *et al.* (2005). *EMBO J* (in press).

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC10

Defining electrical communication in skeletal muscle resistance arteries: a computational approach

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Vascular cells communicate electrically to coordinate their activity and control tissue blood flow. To foster a quantitative understanding of this fundamental process, we developed a discrete computational model that was structured to mimic a skeletal muscle resistance artery. Each endothelial cell and smooth muscle cell in our virtual artery was treated as the electrical equivalent of a capacitor coupled in parallel with a non-linear resistor representing ionic conductance; intercellular gap junctions were represented by ohmic resistors. Simulations revealed that the vessel wall is not a syncytium in which electrical stimuli spread equally to all constitutive cells. Indeed, electrical signals spread in a differential manner among and between endothelial cells and smooth muscle cells according to the initial stimulus. The predictions of our model agree with physiological data from the feed artery of the hamster retractor muscle. Cell orientation and coupling resistance were the principal factors that enable

electrical signals to spread differentially along and between the two cell types. Our computational observations also illustrated how gap junctional coupling enables the vessel wall to filter and transform transient electrical events into sustained voltage responses. Functionally, differential electrical communication would permit discrete regions of smooth muscle activity to locally regulate blood flow and the endothelium to coordinate regional changes in tissue perfusion.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC11

Maxi-K channel expression in myometrial smooth muscle is regulated by endoproteolytic cleavage

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Understanding the regulation of myometrial smooth muscle excitability is important for developing an effective treatment for preterm labor. Large-conductance Ca^{2+}- and voltage-sensitive potassium channels (maxi-K) promote uterine quiescence by generating repolarizing current. Our laboratory identified an alternatively spliced maxi-K channel isoform (mK44) that contains a 44 amino acid (aa) insertion in the first intracellular loop and is expressed predominantly in myometrial and aortic smooth muscle. Endogenous mK44 channels are localized in the endoplasmic reticulum (ER) in human myometrial smooth muscle cells (hMSMCs). This finding indicated that mK44 current does not contribute to hMSMC K^+ current in the quiescent state. We hypothesized that mK44 channels express functional current on the cell membrane when induced by contractile stimuli. In hMSMCs after incubation with 20 mM caffeine, mK44 translocates to the cell membrane and induces hMSMC repolarization. To understand channel trafficking, mK44 was fused with a c-myc tag at the N-terminus (myc/mK44) and transiently expressed in hMSMCs. The N- and C-termini were found to have disparate localization with the C-termini localized in the ER, and the N-termini localized to the cell membrane. Upon incubation with caffeine, the C-termini translocated to the cell membrane and co-localized with the N-termini. Sequence analysis of the mK44 specific insert suggested that mK44 has a putative recognition motif (-RK-, aa 62-63) for the M16 family of peptidases and may undergo an endoproteolytic digest. An N-terminal fragment of ~10 kDa, corresponding to the predicted N-terminal peptide that would result from endoproteolytic cleavage of mK44, was detected in hMSMCs lysates. Mutating this motif generated a protein (R62A/V5) that localized to the ER in hMSMCs, like wild-type mK44, but failed to translocate to the cell membrane in response to caffeine. Heterologous expression of the N-termini (aa 1-62) and C-termini (aa 69-1157) of mK44 reconstituted a functional channel sensitive to IbTX on the cell membrane in HEK293F cells. The myc/mK44 channels did not undergo endoproteolytic digest when expressed in HEK293F cells or mouse fibroblasts suggesting a posttranslational modification specific for a myometrial smooth muscle. Thus, we have identified a novel mechanism to regulate hMSMCs excitability in response to prolonged exposure to certain contractile agents. This mechanism includes a specialized pool of K^+ channels (mK44) that are normally retained in the ER and translocate to the cell membrane in response to sustained depolarization to generate repolarizing current and maintain hMSMC quiescence.

Korovkina VP et al. (2001). Am J Physiol 281, C361-C367.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC12

Facilitation of the stretch-activated non-selective cation channels by P2Y receptors in rabbit pulmonary arterial myocytes

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The active contraction of vascular smooth muscle in response to elevation of luminal pressure, or stretch, is termed as the myogenic response. The mechanosensitive nonselective cation channels activated by membrane stretch (NSC_{MS}) have been considered as a candidate transducing mechanical events into the contractile response of the cells. Using the patch clamp technique, we recorded single-channel currents of NSC_{MS} with unitary conductance of about 25 pS in enzymatically dispersed pulmonary (PASMCs) and coronary arterial smooth muscle cells (CASMCs) from humanely killed rabbits (Park et al. 2003). Both the density and the pressure-sensitivity were higher in PASMCs than CASMCs. Considering the vaso-regulatory role of ATP released during hypoxic stimuli, we investigated whether the purinergic stimulation affects the activity of NSC_{MS}. When ATP (1 μM) was included in the pipette for the cell-attached configuration, the NP_O and the chance of recording the NSC_{MS} was largely increased in PASMCs while not in CASMCs. Similar effects were observed with UTP (1 μM) applied through the patch pipette. However, the presence of α,B-methylene-ATP, an P2X1/3 agonist, in the pipette did not show significant effect on NSC_{MS} in PASMCs in spite of the known expression of P2X1/3 receptors in PASMCs. The bath application of ATP or UTP in the cell-attached configuration had no significant effect on the NSC_{MS}, suggesting a membrane-delimited regulation mechanism. In summary, we found that the NSC_{MS} is more abundantly expressed in PASMCs than CASMCs, which might reflect that the pulmonary circulation has adapted to its low-pressure environment. In addition, the higher expression of NSC_{MS} and the positive regulation by purinoceptors (P2Y) suggest that these channels might play a specific role in the regulation of pulmonary circulation.

Park KS, Kim Y, Lee YH, Earm YE & Ho WK (2003). Circ Res 93, 557-564.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC13

Activation of inward rectifier K^+ channels by hypoxia in rabbit coronary arterial smooth muscle cells

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We examined the effects of acute hypoxia on Ba^{2+}-sensitive inwardly rectifying K^+ (Kir) current in coronary arterial smooth muscle cells from humanely killed rabbits. The amplitudes of Kir current was definitely higher in the cells from small-diameter (o.d. < 100 μm) coronary arteries (SCASMC, -12.8±1.3 pA/pF at -140 mV) than those in large-diameter (o.d.> 200 μm, LCASMC, -1.5± 0.1 pA/pF). Western blot analysis confirmed that Kir2.1 protein was expressed in SCASMC but not in LCASMC. The hypoxic condition was attained by perfusing the bath with normal Tyrode solution bubbled with 100% N_2. In SCASMC, the Kir current was significantly increased by the hypoxic stimulation. This effect was blocked by the adenylyl cyclase inhibitor SQ 22536 (10 μM) and mimicked by forskolin (10 μM) and dibutyryl-cAMP (500 μM). The production of cAMP in SCASMC was measured by ELISA, which increased 5.7-fold following 6 min of hypoxia. Hypoxia-induced increase in Kir currents was abolished by the protein kinase A (PKA) inhibitors Rp-8-CPT-cAMPs (10 μM) and KT 5720 (1 μM). The inhibition of G-protein with GDPβs (1 mM) partially reduced (~50%) the hypoxia-induced increase in Kir currents. In Langendorff-perfused rabbit hearts, hypoxia increased coronary blood flow, an effect that was inhibited by Ba^{2+}. In summary, the hypoxia augments the Kir currents in SCASMC via cAMP and protein kinase A-dependent signalling cascades, which might, at least partly, explain the hypoxia-induced coronary vasodilatation.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC14

Properties of single muscarinic receptor-gated cation channels in murine intestinal myocytes

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Muscarinic receptor cation current in gastrointestinal smooth muscles is strongly modulated by both external and intracellular Ca^{2+}, but the mechanisms of this dependence at the single channel level have not been studied yet. In single ileal single smooth muscle cells, isolated from humanely killed mice, external application of 50 μM carbachol (CCh) or internal application of 200 μM GTPγS evoked an inward current of 0.60±0.05 nA at −40 mV (n=50). The current reversed close to 0 mV and its current-voltage relationship was U-shaped at negative potentials. Single channel recordings in outside-out patches revealed that CCh-induced current was carried via two types of monovalent selective cation channels with unitary conductances of about 17 and 70 pS (n=12). The behaviour of the 70-pS channel was consistent with the whole-cell muscarinic current properties implying its major contribution to the integral current. We therefore investigated the effects of changes in the external (2.5 or 10 mM) and internal (30, 100, 500 nM) Ca^{2+} concentration on the single 70 pS-channel properties. Ca^{2+} added to a nominally Ca^{2+}-free external solution at 2.5 or 10 mM reduced the unitary channel conductance from 70 pS to 46 pS and 42 pS, respectively, but had no significant effect on its open probability (P_O). When $[Ca^{2+}]_i$ was clamped at 30, 100, or 500 nM using a BAPTA-Ca^{2+} mixture the amplitude of the CCh- or GTPγS-evoked whole-cell current at 100 nM $[Ca^{2+}]_i$ was several times greater compared with either 30 or 500 nM $[Ca^{2+}]_i$. Correspondingly, with 30 or 500 nM Ca^{2+} in the pipette solution P_O of the 70 pS-channel was reduced from 0.2-0.5 (at 100 nM) to 0.02-0.1. In contrast, the variations in $[Ca^{2+}]_i$ had no effect on the unitary conductance. Using the fura-2-based calcium imaging system and simultaneous patch-clamp current recordings we found that the most potent blocker of the 70-pS single channel activity, quinine (20 μM), produced a strong decrease of the steady-state component of the CCh-evoked $[Ca^{2+}]_i$ rise. Other common blockers of various cation channels, SK&F 96365, LOE908, 2-APB and La^{3+} applied at micromolar concentrations, produced similar effects. It is concluded that in murine ileal smooth muscle cells the 70-pS cation channel mainly mediates cholinergic depolarisation and is important for maintaining the elevated intracellular calcium concentration during the activation of the muscarinic receptors.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC15

Glucose alters regulation of voltage-gated potassium channels in rat isolated mesenteric arterial smooth muscle cells

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Long term elevation of glucose (24 hours) has been shown to reduce Kv currents in cultured vascular smooth muscle cells (Li et al., 2003). We have examined the acute effects of altering glucose concentration on the activity and on the inhibition by endothelin-1 (ET-1) of Kv and K_{ATP} currents of rat arterial myocytes. Smooth muscle cells were isolated enzymatically from small mesenteric arteries obtained from humanely killed adult Wistar rats. Currents were recorded using conventional whole-cell recording with physiological K^+ gradients (6/140mM) for

Kv and symmetrical 140mM K^+ for K_{ATP} currents. In all experiments the total external concentration of glucose and mannitol was kept at 10mM to maintain osmolarity.
Kv currents elicited by pulsing from −65 to +40mV in glucose free solution, were reduced by 61 ± 8% within 2 minutes of adding 4 mM glucose (n = 6 cells). This reduction in current was reversed by removing the glucose. In 4 mM glucose, 10nM ET-1 decreased peak Kv current amplitude by 33 ± 4% (n = 6) and increased the rate of inactivation, decreasing its time constant at +40mV from 1496 ± 90ms in control to 380 ± 80ms in ET-1 (n = 6). Pre-incubation of the cells for 15 min with 50 μM of the membrane permeable myristoylated PKC inhibitor peptide 19-27 (PKC-IP) completely abolished the effects of ET-1 in 6 of 6 cells. Surprisingly, these alterations in Kv current kinetics were not observed when cells were bathed in 10 mM glucose. To test whether the endothelin receptors were still functional we examined the inhibition of K_{ATP}currents by ET-1. K_{ATP} currents were activated by 100 μM pinacidil and recorded at −60mV. Application of 10nM ET-1 reduced K_{ATP} current by 81 ± 4% (n = 6). Furthermore, in the presence of PKC-IP, ET-1 inhibited the K_{ATP} current by only 7 ± 3% (n=6), indicating that the endothelin receptors were still able to activate PKC in 10mM glucose. These results indicate that changes in extracellular glucose concentration within the physiological range can have rapid and profound effects on the ability of vasoactive compounds such as ET-1 to regulate Kv channels. These mechanisms may have an important role in the pathophysiological effects on the vasculature observed in diabetic patients, where glucose concentrations vary much more than in normal circumstances.

Li H, Chai Q, Gutterman DD & Liu Y (2003). Am J Physiol 285 H1213-1219.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC16

In vitro assay for identifying modulators of Cav channel α1–β subunit interactions

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Voltage-dependent calcium channels (VDCC) are multi protein complexes consisting of at least three subunits (α1, β, α2 δ). VDCC β subunits are cytosolic proteins involved in surface expression of α1 subunit. Co-expression of β subunits increase current amplitude manifold due to the increase in availability of α1 subunits at the membrane (Brice *et al.* 1997). In addition, β subunits have modulatory effects on channel properties, i.e. they hyperpolarize the voltage dependence of activation and differentially affect inactivation kinetics of the channel (Olcese *et al.* 1996).
VDCC play an important role in neurotransmitter release, gene expression and excitation-contraction coupling. Therefore, they are attractive targets for treating disorders associated with these functions. In recent history, the search for calcium channel blockers has centred around the α subunit. However, due to poor selectivity, many such compounds have not succeeded in development. Hence, the requirement is for new or next-generation calcium channel modulators or inhibitors. Here, we report the development of an assay to identify a novel class of compounds that inhibit protein-protein interactions between α1 subunit and the β subunit that can be used to treat diseases associated with smooth muscle constriction, such as urinary incontinence, hypertension and angina.
The full-length Cav β3 subunit and the α interaction domains (AID) of Cav 1.2 and Cav 2.2 were amplified from rat brain cDNA. Cav β3 subunits were subsequently expressed in E. coli and immobilised onto streptavidin substrates via the affinity tag, biotin carboxyl carrier protein. The purified AID proteins were Cy3-labelled and used in binding experiments to demonstrate assay principle. Cav β3 protein was successfully immobilised on streptavidin-coated plates. Furthermore, an anti-Cav β3 antibody directed towards the C-terminal epitope demonstrated that the immobilised Cav β3 subunits were full-length. Increasing concentrations of Cy3-Cav 1.2 or 2.2 AID proteins were incubated with Cav β3, a saturable binding was observed with good signal to noise ratio. In order to define the assay principle, binding affinity and kinetics between Cav AIDs and Cav β3 subunits were characterised further (Table 1).
In summary, an assay has been developed that allows the determination of the affinity and binding kinetics of the interaction between Cav1.2 and Cav2.2 AIDs to the full-length immobilised Cav β3 subunit to be studied in a micro-well format. Utilising this assay for high-throughput screening has the potential to identify compounds that modulate VDCCs through a novel mode of action.

Table 1. Binding parameters of Cav 1.x AID domains to immobilised Cav β3 proteins

Cav AID	K_d (nM)	K_{obs} (min-1)	$t_{1/2}$ (min)
1.2	27.4 ±2.1	0.0225	27.76
2.2	12.0±1.6	0.0183	26.14

K_d, equilibrium dissociation constant (means±S.E.M.; K_{obs}, observed association rate constant; $t_{1/2}$, amount of time required for the ligand to occupy 50% of the binding sites. n=3.

Brice *et al.* (1997). Eur J Neurosci 9, 749-759.

Olcese *et al.* (1996). J Physiol 497, 675-686.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC17

Further evidence for the neonatal splice variant of Nav1.5 potentiating *in vitro* metastatic behaviour of MDA-MB-231 human breast cancer cells: application of RNAi and a novel antibody

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We have shown previously that functional voltage-gated Na+ channels (VGSCs) are expressed in metastatic human breast cancer (BCa) *in vitro* and *in vivo* (Fraser et al. 2005). The predominant VGSC isotype is the TTX-resistant 'neonatal' splice variant of Nav1.5 (nNav1.5). Furthermore, TTX-resistant VGSCs

can potentiate metastatic behaviour in MDA-MB-231 cells (Fraser et al. 2005). The aim of the present study was to ascertain the specific involvement of the nNav1.5 in the VGSC-dependent metastatic behaviour of MDA-MB-231 cells. All data are presented as mean ± S.E.M. Statistical significance was evaluated with Student's t tests.

A polyclonal antibody (NESO-pAb), raised against an external epitope on nNav1.5, reduced both VGSC current density (Chioni et al. 2005) and *in vitro* migration through 12 μm-pore Transwell filters. The reduction in migration was dose-dependent, reaching 38 ± 6% at 1 μg/ml ($P < 0.001$; $n = 7$). NESO-pAb also reduced Matrigel invasion by 49 ± 8% compared to control ($P < 0.001$; $n = 7$), but had no effect on proliferation. There was no effect on proliferation or invasion of PC-3M prostate cancer cells, where Nav1.7 is predominant (Diss et al. 2001). IgG and an unrelated polyclonal anti-laminin antibody had no effect on invasion or proliferation of either cell line.

A small interfering RNA (siRNA) was used to specifically silence the nNav1.5 gene. A non-targeting siRNA was used as control. Five days post transfection, the siRNA reduced the level of nNav1.5 mRNA, quantified using real-time PCR normalised to cytochrome b5 reductase gene, by 91 ± 6%, compared to the control ($P < 0.001$; $n = 3$). Surprisingly, protein level reduction was much smaller, even after 13 days; Western blots indicated reductions of only 25 ± 10% ($P = 0.07$; $n = 3$), and 26 ± 3% ($P = 0.006$; $n = 3$) for NESO-pAb and pan-VGSC antibodies, respectively. Nevertheless, the siRNA significantly reduced peak VGSC current density by 33%, from 21 ± 3 pA/pF to 14 ± 2 pA/pF ($P = 0.02$; $n > 20$). Importantly, migration was suppressed by 43 ± 18% ($P = 0.037$; $n = 5$), and was not further reduced by TTX (10 μM).

We conclude that nNav1.5 is indeed primarily responsible for the VGSC-induced enhancement of metastatic cell behaviour in MDA-MB-231 cells and that targeting nNav1.5 expression/activity may be useful in clinical management of metastatic BCa.

Chioni A-M et al. (2005). J Neurosci Methods (in press).

Diss JKJ et al. (2001). Prostate 48, 165-178.

Fraser SP et al. (2005). Clin Cancer Res (in press).

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC18

Molecular expression of ion channel subtypes in human endometrium

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The adult human endometrium, a unique tissue undergoing monthly cellular remodelling in preparation for implantation, consists predominantly of epithelial and stromal cells. Ion channels of non-excitable cells are involved in diverse cellular functions that include proliferation and secretion. Endometrial function is thus likely to involve stage specific expression of ion channel. The four transmembrane domain two pore potassium (K2P) channels are a family of K^+ channels that contribute to the resting membrane potential and may be regulated by various factors including heat, stretch and pH. A second class of channels of interest is the transient receptor potential vanilloid (TRPV) subfamily, in particular TRPV5 and TRPV6 which mediate Ca^{2+} entry in epithelial cells. The aim of this investigation was to determine ion channel expression of known subtypes in human endometrium.

This study had local ethics committee approval. Endometrial samples were obtained by pipelle biopsy from healthy, non-pregnant, fertile women (<45 years of age), during clinical investigation for benign disorders. All women gave fully informed written consent. Total RNA was extracted from snap-frozen endometria using TRIzol reagent and reverse-transcribed to cDNA. Reverse transcriptase-polymerase chain reaction (RT PCR) was carried out using primers specific to members of the K2P channel family, TRPV5, TRPV6 and the amiloride sensitive Na^+ channel. For immunofluorescence, endometrial samples were separated into stromal and glandular cells by selective filtration and cultured overnight. Thereafter cells were fixed and processed for cytokeratin, vimentin and TWIK-2 immunostaining using the avidin-biotin complex (ABC) technique.

Of the K2P channels tested, PCR products of the expected size were observed for TWIK-2 (n = 16/16), TREK-1 (n = 3/6), KCNK7 (n = 19/23). There was no expression for TRAAK or TASK-1. TRPV6, but not TRPV5, was expressed in all samples (n = 6/6) as was the case for the amiloride-sensitive Na+ channel (n = 6/6). All samples tested positive for the endometrial epithelial cell marker E cadherin and the housekeeping gene β-actin. Cytokeratin and vimentin immunoreactivity was observed in epithelial and stromal cells respectively. TWIK-2 specific immunofluorescence was apparent in both cell types.

Our findings demonstrate the presence of mRNA for TREK-1, KCNK7, TWIK-2, and TRPV6 in the human endometrium. Immunofluorescence revealed translation of TWIK-2 mRNA into protein in both stromal and glandular endometrial cells. Expression of TRPV6 is in agreement with findings from other epithelial cells possibly indicating their importance in regulating Ca^{2+} entry in endometrium (1). Studies are continuing to determine if expression is temporally and functionally associated with samples obtained during the proliferative or secretory phase of the menstrual cycle.

Peng JB *et al.*(2003). *J Physiol.***551**,729-740.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC19

Ion channel currents in isolated human urothelial cells

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Urothelial cells are reported to express a diverse range of receptors and ion channels similar to those found in sensory nerves, with TRPV1 the most strongly implicated in normal bladder function (1). In addition, these cells are known to release a variety of transmitter/modulator substances, including ATP (2),

which can alter bladder afferent nerve activity. Stimulation of sub-urothelial neurones may also release a number of factors that act upon urothelial cells (2). With the potential involvement of these cells in bidirectional signalling with bladder afferent nerves and their possible influence on sub-urothelial myofibroblasts and smooth muscle cells, their complement of channel and receptor expression could have major consequences for normal bladder function.

Urothelial cells were isolated from human tissue bank and post-operative samples (3) obtained in accordance with ethical guidelines. Currents were recorded using the whole-cell patch clamp technique at a holding potential of –60mV. Experiments were performed in a physiological bath solution and with an ATP-supplemented pipette solution with a free-calcium concentration of 100nM. All experiments were performed at room temperature (20-22°C).

Urothelial cells were exposed to ATP (50μM); forskolin (10μM, adenylate cyclase activator); capsaicin (1μM, TRPV1 activator); 4α-PDD (1μM, TRPV4 activator) and menthol (2mM, TRPM8 activator).

Responses were observed in 6/13 cells to ATP (n=5 samples); 5/10 cells to forskolin (n=2 samples); 8/8 cells to menthol (n=3 samples); 0/12 cells to capsaicin (n=3 samples) and 0/15 to 4α-PDD (n=4 samples).

Application of ATP elicited a peak increase in normalised current of 3.87±0.95 pA/pF (mean±S.E.M.), that declined to a low plateau. The response to forskolin peaked at 5.57±3.96 pA/pF then desensitised in the maintained presence of agonist. The menthol response peaked at 3.56±1.48 pA/pF and was maintained in the presence of the activator.

These data demonstrate urothelial responses to ATP, forskolin and menthol, but not to capsaicin or 4α-PDD. TRPV1 (4) and TRPM8 (5) are expressed on urothelial cells; however, antibody staining of TRPV1 shows it is located mostly in the cytoplasm (4) and not at the plasma membrane. Results presented here suggest that TRPV1, unlike TRPM8, may be non-functional in these cells or that it may play an alternative role in intracellular signalling but not as a plasma membrane localised channel.

Further work is required to identify the receptors/channels mediating responses to ATP and forskolin in these cells.

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Stein et al. (2004). J Urol 172, 1175-1178.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC20

Contribution of Kvα2 subunits to the Kv current of rat cerebral small artery smooth muscle cells

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Voltage-dependent potassium channels (Kv channels) have been reported to be involved in a variety of contractile responses of small arteries. In small arteries of the systemic circulation the expression of Kvα1 and Kvα2 subunits has been detected. However, due to a lack of specific inhibitors the contribution of these subunits to the Kv current is still unknown. Recently, a specific inhibitor of Kvα2 subunits, stromatoxin, was discovered. Thus, the hypothesis was tested that Kvα2 subunits are a major component of the Kv current in smooth muscle cells freshly isolated from Wistar rat posterior cerebral arteries employing the patch-clamp technique. Animals were humanely killed.

An outward current with biophysical and pharmacological properties similar to recently described Kv currents of rat and mouse small arteries was found in the cells studied. Stromatoxin inhibited the Kv current in a concentration-dependent manner with an ED_{50} of 36 nM and a maximum effect of 61.2%. At 100 nM stromatoxin produced a fast, reversible inhibition of the Kv current. The voltage dependence of activation of the Kv current in the presence of 100 nM stromatoxin was characterized by a potential of half-maximal activation of -6.2±2.3 mV (n=10; mean±S.E.M.) and a slope of 12.3±1.0 mV (n=10), which are not different from the potential of half maximal activation of -4.4±1.9 mV (n=10) and the slope of 12.1±0.9 mV (n=10) obtained in the absence of stromatoxin. The inactivation of the Kv current in the presence of 100 nM stromatoxin was characterized by a potential of half maximal inactivation of -45.1±2.5 mV (n=10), a slope of 7.7±0.7 mV (n=10) and a sustained current at maximal inactivation of 12±2% of the initial current (n=10). These parameters show some difference compared to the characteristics obtained in the absence of stromatoxin, where the potential of half maximal inactivation was -37.9±1.4 mV (n=10; $p<0.05$, t test), the slope was 9.3±0.5 mV (n=10) and the sustained current at maximal inactivation was 9±2% of the initial current (n=10). The recovery from inactivation in the presence of 100 nM stromatoxin had a time constant of 2.12±0.20 s (n=10), which is not different from the time constant for recovery from inactivation in the absence of stromatoxin of 2.14±0.24 s (n=10).

Thus, the data show that a stromatoxin-sensitive current, most probably carried by Kvα2 subunits, contributes considerably to the Kv current of smooth muscle cells isolated from Wistar rat posterior cerebral arteries. The presence of the stromatoxin-sensitive current affects inactivation of the Kv current resulting in a larger current at physiological membrane potentials.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC21

REST regulates IKCa gene expression in vascular smooth muscle cells

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Repressor Element-1 Silencing Transcription factor (REST) is a negative regulator of neuronal genes that contain a 23 bp repressor element (RE1) (Schoenherr & Anderson, 1995). Although REST has been associated almost exclusively with neuronal phenotype we provide evidence for a role for REST in regulating the

expression of KCNN4 in vascular smooth muscle cells. Male 8-week-old mice were humanely killed. Using a rabbit polyclonal antibody targeted to REST we detected a 115kDa protein band in mouse aorta. This is close to the predicted molecular mass of full-length REST (117kDa). Using RT-PCR we also detected REST mRNA in isolated smooth muscle cells from mouse aorta. To link REST expression to function, we used a bioinformatics search and identify conserved RE1 sites within the promoter region of human, mouse and rat KCNN4 gene, which encodes the intermediate calcium-activated potassium channel IKCa. To determine whether REST has a functional role in regulating the KCNN4 promoter, we cloned a region of the KCNN4 gene into a luciferase reporter plasmid. When transfected in REST-expressing HEK cells, the promoter containing a mutation of the RE1 site drove a 27-fold higher luciferase expression than the wild type promoter. Gel electromobility shift assays show that the RE1 site of the KCNN4 formed a DNA-protein complex and that vascular smooth muscle nuclear extracts contain REST protein bound to this RE1 site. Furthermore introduction of a dominant-negative form of REST to isolated aorta smooth muscle cells in primary culture induces the upregulation of KCNN4 gene expression. Together, these data indicate a novel role for REST in regulating KCNN4 gene expression in vascular smooth muscle cells.

Schoenherr CJ & Anderson DJ (1995). Curr Opin Neurobiol 5, 566-571.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC22

Regulated alternative splicing of TRPC1 transcripts

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TRPC1 is a store-operated cationic channel of the transient receptor potential family. Functionally, TRPC1 is implicated in several physiological roles including endothelial cell permeability, mechanosensitive calcium entry, glutamatergic signalling and smooth muscle cell proliferation. The TRPC1 gene expresses a protein of 793 amino acids, derived from an mRNA transcript of 13 exons.

Exon spanning long PCR of TRPC1 in human aorta, saphenous vein, brain and HEK 293 cells was carried out with ethical approval. 11 novel splice variants were sequenced; Exons 2,3, 5, 6, 7, 8 and 9 are all revealed to be cassette exons which can be alternatively spliced. Exon 4 appears to be constitutively spliced, as it is present in all transcripts.

In 9 of the variants, splicing events lead to a frameshift deletion and introduction of an early stop codon. Translation of these mRNA transcripts would lead to premature termination and production of a truncated protein. Any truncated protein arising from mRNA containing an early stop codon is potentially harmful to the cell, and nonsense mediated decay (NMD) is a cellular process which has been thought to be in place to degrade aberrant mRNA products containing premature stop codons. Many TRPC1 isoforms identified are potential candidates for NMD. Cycloheximide, an inhibitor of NMD was used to treat HEK 293 cells. Inhibition of NMD increases the stability of transcripts normally degraded by this pathway and this can be detected by PCR and gel electrophoresis. Cycloheximide treated cells showed an increase in the abundance of NMD vulnerable TRPC1 transcripts, indicating alternative splicing of TRPC1 can produce isoforms susceptible to degradation by this pathway.

Physiological regulation of TRPC1 splicing was also investigated: HEK 293 cells were store-depleted with thapsigargin and PCR of TRPC1 transcripts six hours later showed an increase in TRPC1 alternative splicing. Exon 4 levels were not changed indicating no alteration of total mRNA turnover. A decrease in the amount of full length TRPC1 and a significant increase in the presence of splice variants, many of which are degraded by NMD was observed. We suggest the alternative splicing and NMD pathways represent a mechanism of regulating TRPC1 dependent calcium entry.

Work supported by the Wellcome Trust

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC23

Proliferation of human uterine artery smooth muscle cells modifies the expression profile of Kv channels

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Vascular smooth muscle cells (VSMCs) express a large repertoire of ion channels and membrane receptors, which are a key determinant of the electrical and contractile responses of the cells. Contractile activity of VSMCs is triggered by Ca^{2+} influx through voltage-activated Ca^{2+} channels, and because of this voltage dependence, membrane potential is the primary determinant of VSM tone. K channels, as key players in controlling membrane potential, may have an important role in dedifferentiation and proliferation processes in VSMCs, and it is becoming clear that their contribution to cell proliferation is a complex modulatory activity that only certain K channels at specific times and locations can perform. This fact, together with the broad diversity of functional K currents among different vascular beds, that results largely for the great diversity of K channels expressed, has ravelled the analysis of the role of K channels on VSMCs proliferation. Among the different types of K channels found in VSMCs, voltage-dependent (Kv) and large-conductance, calcium activated (BKCa) K channels are present in virtually all vascular myocytes and have been shown to strongly influence contractile responses. In this study we characterised the expression profile and the functional involvement of Kv channels in VSMCs from human uterine artery in contractile and proliferating phenotype, in order to evaluate their functional role in VSMCs from human resistance arteries and their possible contribution to the phenotypic modulation of the cells. Uterine arteries were obtained from women subjected to hysterectomy. Enzymatic dispersion of small pieces of the muscular layer of these arteries

provide VSMCs in contractile phenotype, while proliferating VSMCs were obtained from explants of uterine arteries kept in culture for several passages. We found that the proliferation process in uterine artery leads to a decreased expression of BKCa channels without a significant change in the outward current density, implying an increase in the proportion of Kv currents in dedifferentiated VSMCs. The molecular identification of the functional Kv channels in both preparations shows that while Kv1 members are the main contributors to the Kv currents in contractile VSMCs, Kv3.4 expression is up-regulated under proliferating conditions and represents the largest proportion of the Kv current in dedifferentiated VSMCs. This functional characterization was supported, with slight discrepancies, by the determination of the expression levels of the corresponding mRNAs by quantitative RT-PCR. Finally, we demonstrate a direct relationship between Kv3.4 channel function and VSMCs proliferation rate, showing that selective blockade of Kv3.4 channels leads to a decrease in the number of VSMCs without increased apoptosis.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC24

Two distinct Ca^{2+} influx pathways induced by $PGF_{2\alpha}$ in rat intrapulmonary arteries

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Using Fura PE3-loaded rat intrapulmonary arteries (IPA) from humanely killed rats, we have examined the mechanisms by which prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) increases the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ with simultaneous recording of isometric force. Values are means ± S.E.M.

A low concentration (0.1μM) of $PGF_{2\alpha}$ caused a marked transient rise in $[Ca^{2+}]_i$ followed by a sustained plateau, which was not associated with contraction. The plateau was abolished by removal of extracellular Ca^{2+}, pre-treatment with La^{3+} (10μM), the putative store-operated channel antagonist 2-APB (75μM), and the SERCA inhibitor thapsigargin (0.1 or 1.0μM), and reduced by 87 ± 5% by the phospholipase C antagonist U-73122 (3μM). It was unaffected by the L-type Ca^{2+} channel blocker diltiazem (10μM) and by the TP receptor antagonist SQ-29548 (1μM). The selective FP receptor agonist fluprostenol (0.1μM) caused an identical response. A higher concentration (10μM) of $PGF_{2\alpha}$ caused a contraction which was abolished by SQ 29548 and associated rise in $[Ca^{2+}]_i$ which was partially inhibited by diltiazem. The diltiazem-resistant component of this increase in $[Ca^{2+}]_i$ was reduced by 78 ± 3% and 47 ± 17 % by 75μM 2-APB and 10μM La^{3+}, respectively, but by 31 ± 10% by U73122. The rise in $[Ca^{2+}]_i$ in response to the TP receptor agonist U-46619 (50nM) was similarly (44 ± 11%) inhibited by 10μM diltiazem, and was inhibited by 43 ± 12% by thapsigargin pre-treatment. When Ca^{2+} was replaced by Sr^{2+} (4mM), only the transient increase in the Fura PE-3 signal occurred following application of 0.1μM $PGF_{2\alpha}$. Conversely, both 10μM $PGF_{2\alpha}$ and 50nM U-46619 caused a sustained increase in Fura PE3 signal similar to that observed in Ca^{2+}-containing PSS. These results suggest that at low concentrations $PGF_{2\alpha}$ acts via FP receptors to cause IP_3-dependent Ca^{2+} release and store operated Ca^{2+} entry (SOCE), resulting in a large rise in $[Ca^{2+}]_i$ which is not associated with contraction. U-46619 and higher concentrations of $PGF_{2\alpha}$ cause a TP receptor-mediated contraction and additional Ca^{2+} influx involving both L-type Ca^{2+} channels and a receptor-operated pathway, which differs from SOCE in that it is less La^{3+} and thapsigargin sensitive, does not require PLC activation, and is Sr^{2+} permeable.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC26

p38 mitogen-activated protein kinase (p38 MAPK) modulates $PGF_{2\alpha}$-mediated constriction and NO-mediated relaxation of rat pulmonary artery

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Male Wistar rats were humanely killed and IPA (internal diameter 200-500μm) were used in all experiments. Student's t test was used for all statistical comparisons, and data are presented as mean ± S.E.M.

IPA constricted with 20 μM $PGF_{2\alpha}$ were relaxed by SB203580 with an apparent IC_{50} of 1.6 ± 0.4 μM (n = 12) and a near-maximal effect at 30 μM. The inactive analogue SB202474 was approximately 30-fold less potent (only 2.8 ± 1.4% relaxation at 1 μM and only 39 ± 6% relaxation at 20 μM, n = 10). SB203580 also inhibited the second phase contractile response to hypoxia (~50% inhibition, n = 8, $P < 0.05$), while SB203474 had no effect. $PGF_{2\alpha}$ significantly increased levels of the phosphorylated forms of p38 MAPK (168 ± 20% of control, n = 16, $P < 0.01$) and of its downstream effector, heat shock protein 27 (HSP27) (278 ± 77% of control, n = 21, $P < 0.05$) in IPA. Both increases were reversed by co-incubation with 2 μM SB203580 (77 ± 7% of control for p38 MAPK, n = 5; 71 ± 16% of control for HSP27, n = 8), but not by 2μM SB202474 (194 ± 53% of control for p38 MAPK, n = 7; 276 ± 66% of control for HSP27, n = 8).

The sensitivity of the $PGF_{2\alpha}$ contraction to SB203580 was greatly reduced by endothelial denudation (IC_{50} 16 ± 2.3 μM, n = 10, $P < 0.001$ vs. control). The response to SB202474, however, was not affected by endothelial denudation. The sensitivity of $PGF_{2\alpha}$ contraction to SB203580 was also inhibited by 1 mM L-NAME (IC_{50} = 12 ± 3 μM, n = 13, $P < 0.01$ vs. control), indicating the involvement of nitric oxide in the relaxation response. Similarly, L-NAME prevented the inhibition of the second phase of HPV by SB203580.

As an indication of NO bioavailability, we measured cGMP production in HUVECs. Levels of cGMP were increased 2- to 3-fold

by both SB203580 and SB20474 (2 μM, n = 8), although this was not observed in the presence of $PGF_{2\alpha}$, which itself also increased cGMP levels. This suggests that p38 MAPK is not specifically involved in either NO production or regulating guanylate cyclase activity.
As an indicator of smooth muscle sensitivity to NO, the influence of p38 MAPK inhibition on relaxation of $PGF_{2\alpha}$-contracted endothelium-denuded arteries to the NO donor SNAP was examined. 2 μM SB203580, but not SB202474, enhanced the relaxation response to SNAP (IC_{50}: control, 0.28 ± 0.11 μM, n = 8, +SB203580, 0.12 ± 0.03 ± 0.03 μM, $P < 0.05$; maximum relaxation: control, 56 ± 8.4%, +SB203580, 82.8 ± 6%, $P < 0.05$).
These results suggest that p38 MAPK activity contributes to $PGF_{2\alpha}$-induced and hypoxia-induced contraction of IPA partly via inhibition of the relaxing actions of nitric oxide.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC27

Reversal of vasoconstriction in chronically hypoxic hypertensive lungs does not alter the alveolar arterial oxygen gap

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Acute hypoxic pulmonary vasoconstriction is an adaptive response that optimizes the ratio of alveolar ventilation to regional pulmonary perfusion thereby improving oxygen uptake. However, sustained exposure to hypoxia produces a chronic hypoxic vasoconstriction, which is no longer immediately reversible with correction of alveolar hypoxia. Thus, the vasoconstriction in chronically hypoxic hypertensive lungs is distinct from acute hypoxic pulmonary vasoconstriction. This chronic hypoxic vasoconstriction is associated with the development of pulmonary artery hypertension and right ventricular strain.
We recently demonstrated that Rho-A/Rho-Kinase inhibition returns pulmonary artery pressures to near normal values in chronically hypertensive lungs [1]. While inhibition of the Rho-A/Rho-Kinase pathway reverses much of the chronic hypoxic vasoconstriction, it is unknown if chronic hypoxic vasoconstriction has a role in V/Q matching in the lung.
Adult male rats (n=6 per group) were housed in a normobaric hypoxic chamber (FiO2 0.10) for 3 weeks. Anaesthesia was induced with pentobarbitone (60mg/kg, i.p.), venous and arterial cannulae were placed for drug administration, blood gas sampling, heart rate and blood pressure monitoring. Adequate depth of anaesthesia maintained with Saffan (Alfaxalone 0.3% and Alfadalone 0.1%,0.1-2 ml/h i.v.) and monitored throughout the experiment by the haemodynamic response to paw pinch. A tracheostomy was performed and mechanical ventilation (FiO2 0.3) was commenced after neuromuscular blockade with pancuronium (2mg i.v.).
The rats were allowed to stabilise and arterial blood gas measurements were then made 30 min before and after i.v. administration of the Rho-Kinase inhibitor Y-27632 (15mg/kg, i.v.) as previously described [1] or vehicle. Animals were humanely killed at the end of the experiments.
The (mean±S.E.M.) alveolar-arterial oxygen (A-aO2) gap was not significantly different (p=0.56, t test) following the administration of Y-27632 (38.1±9.1mmHg) from the initial value (40.5±3.2mmHg). The partial pressure of oxygen in arterial blood (PaO2) was not significantly different (p=0.31, t test)) following the administration of Y-27632 (137.8±5.9mmHg) from the initial value (132.2±2.8mmHg). There was no difference in A-aO2 gap and PaO2 following injection of vehicle. Y-27632 caused a significant attenuation of right ventricular systolic pressure max in chronic hypoxia when compared to vehicle ($p<0.01$).
These findings suggest that unlike acute hypoxic pulmonary vasoconstriction, chronic hypoxic pulmonary vasoconstriction is not important in maintaining arterial oxygenation. Reversal of the chronic hypoxic vasoconstriction does not alter the A-aO2 gap in the lung. These results support the view that chronic hypoxic vasoconstriction is a maladaptive response which overloads the right ventricle with no obvious benefit to gas exchange.
Hyvelin JM et al. (2005). Circ Res (in press).

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC28

Selective impairment of nitrergic neurotransmission in lower esophageal sphincter (LES)circular smooth muscle (CSM) of *W/W^v* Interstitial Cells of Cajal (ICC)-deficient mice

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ICCs appear to be involved in neurotransmission to GI smooth musclemuscle (Ward & Sanders, 2001; Hirst & Edwards, 2004; Sivarao et al. 2001). To determine whether this is neurotransmitter specific, LES CSM in *W/W^v* wild-type (+/+) and mutant (−/−) mice were studied using intracellular recordings *in vitro*. LES strips were prepared after mice of either sex were humanely killed. In the presence of nifedipine, guanethidine and substance P, resting membrane potential (MP) of LES was −44.5±2.3 mV (n=6) in wild-type *vs.* −51.6±1.6 mV in mutant mice (n=15, $P\langle 0.05$). In wild-type mice nerve stimulation induced a biphasic IJP consisting of an initial fast IJP (fIJP) with amplitude of 7.7±2.1 mV and duration of 644±97 ms followed by a second slow IJP (sIJP) with amplitude of 1.9±0.7 mV and duration of 11048±1779 ms (n=6). The fIJP in mutant mice was no different than wild-type, but the sIJP was impaired (amplitude 0.1 ±0.5 mV; n=15, $P\langle 0.05$). Atropine increased the aplitude of both IJP components to 10.9±1.7 mV and 4.2±0.7 mV in wild-type (n=6, $P\langle 0.05$), and to 8.2±1.6 mV (n=15, $P\rangle 0.05$) and 1.5±0.4 mV (n=15, $P\langle 0.05$) in mutant mice, respectively. Concomitant application of apamin in wild-type mice depolarized MP to −37.3±1.9 mV and decreased the amplitude of the fIJP to 7.3±2.0 mV (n=5, $P\langle 0.05$), but had no effect on the sIJP. In mutant mice, apamin depolarized MP to −43.4±1.8 mV and decreased the fIJP

amplitude to 3.0±1.3 mV (n=14, $P\langle 0.05$), but had no effect on sIJP amplitude. Subsequent application of L-NAME markedly inhibited the biphasic IJP amplitudes to 1.3±0.5 mV and 0.1±0.1 mV, respectively, in wild-type mice (n=4, $P\langle 0.05$). However, in mutant mice L-NAME did not significantly suppress the apamin-resistant component of the fIJP (n=12, $P\rangle 0.05$) but completely eradicted the sIJP (n=12, $P\langle 0.05$). Moreover, application of caffeine hyperpolarized MP to −52.8±1.7 mV, inhibited the fIJP to 6.6±1.6 mV, and completely abolished the sIJP in wild-type mice (n=6, $P\langle 0.05$). In contrast, caffeine depolarized MP to −42.4±1.8 mV and abolished the sIJP (n=9, $P\langle 0.05$), but did not affect the fIJP (n=9, $P\rangle 0.05$) in mutant mice. These data suggest that purinergic and cholinergic neurotransmission is preserved whereas nitrergic neurotransmission is impaired in LES CSM of W/W^v mutant mice. The impaired nitrergic neurotransmission is associated with evidence for dysfunction of sarcoplasmic reticulum in mutant mice.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC29

The effect of caffeine on excitation-contraction (EC) coupling in guinea-pig ureteric smooth muscle

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Caffeine is a potent facilitator of Ca^{2+}-induced Ca^{2+} release (CICR) and is widely used to study the role of ryanodine receptors (RyRs) in the control of smooth muscle function. Studies of the role of caffeine in intact tissues can be complicated to interpret if Ca^{2+} released by CICR also activates IP_3 receptors. We have therefore used guinea-pig ureter, which only expresses RyRs (1) to elucidate the effects of caffeine in intact smooth muscle. Adult guinea-pigs were humanely killed and the ureters dissected and loaded with Fluo-4 or Indo 1 (15μM) for 120 min at room temperature, and force and membrane potential recorded as previously described (2). Single cells were also produced by enzymatic digestion and voltage clamped. Data are means ± S.E.M.s, and n is number of animals or cells and is between 5 and 25. Significance was tested by t tests, taking significance at $P<0.05$. In intact ureteric muscle caffeine (1mM) produced selective inhibition of the plateau component of the action potential, and the amplitude and duration of the Ca^{2+} transient and phasic contractions. It also significantly increased the length of the refractory period from 40-90s to 3-4 min, when tested by the ability of the ureteric muscle to produce an action potential in response to suprathreshold depolarising pulses (3-5V, 100ms in duration). Confocal microscopy demonstrated that caffeine initiated Ca^{2+} sparks in both isolated cells and cells in situ. In voltage-clamped cells dialysed with the KCl pipette solution, spontaneous transient outward currents (STOCs) were elicited by caffeine, and it also significantly increased the ratio of outward to inward currents evoked by depolarising steps from a holding potential of -70 mV to 0 mV. Using the same protocol of stimulation but with CsCl in the pipette solution, caffeine had no effect on inward current but slightly potentiated Ca^{2+} transients evoked by depolarising voltage steps. Ryanodine (50μM) and cyclopiazonic acid (20μM) both inhibited Ca^{2+} sparks and reversed the inhibitory action of caffeine on the action potential and excitability. The inhibitory actions of caffeine on the action potential and excitability were also abolished by TEA (2mM) or iberiotoxin (200nM), blockers of Ca^{2+}-activated K^+ channels.

These data suggest that in the guinea-pig ureter the inhibitory action of caffeine on the action potential and excitability is mediated by activation of a Ca^{2+} spark/STOCs coupled mechanism.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC30

Mechanisms underlying pacemaker Ca^{2+} activity in gastrointestinal tract

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c-Kit-immunopositive interstitial cells (equivalent to interstitial cells of Cajal: ICC) are currently recognised to play a crucial role in gastrointestinal (GI) pacemaking (Takaki, 2003). One of the characteristic features of GI spontaneous rhythmicity is Ca^{2+} dependence (Tomita, 1981). Also, it has been recently suggested that activation of a Cl^- conductance is responsible for pacemaker potentials (Dickens et al. 1999; Huizinga et al. 2002). It is therefore speculated that cytosolic Ca^{2+} ($[Ca^{2+}]_i$) oscillations are the primary mechanism in GI pacemaker cells, i.e. Ca^{2+}-activated Cl^- channels and/or other Ca^{2+}-activated channels (Walker et al. 2002) are periodically activated by $[Ca^{2+}]_i$ oscillations in ICCs. Cell cluster preparations enzymatically isolated from GI tract contain smooth muscle, enteric neurones and ICCs, and are therefore considered to consist of the essential minimum cell members necessary to investigate mechanisms underlying GI motility and pacemaker function. In this communication, we show properties of $[Ca^{2+}]_i$ oscillations in ICCs, using cell cluster preparations from mouse stomach and small intestine after incubation in culture medium for several days. The mice were humanely killed. Dihydropyridine Ca^{2+} antagonists (1 μM nifedipine) were used to monitor Ca^{2+} signals from ICCs. Pacemaker $[Ca^{2+}]_i$ oscillations in both stomach and small intestine were affected by drugs interacting with ryanodine receptors (RyR) (e.g. ryanodine 1-10 μM), inositol 1,4,5-trisphosphate

(InsP_3) receptors (e.g. 10 μM xestospongin C) and TRP homologues (e.g. 40 μM SK&F96365). It is speculated that coordinating actions of different types of intracellular Ca^{2+} release channels produce $[Ca^{2+}]_i$ oscillations in ICCs, supported by Ca^{2+} influx via TRP family channels. RT-PCR examinations detected the cDNA of the molecular component of these channels in isolated ICCs identified by c-Kit-immunoreactivity. Involvement of RyR was also suggested in spontaneous rhythmicity of gut-like organ formed from mouse embryonic stem cells. We further investigated the effects of K^+ channel openers (e.g. 1-10 μM cromakalim) and sulphonylurea compounds (1 μM glibenclamide). The results suggest that sulphonylurea receptors differentially modulate pacemaker $[Ca^{2+}]_i$ oscillations in ICCs and smooth muscle contractility.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC31

Identification of a novel transient outward current in murine portal vein myocytes

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There is a plethora of data on transient outward currents (A-type, I_A) in various cell types (see review by Amberg *et al.* 2003) but relatively less is known about I_A in vascular smooth muscle cells. Here we describe the pharmacological, electrophysiological and molecular properties of IA in isolated mouse portal vein (mPV) myocytes.

Female BALB/c mice aged 6 to 8 weeks were humanely killed and single mPV cells were obtained by enzymatic dispersion. Recordings were made using the whole-cell voltage-clamp techniques with an external solution of (mM): NaCl 126, KCl 5, $MgCl_2$ 1, $CaCl_2$ 0.1, glucose 11, Hepes 10, adjusted to pH 7.2 with NaOH. The internal solution contained (mM): KCl 130, $MgCl_2$ 1, ATP 2, Hepes 10, EGTA 5, adjusted to pH 7.2 with KOH. Currents were evoked by stepping to various test potentials (VT) from a holding potential of –60 mV or –90 mV. I_A was isolated by subtracting those currents at VT evoked from –60 mV from those with a –90 mV prepulse. Values are mean ± S.E.M.

I_A was evoked with an apparent threshold of approximately –40 mV and inactivated rapidly. Peak amplitude at +20 mV was 568 ± 41 pA (n=42). Half voltage of activation was –11 ± 2 mV (slope (k) = 12 ± 3 mV, n=29); and –85 ± 2 mV (k = 4 ± 0 mV, n=11) for inactivation. Time to peak was also voltage dependent, becoming faster with stronger depolarizations, e.g. 4.2 ± 0.2 ms at VT = +20 mV (n=25) but 7.8 ± 0.4 ms at –20 mV (n=26). Current decay was described by a double exponential with fast (τfast) and slow (τslow) components. τfast was 6.3 ± 1.6 ms at +20 (n=13) and 24.2 ± 4.6 ms (n=8) at –40 mV.

Application of 5 mM 4-AP had a small effect on I_A and the block was voltage dependent (inhibition was 54.4 ± 5.2% at –40 mV and 29.8 ± 5.9% at +20 mV, n=11). In comparison, flecainide was an effective inhibitor of I_A (IC_{50} at +20 mV was 0.13 μM, n=4). The KCNQ blocker XE991 (10 μM) also reduced peak current at +20 mV by 32.9 ± 7.5% (n=6).

A number of different protein combinations underlie I_A. Quantitative PCR identified relatively abundant transcripts for Kv1.7, Kv4.3 and the accessory genes Kvb3, KCNE2 and KCNE3 (Ohya *et al.* 2003). These pharmacological and molecular data suggest that the I_A in murine portal vein may reflect a novel combination of gene products.

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PC32

Regulation of capillary diameter in rat retina

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Neuronal activity increases local blood flow in the central nervous system (CNS), and this increase is the basis of BOLD and PET functional imaging techniques. Capillaries adjacent to active neurons are devoid of smooth muscle, and it is generally assumed that blood flow is regulated by smooth muscle associated with precapillary arterioles. However, most (65%) noradrenergic innervation of the CNS vasculature is of capillaries rather than arterioles (Cohen *et al.* 1997), and in both skeletal muscle and brain a retrograde vasodilatory signal passes from capillaries located near metabolically active cells to precapillary arterioles (Dietrich *et al.* 1996; Berg *et al.* 1997; Iadecola *et al.* 1997), suggesting that blood flow control signals are initiated at the capillary level. Pericytes, which are apposed to CNS capillaries and contain muscle actin and non-muscle actin and myosin, are potential initiators of such signalling.

Using video imaging techniques we have studied the potential for pericytes to regulate the diameter of capillaries on the vitreal surface of the isolated P21 rat retina (animals were humanely killed). Pericytes are labelled by an antibody directed at the membrane chondroitin sulfate proteoglycan NG2, and are separated by a distance of 37.7±3.8 μm (mean±s.e.m, n=24) along retinal capillaries. Electrical stimulation with an electrode touching a pericyte evoked a contraction, which constricted capillaries at localized points. Electrical stimulation of pericytes resulted in an average constriction of 75.3±3.3% (mean±s.e.m, n=22) of the capillary at these points. In some cases constriction propagated to more distant sites of the vessel, close to other pericytes. Stimulation of pericyte-free zones on the capillaries evoked an average constriction of 5.0±4.7% (mean±s.e.m, n=11), which is significantly different to the constriction observed at pericyte sites ($p \leq 0.0001$, Student's t test).

Similar changes in capillary diameter were observed following superfusion of retinal capillaries with 100 μM UTP. At sites occupied by pericytes, capillaries constricted by 59.4±8.7, (mean±s.e.m, n=7), suggesting a possible role for purinergic P2 receptors in the control of capillary diameter.
These experiments identify pericytes as possible mediators of the vascular response to changes in neural activity at the capillary level, and pericytes could therefore play a key role in CNS disease and its therapy.

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PC33

Purinergic neurogenic transmission is absent in small mesenteric arteries of $P2X_1$ null mice

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ATP is co-released with nor-adrenaline from sympathetic nerves and acts through P2X receptors on smooth muscle cells to mediate membrane depolarization and contraction. The $P2X_1$ subtype is the predominant P2X receptor found in vascular smooth muscle.
In earlier studies we have shown novel spatially localized Ca^{2+} signals in the smooth muscle adjacent to perivascular nerves (Lamont & Wier, 2002; Lamont et al. 2003). We hypothesized that these novel Ca^{2+} transients represented Ca^{2+} entering smooth muscle cells through $P2X_1$ receptors activated by ATP released from sympathetic nerves and we therefore called them 'junctional Ca^{2+} transients' or jCaTs. Owing to the lack of potent and subtype-selective P2X-receptor antagonists the physiological role of $P2X_1$ receptors has been difficult to determine. Recently $P2X_1$ receptor-deficient mice have been developed (Mulryan et al. 2000; Vial & Evans, 2002). Using small pressurized mesenteric arteries from these mice we have examined the contractile responses to sympathetic nerve stimulation and the underlying Ca^{2+} signals. Mice were humanely killed by a lethal overdose of CO_2. Isolated arteries were mounted between two glass pipettes and the intra-luminal pressure controlled (30mmHg). Arteries were loaded with the Ca^{2+} indicator Fluo-4 AM. Brief pulses (< 0.2ms) of electrical field stimulation (EFS) preferentially stimulated the perivascular nerves. The neurogenic origin of all responses was confirmed by their abolition by TTX (1μM). In wild type (WT) arteries confocal microscopy of Fluo-4 fluorescence during EFS (just sub-threshold for contraction) revealed jCaTs in the smooth muscle cells adjacent to the perivascular nerves (18.63±1.96 Ca^{2+} transients were observed during 10s of 3Hz EFS in an area 75 by 50μm, 6 arteries, n=12, all data expressed as mean±S.E.M.). In $P2X_1$ null (KO) arteries the same stimulation protocol produced no jCaTs. Some Ca^{2+} transients were observed (0.7193±0.227, protocol as above); these were not jCaTs but Ca^{2+} sparks, as assessed by their resistance to TTX, temporal spatial characteristics and abolition by ryanodine. In addition in KO arteries no contraction was produced by αBmethylene ATP (a potent P2X receptor agonist, 10μM). In the WT arteries the frequency of jCaTs increased with nerve stimulation frequency (10.77±2.42 events at 1 Hz, 27.90±7.90 events at 10 Hz, protocol as above). Application of α-latrotoxin (2.5nM), an agent which evokes exocytotic release of neurotransmitters from a variety of nerve terminals, produced a significant (t test, $p<0.05$) increase in the frequency of spontaneous events from 1.143±0.553 events/30s, to 9.909±0.553 events/30s in WT arteries (3 arteries, n=6). Only sparks were observed in the KO arteries (3.300±1.012 events/30s,) and there was no significant change in spark frequency in α-latrotoxin (3.142±1.933, 3 arteries, n=6). With stronger (levels producing contraction) EFS a biphasic contraction of the artery was observed in the WT arteries. In the $P2X_1$ null arteries, however, the initial rapid phase was absent; this phase has previously been attributed to be the purinergic component by pharmacological methods. The data support the hypothesis that jCaTs represent Ca^{2+} entering smooth muscle cells through $P2X_1$ receptors and that the initial rapid component of the neurogenic contraction in these small arteries is purinergic in origin and more specifically originates from $P2X_1$ receptors.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC34

Immunocytochemical staining of arterial ICC-like cells in guinea-pig mesenteric arteries suggests their close relation to the contractile phenotype of smooth muscle cells

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Cells with irregular elongated shape and numerous long thin processes were described earlier in guinea-pig mesenteric arteries (Pucovsky et al. 2003). Due to their morphological similarity with the Interstitial Cells of Cajal (ICCs), they were named the arterial ICC-like (AIL) cells. The ontogenic origin of these cells, suggestive of their physiological role, is as yet unknown. This work investigates the AIL cells for the presence of smooth muscle cell (SMC) and neural marker proteins in order to elucidate which lineage they belong to.
Guinea-pigs were humanely killed. Single cells were obtained by enzymic digestion of mesenteric arteries. The cells were fixed, labelled by incubation with primary antibodies against a target molecule and the labelling was visualised using fluorescent secondary antibodies. The images were obtained with a laser scanning confocal microscope. Statistical significance was tested with Student's t test.

The staining for α-smooth muscle actin, an early marker of SMC lineage, was confined to the subplasmalemmal cytoplasm and the filopodia of the AIL cells (signal registered from 89.1±2.4% of pixels within 1 μm of plasma membrane and only from 15.4±6.5% of pixels more than 1 μm away from the plasma membrane, n=10, $p=7\times10^{-7}$). Similar distribution was found in SMCs (78.8±4.5% of fluorescent pixels within 1 μm of plasma membrane and 4.3±2.0% more than 1 μm away, n=13, $p=1.9\times10^{-9}$). Neither AIL cells nor SMCs stained for neuronal marker PGP 9.5. When pairs consisting of one SMC and one AIL cell were stained for smooth muscle myosin heavy chain, a marker of differentiated SMCs, the AIL cells produced a fluorescence signal of similar intensity to the SMCs (98.9±5.8% of the signal from the SMCs; n=7 pairs, p=0.852). Staining of the SMC/AIL cell pairs for smoothelin, a component of cytoskeleton and the most specific marker of contractile SMCs to date, showed significantly weaker signal in AIL cells (41.4±6.7% of the signal from the SMCs; n=10 pairs, $p=10^{-5}$). In all the experiments the primary or secondary antibodies alone produced none or negligible fluorescence.
The data suggest that the AIL cells are closely related to contractile SMCs and are not fibroblasts, myofibroblasts or cells of neural origin.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC35

The differential effect of CNP in superior and resistance mesenteric arteries

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C-type natriuretic peptide (CNP), a member of the natriuretic peptide family which also includes ANP and BNP, is a putative endothelium-derived hyperpolarising factor (EDHF) in rat mesenteric resistance arteries (Chauhan, 2002) and the coronary vasculature as assessed in isolated perfused rat hearts (Brunner, 2001; Hobbs, 2004). However, there is also limited evidence to suggest that CNP also increases vascular resistance in dog mesenteric (Woods, 1999). We therefore investigated the effects of CNP in rat mesenteric and coronary arteries. Third-order (179 ± 3μm) and superior mesenteric arteries (576 ± 12μm) were dissected from humanely killed 12-week male Wistar rats and mounted on a small vessel wire myograph. All experiments used 300μmM L-NAME to block endothelial nitric oxide synthase. Results are shown as mean±S.E.M. and statistical analysis was performed with Student's paired t test.

In contrast to published work, we observed that CNP caused a further dose-dependent and endothelium-independent vasoconstriction when applied to U46619-preconstricted rat mesenteric resistance arteries (27 ± 4% contraction, n=6, P<0.001). This effect was observed in both Wistar and Sprague Dawley rats. The CNP-induced vasoconstriction was insensitive to blockers of PKC, PKA and tyrosine kinase but was sensitive to diltiazem (10μM), and was associated with a rise in the smooth muscle intracellular calcium concentration as measured using fura PE3. CNP up to 1μM had no effect on isolated mesenteric resistance arteries preconstricted with noradrenaline, and also had no effect on U46619-preconstricted rat coronary arteries.
Conversely, CNP induced a vasodilatation in U46619-preconstricted rat superior mesenteric arteries (78 ± 3% relaxation, n=4, P<0.001) and had a similar effect in aorta. The relaxation was blocked when $[K^+]$ was raised to 25 mM (15 ± 14% contraction, n=4, P<0.001) and also by the combination of the EDHF inhibitors Ba^{2+} (50 μM) and ouabain (100μM), which block K_{ir} and the Na^+, K^+-ATPase, respectively. The relaxation was insensitive to the combination of TRAM-34 and apamin (2 ± 3% inhibition, n=4, n.s.), which block endothelial K_{Ca} channels.
The NPR-C agonist, cANF (1μM), had no effect on either resistance (0 ± 2% n=4) or superior arteries (1 ± 1%, n=4) indicating that the action of CNP is not mediated through this receptor.
These results show that CNP has opposing effects in the same arterial bed depending on the order/size of the artery in question. The relaxation observed in the larger arteries seems to occur via an EHDF mechanism, and the contraction in the resistance arteries appears to involve the activation of L-type Ca^{2+} channels. These effects were not mimicked by a selective NRP-C receptor agonist, suggesting that they could involve NPR-B receptors.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC36

c-kit-positive interstitial cells involved in the regulation of mouse mesenteric artery?

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The presence of interstitial cells (ICs) of Cajal, or morphologically similar c-kit-positive/c-kit-negative cells in a range of visceral and vascular tissues throughout the bodies of different

species has been reported in a number of recent publications [1]. The present study provides initial data about the presence of ICs in the wall of branches of the mouse mesenteric artery (MMA) and about some features of single ICs freshly isolated from this blood vessel.

Experiments were performed on segments (about 2 mm in length) of branches of MMA (from 1st to 5th order) from humanely killed BALB/C mice and on single cells from these within a few hours of enzyme and mechanical dispersion. MMA segments were mounted on glass pipettes, secured by 10-0 sutures, pressurized to 60 mmHg and then the living tissue was stained with Phycoerythrin-Cy7 conjugated anti-mouse CD117 (c-kit) antibodies (eBioscience) or with rat anti-mouse primary monoclonal c-kit antibodies (RDI), followed by Alexa Fluor 633 conjugated goat anti-rat secondary antibodies (Molecular Probes). In some experiments electrical field stimulation (EFS; typical parameters: 0.5-10 Hz, 0.5 ms, 50-60 V) was applied via two platinum wires placed parallel to the long axis of unstained arterial segments, loaded with the fluorescent Ca^{2+}-sensitive indicator fluo-4 AM (Molecular Probes). Single cells were imaged also after loading with fluo-4 AM.

ICs were detected using laser scanning confocal microscopy in the adventitial layer of MMA, as was suggested to be the case for the human cerebral arteries [2]. Only larger branches (> 200 μm) showed cells with strong positive c-kit staining, while smaller ones did not stain. Confocal microscopy of fluo-4-loaded pressurized segments of this blood vessel revealed junctional Ca^{2+} transients (similar to [3]: mean peak ratio F/F_0, 2.74; FWHM, 5.4 μm; t_{peak}, 80 ms; $t_{1/2}$, 510 ms) accompanied sometimes by contraction in response to low-frequency EFS only in the case of large branches, but not the smaller ones. X-Y fluorescent confocal imaging of single fluo-4-loaded cells enzymatically isolated from the whole MMA tree showed (among numerous myocytes) a small population (about 5%) of branching cells morphologically closely resembling ICs of Cajal, widely described in the gut [4]. Similar to the recently described rabbit portal vein ICs [5], ICs freshly isolated from the MMA varied in their morphology (from 18 to 62 μm in length) but always had numerous thin processes up to several tens of microns in length, and, unlike myocytes, these cells did not contract in response to noradrenaline (1-10 μM), caffeine (1-10 mM) or high K^+ solution (60 mM), although they responded with an increase in $[Ca^{2+}]_i$. The functional role of ICs in the MMA is not yet clear; they may play a role as intermediaries between the nerves and the smooth muscle cells.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC37

M_2 and M_3 muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice

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In gastrointestinal smooth muscles, M_2 and M_3 muscarinic receptors exist with a preponderance of the former subtype. These receptors are believed to mediate the action of the parasympathetic neurotransmitter acethylcholine. However, pharmacological analyses of the contractile responses to muscarinic agonists including carbachol (CCh) have revealed that they are mediated extensively by the minor M_3 subtype; the role of M_2 subtype is unclear. To explore the role of M_2 and M_3 subtypes in the muscarinic contraction, we characterized isometric contractions to CCh in ileal longitudinal smooth muscle strips from humanely killed wild-type mice and mice genetically lacking M_2 or M_3 subtypes. Single applications of CCh (0.1-100 μM) produced concentration-dependent contractions in preparations from M_2-knockout (KO) and M_3-KO mice, mediated via M_3 and M_2 subtypes, respectively, as judged by the sensitivity of contractile responses to blockade by the M_2-preferring antagonist methoctramine (300 nM) or the M_3-preferring antagonist 4-DAMP (30 nM). The M_2-mediated contractions were mimicked in shape by submaximal stimulation with high K^+ (up to 35 mM), almost abolished by voltage-dependent Ca^{2+} channel (VDCC) antagonists (n=8) or depolarization with 140 mM K^+ medium (n=7), and greatly reduced by pertussis toxin (PTX) treatment; the maximum contractile response to CCh expressed as a percentage of the 70 mM K^+ value in PTX-treated and -untreated preparations was 26.9 ± 6.3% (n=8) and 94.8 ± 4.9% (n=12), respectively, and the difference was statistically significant (unpaired t test: $P<0.05$). The M_3-mediated contractions were only partially inhibited by VDCC antagonists (82.7 ± 3.4% (n=6) of the control response at 10 μM CCh) or 140 mM K^+ medium (23.5 ± 4.3% (n=6) of the control response at 10 μM CCh). The contractions observed during high K^+ medium consisted of different components, either sensitive or insensitive to extracellular Ca^{2+}. PTX treatment had little or no effect on the M_3-mediated contractions (the maximum contractile response to CCh was 136.2 ± 4.0% (n=6) in PTX-treated and 142.4 ± 9.7% (n=7) in PTX-untreated preparations). The CCh contractions observed with wild type preparations consisted of PTX-sensitive and -insensitive components. The PTX-sensitive component was functionally significant only at low CCh concentrations (0.01 to 3 μM). The results suggest that the M_2 receptor, through PTX-sensitive mechanisms, induces ileal contractions which depend on voltage-dependent Ca^{2+} entry, especially associated with action potential discharge, and that the M_3 receptor, through PTX-insensitive mechanisms, induces contractions which depend on

voltage-dependent and -independent Ca^{2+} entry and intracellular Ca^{2+} release. In intact tissues co-expressing M_2 and M_3 receptors, M_2 receptor activity appears functionally relevant only when fractional receptor occupation is relatively small.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC38

Membrane potential and current responses to carbachol in longitudinal smooth muscle cells of mice ileum genetically lacking M_2 or M_3 subtype of muscarinic receptors

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Membrane potential and current responses to the muscarinic agonist carbachol (CCh) were investigated in single ileal smooth muscle cells isolated from humanely killed M_2 or M_3 knockout (KO) mice as well as wild-type (WT) mice. Membrane potential responses were recorded using the nystatin-perforated current-clamp technique, and membrane current responses using the conventional whole-cell voltage-clamp technique. Data are expressed as mean ± S.E.M. Unpaired Student's t test was used for statistical comparison.

In cells from M_2 KO mouse, 1 μM CCh produced a sustained depolarization, on which an increased frequency of action potential discharge was often superimposed. The size of depolarizations evoked had a mean amplitude of 12.2 ± 1.2 mV (n=4), much smaller than that in WT cells (41.8 ± 1.5 mV, n=4, $P<0.05$). In M_3 KO cells, CCh at 1 μM was almost without effect (n=5) and even at 100 μM produced no more than 10 mV depolarization (n=4).

In M_2 KO cells bathed in a Na^+-rich physiological medium and dialysed intracellularly with a CsCl-based solution, application of CCh (100 μM) under voltage-clamp at -50 mV evoked a biphasic inward current consisting of an initial transient followed by a sustained component, as seen in WT. However, the respective components (67.5 ± 28.3 pA and 3.7 ± 1.0 pA, n=10) were significantly smaller than those in the WT (161.9 ± 26.6 pA and 23.1 ± 6.2 pA, n=11, respectively, $P<0.05$). Replacement of the intracellular Cl^- with glutamate$^-$ (n=6) or cell treatment with the Cl^- channel blocker niflumic acid (n=3), resulted in abolition of the transient component alone. In M_3 KO cells, CCh (100 μM) evoked a slight sustained inward current (4.0 ± 0.4 pA, n=7), and the current response become more clear (11.5 ± 1.5 pA, n=15) if the extracellular Na^+ was replaced with Cs^+. Non-stationary noise analysis of CCh (100 μM)-evoked currents revealed that the unitary channel conductances underlying the sustained inward currents in M_2 KO and M_3 KO cells were 10.8 ± 1.5 pS (n=8) and 2.2 ± 0.2 pS (n=8), respectively. These values clearly differed from the corresponding value (40.1 ± 4.4 pS, n=6, $P<0.05$) estimated for the WT.

These results suggest that M_2 receptor stimulation depolarizes the cell via activating 2-pS cationic channels, and M_3 receptor stimulation does so via activating both 10-pS cationic channels and Ca^{2+}-activated Cl^- channels. In intact cells, beside these channels, the 40-pS cationic channels which are activated synergistically by both M_2 and M_3 subtypes may play a crucial role in the muscarinic depolarization.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC39

Sphingolipids differentially regulate mitogen-activated protein kinases and intracellular Ca^{2+} in vascular smooth muscle: effects on CREB activation

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Structurally related sphingolipids, sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), may be potential mitogenic factors in the development of vascular disease. The aim of this study was examine the intracellular pathways regulated by S1P, in comparison to SPC, in rat cerebral artery smooth muscle.

Male Sprague Dawley rats (6 weeks old, 300-350 g) were humanely killed. Denuded rat cerebral arteries were stimulated with either 5 μM S1P or 10 μM SPC and homogenates subjected to SDS-polyacrylamide electrophoresis followed by immunoblotting. S1P significantly increased extracellular signal-regulated kinase (ERK)1/2 phosphorylation (5.2 ± 1.4-fold increase in band density compared to controls, mean ± s.e.m., n=6, $p<0.05$) but did not activate p38 mitogen-activated protein kinase (p38MAPK) as assessed with phospho-specific antibodies. In contrast, SPC significantly increased p38MAPK phosphorylation (3.0 ± 0.3-fold increase, n=6, $p<0.05$) but did not stimulate ERK1/2 activation. This differential activation was confirmed by measuring phosphorylation of heat shock protein (HSP) 27, a known target of p38MAPK. Only SPC, but not S1P, activated HSP27 (2.8 ± 0.3-fold increase, n=5, $p<0.05$).

Dispersed cerebral artery myocytes were prepared by enzymatic digestion and loaded with the fluorescent Ca^{2+} indicator, fura-2. SPC (0.1 - 30 μM) increased $[Ca^{2+}]_i$ in a concentration-dependent manner (peak response at 10 μM - 4.1 ± 0.2 ratio 340/380 nm, n=41). In contrast to S1P, the SPC-induced $[Ca^{2+}]_i$ increase did not involve release from intracellular stores as determined by a lack of effect of thapsigargin. However, removal of extracellular Ca^{2+} (n=20) or pretreatment with 2 μM nifedipine (n=11) completely blocked the SPC-induced increase in $[Ca^{2+}]_i$ ($p<0.05$). Despite differences in signalling, both S1P and SPC produced increased phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in cerebral artery homogenates as assessed by phospho-specfic anti-

bodies (S1P: 3.2 ± 0.5-fold increase, SPC: 3.3 ± 0.8, n=5). S1P-induced CREB activation was significantly inhibited by pre-treatment with PD98059 (ERK1/2 inhibitor) and the Ca^{2+}-calmodulin-dependent protein (CaM) kinase inhibitor KN93. CREB activation by SPC was not inhibited by PD98059, but was inhibited by SB203580 (p38MAPK inhibitor) and KN93.
In conclusion, S1P and SPC activate distinct members of the MAP kinase family and increase $[Ca^{2+}]_i$ via different mechanisms in rat cerebral artery. This does not affect the ability of either S1P or SPC to activate CREB, although this occurs via different pathways.

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PC40

Vasopressin and 5-HT pathways in A7r5 vascular smooth muscle cells

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In A7r5 cells, vasopressin (AVP) and 5-hydroxytryptamine (5-HT) share the ability to stimulate phospholipase C and so production of inositol 1,4,5-trisphosphate (IP3). AVP can also reciprocally regulate capacitative and non-capacitative Ca^{2+} entry pathways via its ability to stimulate nitric oxide synthase-III (NOS-III), but 5-HT appears not to activate NOS-III and stimulates only capacitative Ca^{2+} entry (Moneer et al. 2005). The IP3-evoked Ca^{2+} release promoted by AVP causes a long-lasting inhibition of adenylyl cyclase (AC) dependent on activation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) (Dyer et al. 2005). These observations suggest rather precise associations between receptors, Ca^{2+} signalling pathways, and proteins that are regulated by Ca^{2+}. Our aim was to examine the associations between these signalling proteins.
cDNA was synthesised from RNA and quantitative real-time PCR (RT-PCR) was performed. Each reaction included LUX primers for a specific CaMKII or AC subunit and primers for a housekeeping gene (beta-actin). Non-detergent discontinuous sucrose gradients were used to separate membrane fractions (Ostrom et al. 2002). Immunoprecipitation of solubilized membranes was performed using appropriate primary antibodies and Protein A/G Plus-agarose beads. Proteins in immunoprecipitates or sucrose gradient fractions were identified by immunoblotting. Proteins were separated by SDS-PAGE on 4-12% Bis-Tris gradient gels, transferred to PVDF membranes, blocked and incubated with primary and secondary antibodies using standard methods. All experiments were performed at least 3 times.
Immunoblotting established that AC3, AC5/6, CaMKII, type 1 and type 3 IP3 receptors and NOS-III were expressed in A7r5 cells. From RT-PCR, d-CaMKII was the major expressed isoform, and AC3 and AC6 were each expressed at similar levels. Both V1A receptors for AVP and 5-HT2A receptors were expressed predominantly in non-caveolar membranes, whereas NOS-III and type 1 and 3 IP3 receptors were more abundant in fractions that included caveolae. Immunoprecipitation established an association between caveolin 3 and IP3 receptors, caveolin 3 and NOS-III, and between type 3 IP3 receptors and CaMKII. Our results suggest that the selective ability of AVP, but not 5-HT, to activate NOS-III is unlikely to result from colocalization of V1A receptors with NOS-III in caveolae. An association of type 3 IP3 receptors with CaMKII may underlie the selective ability of IP3-evoked Ca^{2+} signals to inhibit AC activity via CaMKII.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC41

Pre-synaptic consequences of ecto-ATPase inhibition: modulation of transmitter release in guinea-pig vas deferens

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At sympathetic neuromuscular junctions in smooth muscle, ecto-ATPase is believed to curtail the actions of ATP in the synaptic cleft, although details of its modulation of purinergic transmission are not yet clear. An accepted method of evaluating the role of transmitter inactivation is to assess its effect on synaptic potentials following inhibition of the transmitter removal mechanism. Here, we have studied the role of ecto-ATPase in purinergic transmission using ARL 67156, a selective inhibitor of the enzyme. Intracellular recordings of spontaneous excitatory junction potentials (SEJPs), which reflect the underlying transmitter-activated conductance changes (Tomita, 1975), were made from isolated vasa deferentia isolated from humanely killed guinea pigs. 100 μM ARL 67156 significantly increased the mean amplitude, rise time and decay time constant of SEJPs (Student's t test; $P < 0.01$), as expected from analogy with inactivation of transmitter-degrading enzymes at other synapses. Interestingly, however, the frequency of occurrence of SEJPs was found markedly to increase (Fig. 1), from 0.28 ± 0.13 Hz in control to 0.90 ± 0.26 Hz in the presence of ARL 67156 (n = 4 cells each; Student's t test; $P < 0.01$). Although the inter-event interval (IEI) for SEJPs decreased significantly in the presence of ARL 67156 (K-S test; $P < 0.01$), its distribution remained exponential, indicating that the quantal release events continued to be random. An increase in frequency of spontaneous potentials indicates a pre-synaptic effect of ecto-ATPase inhibition (Khakh, 2000). To probe this possibility further, discrete events (DEs), which represent stimulation-evoked quantal release underlying the excitatory junction potential (EJP), were analysed. As seen for SEJPs, DE frequency increased from 0.7 per EJP in control conditions to 1.4 per EJP with ARL 67156 (single cell data). Although ARL 67156

did not significantly change DE amplitude, it elicited the appearance of DEs in previously 'silent' latency bands. These observations suggest that the ARL 67156-induced elevation of SEJP frequency could be due to a combination of (i) increased transmitter release from previously active varicosities and (ii) recruitment of previously 'silent' varicosities. The precise mechanism underlying these effects is unclear, but one possibility is the enhanced activation of pre-synaptic P2X receptors by elevated concentrations of synaptic ATP due to ecto-ATPase inhibition, for which we have obtained supporting evidence (P. Ghildyal & D. Palani, unpublished data). In conclusion, ecto-ATPase may modulate not only post-synaptic transmitter action but also its pre-synaptic release, thus suggesting novel control mechanisms in the regulation of purinergic transmission.

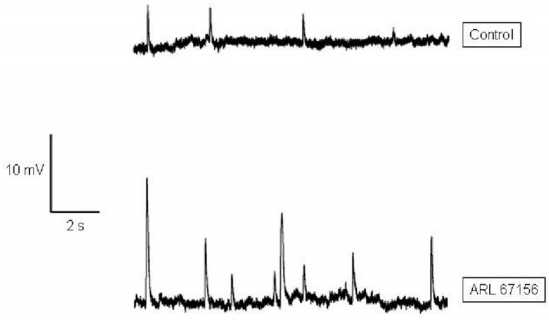

Fig. 1 Typical sEJPs recorded from a single cell. Note both potentiation & increased frequency of sEJPs following ecto-ATPase inhibition.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC42

Inward calcium current in interstitial cells from the guinea-pig bladder

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Inward currents were studied in interstitial cells isolated from bladders of humanely killed guinea-pigs. These cells have previously been shown to be kit-positive (McCloskey & Gurney, 2002) and several components of outward potassium current have recently been characterised (McCloskey, 2005).
Enzymatic dispersal of detrusor tissue produced a heterogeneous yield of cells containing smooth muscle cells and interstitial cells. The latter were readily identified by the presence of several lateral branches and were studied using the whole-cell patch clamp technique and Cs^+-filled pipettes. Data are expressed as means ±S.E.M. and were compared with Student's paired t test. P values less than 0.05 were considered significant. Inward currents were evoked by stepping positively from a holding potential of −80mV. They activated at −50mV, peaked at 0mV and reversed positive to 50mV and were half-maximally activated at −27±1mV (n=15). The inward current displayed voltage-dependence of inactivation with half-maximal inactivation at −36mV. Boltzmann fits of the activation and inactivation data revealed a window current between −40mV and 20mV. The time course of decay of the current evoked at 0mV could be fitted with a single exponential and had a time constant of 79±11ms (n=4). Replacement of external $Ca^{2+}$with Ba^{2+}significantly increased this to 344 ±61ms (n=4, p=0.022). The current amplitude was also augmented by Ba^{2+} (n=6). Removal of extracellular calcium significantly reduced inward currents (n=5). In contrast, they were enhanced by Bay K 8644 (1μM, n=3).
Nifedipine (1μM), significantly reduced current amplitude across the voltage range; however, blockade was more effective on the current evoked at 0mV (−58±7pA to −19±4pA, n=4, p=0.004) than that evoked by a step to −20mV (−39±3pA to −25 ±2pA, n=4, p=0.012) either indicating voltage dependence of the action of nifedipine or another component of inward current. Increasing the concentration of the drug to 10μM did not bring about further significant reduction either at 0mV or at ±20mV. In the presence of 1μM nifedipine, however, the latter current, was significantly reduced by 100μM Ni^{2+} (− 18±4pA to -5±3pA, n=10, p=0.008).
In summary, interstitial cells from the guinea-pig detrusor possess inward currents with typical characteristics of L-type Ca^{2+} current. They also had a component of inward calcium current which was resistant to nifedipine, but sensitive to Ni^{2+}. Further work is required to characterise the latter conductance.

McCloskey KD & Gurney AM (2002). J Urol 168, 832-836.

McCloskey KD (2005). J Urol 173, 296-301.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC43

Modulation of the muscarinic cationic current in guinea-pig ileal myocytes by PLCβ

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Visceral smooth muscles co-express two muscarinic receptor subtypes, M2 and M3, which produce two major effects during cholinergic excitation - adenylyl cyclase inhibition and PLCβ activation, respectively. In our work we studied the relevance of these effects for muscarinic cationic current (mI_{CAT}) generation, which underlies cholinergic excitation. Experiments were performed on single ileal cells, from humanely killed guinea-pigs, using patch-clamp techniques with weakly buffered $[Ca^{2+}]_i$ (50 μM EGTA) or with $[Ca^{2+}]_i$ 'clamped' at 100 nM (10 mM BAPTA/$CaCl_2$ mixture). Application of cAMP-elevating agents (1 μM isoproterenol or 10 μM 8-Br-cAMP) had no effect on mI_{CAT}. In contrast, we found that PLCβ plays one of the major roles in the regulation of mI_{CAT}. Firstly, $InsP_3$-induced Ca^{2+}

release due to M3 receptor activation facilitates mI_{CAT}, an effect which could be inhibited by the PLC blocker U-73122. Secondly, PLCβ activation was important for cationic current generation even when $[Ca^{2+}]_i$ was 'clamped' at 100 nM. U-73122 inhibited mI_{CAT} with an IC_{50} of 0.72 ± 0.18 μM (n=4) by reducing the maximal conductance and shifting the activation curve positively and at 2.5 μM virtually abolished both carbachol- and GTPγS-induced cationic current (n=18). $InsP_3$ (1 μM, n=3) in the pipette or OAG (20 μM, n=7) applied externally had no effect on mI_{CAT} or its inhibition by U-73122. U-73343, a structural U-73122 analogue which inhibits PLC only weakly, had no effect on GTPγS-induced mI_{CAT} (n=4), but weakly inhibited carbachol-induced current (n=5), possibly by competitively inhibiting muscarinic receptors, since the inhibition could be prevented by increasing the carbachol concentration to 1 mM (n=3). Aristolochic acid (n=3), which inhibits PLA2, as well as D-609 (n=7) and NCDC (n=4), which inhibit phosphatidylcholine-specific PLC had no or very small effect on mI_{CAT}, suggesting that these enzymes were not involved. It is concluded that the 'permissive' role of the M3 receptor subtype in mI_{CAT} generation is exerted via PLCβ-dependent modulation of mI_{CAT} both through Ca^{2+} release and independently of $InsP_3$, DAG, Ca^{2+} store depletion or a rise of $[Ca^{2+}]_i$.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC44

Low chloride potentiation of hypoxic pulmonary vasoconstriction

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In the pulmonary artery (PA), hypoxia releases Ca^{2+} from the SR (Dipp *et al.* 2001) and during hypoxia, Ca^{2+} released from the SR may activate the Ca^{2+}-activated Cl^- channel (Cl_{Ca}) in the plasma membrane (Wang *et al.* 1997). Decreasing $[Cl^-]_o$ can increase agonist-induced contraction in the PA (Lamb & Barna, 1998). Here we report the effects of decreasing $[Cl^-]_o$ on the hypoxic pulmonary vasoconstriction (HPV) response in pre-constricted porcine PA.

Porcine lungs were obtained from a local abattoir. Pulmonary arteries (4th-5th order) were dissected free (external diameter 500-800 μm), mounted in a myograph and resting tone set at 2mN. Hepes-based PSS were made hypoxic by aerating with N_2 and P_{O2} monitored continuously. In low Cl^- solutions, NaCl was replaced with equimolar Na-gluconate. Pre-tone was achieved using 2 μM $PGF_{2\alpha}$. Endothelial function was assessed by determining the ability of 20μM ACh to relax KCl-constricted vessels. All drugs were bath applied. Where appropriate, data are expressed as a % of KCl-induced contraction and presented as mean values ± S.E.M. Significance was determined at $P< 0.05$ using a Student's paired or unpaired *t* test as appropriate.

Control contractions were induced using 80 mM KCl (14.4 ± 2.8 mN, n=6). $PGF_{2\alpha}$-induced contractions were 1.4 ± 1% of control response. Hypoxia (P_{O2} < 25 mm Hg) resulted in a slow monophasic increase in tone (13.4 ± 3.6% of control), which was reversible on return to normoxia. In low-Cl^- solution, hypoxia-induced contractions were significantly increased (41.5 ± 9.8% of control; $P< 0.05$ compared to Cl^--containing bath solution, n=6). In Ca^{2+}-free solution, the hypoxic response (15.9 ± 5.3% of control) was not significantly different compared to hypoxic, normal Cl^- solution, ($P> 0.05$, n=6). There was no significant difference between endothelial (+) and endothelial (-) vessels in the contractile response to hypoxia, either in normal Cl^- or low-Cl^- solution ($P> 0.05$). Hypoxia-induced contractions returned to resting tone upon washout with normoxic normal Cl^- solution (1.6 ± 0.3 mN). Washout with normoxic, low-Cl^- solution only resulted in a partial relaxation to baseline (5.1 ± 0.6 mN, $P< 0.05$, n=5). Increased tone in low-Cl^-, normoxic solution was insensitive to the Ca^{2+} channel blocker nifedipine (5μM) but was sensitive to the Cl^- channel blocker niflumic acid (50 μM).

These data suggest a role for Cl_{Ca} in modulating the porcine HPV response and resting tone in pre-stimulated porcine PA.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC45

TRPV4 hyperpolarizes smooth muscle via a novel calcium-induced calcium release mechanism

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Vasodilatory factors produced by the endothelium are critical mediators of blood pressure and flow. We hypothesised that endothelial signals are transduced to underlying smooth muscle by vanilloid transient receptor potential (TRPV) channels. Experiments were performed using cerebral arteries harvested from humanely killed male Sprague-Dawley rats. TRPV4 message was detected in RNA from cerebral artery smooth muscle cells (n=3). In patch-clamp experiments employing freshly isolated cerebral myocytes, whole-cell currents with properties consistent with those of expressed TRPV4 channels were evoked by the TRPV4 agonist 4α-phorbol 12,13-didecanoate (4α-PDD, 5 μM, n=3) and the endothelium-derived arachidonic acid metabolite 11, 12 epoxyeicosatrienoic acid (11,12 EET, 300 nM, n=6). High-speed laser scanning confocal microscopy was used to evaluate the effects of TRPV4 activation on intracellular Ca^{2+} dynamics in cerebral artery smooth muscle cells loaded with the fast Ca^{2+} indicator dye fluo-4. Values are means±S.E.M.

11,12 EET increased the frequency of unitary Ca^{2+} release events (Ca^{2+} sparks) via ryanodine receptors located on the sarcoplasmic reticulum (control, 0.18±0.02 Hz, n = 16; 11,12-EET, 0.29±0.03 Hz, n=13; $P<0.05$, t test). EETs-generated Ca^{2+} sparks

activated nearby sarcolemmal large conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) and increased the frequency of transient K^+ currents (referred to as 'spontaneous transient outward currents' or 'STOCs') (control, 0.475±0.15 Hz, vehicle, 0.472±0.12 Hz, 11,12 EET (100 nM), 1.1±0.34 Hz*, n=9 for all groups *P<0.05 vs. all other groups, one-way repeated measure ANOVA). 11,12 EET-induced increases in Ca^{2+} spark and STOC frequency persisted when voltage-dependent Ca^{2+} channels (VDCCs) were inhibited (n=5 for both groups). Conversely, EETs-induced changes in Ca^{2+} spark and STOC frequency were blocked when external Ca^{2+} was reduced from 2 mM to 10 μM (n=5 for both groups), suggesting that these response require extracellular Ca^{2+} influx via channels other than VDCCs. Antisense-mediated suppression of TRPV4 expression in intact cerebral arteries prevented 11,12 EET-induced smooth muscle hyperpolarization (change in membrane potential in response to 300 nM 11,12 EET: sense, -10.3±0.18 mV, n=6 vs. antisense, -0.5±1.8 mV, P<0.05, two-way ANOVA) and vasodilatation (change in luminal diameter in response to 11,12 EET (100 nM): sense, 37.1±8.2 μm vs. antisense, -6.7±4.4 μm, n=5 for both groups, P<0.05, two-way repeated measures ANOVA). Thus, we conclude that TRPV4 forms a novel Ca^{2+} signalling complex with ryanodine receptors and BK_{Ca} channels that elicits smooth muscle hyperpolarization and arterial dilatation via Ca^{2+}-induced Ca^{2+} release in response to an endothelial-derived factor.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC46

G-Protein knockdown by small interference RNA in intact cerebral arteries

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Pyrimidine nucleotides potently constrict cerebral arteries by activating signal transduction pathways that influence myofilament calcium sensitivity and/or ion channels that control resting membrane potential. G-protein-coupled P2Y receptors are important to this transduction process and recent observations have implicated a prominent role for Gq, G11 and G13. To define which G-proteins control uridine triphosphate (UTP)-induced vasoconstriction, a small interference RNA (siRNA)approach was developed to induce mRNA and protein knockdown in intact cerebral arteries from humanely killed Sprague-Dawley rats. These G-proteins were cloned from rat brain cDNA (humanely killed 12- to 16-week-old Sprague Dawley rats) and siRNAs designed to specifically target Gq but not G11 or G13. Targeted siRNA (20 nM) was introduced into cerebral artery segments using reverse permeabilization and vessels were subsequently cultured in serum-free medium for up to 144 hours. During this period, arteries were removed and assayed for contractile function, mRNA (by real-time PCR) and protein (by Western blotting). A 75% reduction in Gq mRNA was observed within 72 hours post-permeabilization, a response that was sustained up to 120 hours (n=6). G11, a close family member of Gq (79% sequence identity) was unaffected (n=6). Despite reductions in Gq mRNA, protein levels measured between 96-144 hours post-permeabilization were unaffected (n=4). Consistent with the apparent lack of translational knockdown, cerebral arteries treated with Gq-targeted or scrambled siRNAs constricted similarly to UTP (0.01-100 uM) and intravascular pressure (20-100 mmHg) (n=6). Control experiments confirmed antibody specificity (anti Gq, Santa Cruz) and that Gq-targeted siRNA induced protein knockdown in cultured smooth muscle cells (n=3). Our findings demonstrate that mRNA can be successfully knocked down by siRNA in intact cerebral arteries. Selective mRNA down regulation does not, however, ensure effective protein knockdown. Ongoing experiments in intact cerebral arteries are addressing whether the absence of translational knockdown is a function of the turnover rate of the targeted protein.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC47

The role of the sarcoplasmic reticulum (SR) increases with gestation in pregnant rat myometrium: is store-operated calcium entry (SOCE) involved?

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In smooth muscle cells, the sarcoplasmic reticulum (SR) is an important regulator of cytosolic Ca^{2+}, membrane excitability, and a source of Ca^{2+} for contraction. Previous studies on the myometrium have shown that the SR has a role in limiting contraction (1). As the relative quiescence of the uterus clearly changes during gestation, the aim of this study is to investigate the role of the SR throughout pregnancy.

Simultaneous measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and force were made on strips of longitudinal myometrium taken from non-pregnant (NP) and pregnant rats at 9-10 days (10), 16-17 days (17) and 20-22 days (22, term day 22-23) gestation, after humane killing. The effect of the SR on spontaneous uterine activity was compared using 20μM cyclopiazonic acid (CPA), a specific inhibitor of the SR Ca^{2+} pump. The myometrial strips were spontaneously active at 32°C and means ± s.e.m. are normalized to the amplitude of control Ca^{2+} and force transients (100%). Significance was tested using the appropriate Student's t test with significance taken at $p<0.05$; n is number of animals. Inhibiting SR Ca^{2+} uptake with CPA increased contraction frequency and force in all animals (n=25). The CPA- induced rise in basal force was similar in day 17 and 22 rats and was significantly increased in these late gestational groups compared with day 10 and NP rat uteri (42.7 ± 10.2%; n=10 vs 3.01 ±0.6%; n=15, respectively).

To further investigate this gestational difference in response to CPA, the contribution of external Ca^{2+} entry, through voltage-gated (VOCC) or SOCE channels was determined by emptying

the SR in zero Ca^{2+} solutions containing CPA (0-CPA) and then returning tissues to physiological solutions containing 2mM Ca^{2+}.

In NP and day 22 rats, 0-CPA induced a prolonged (>20min) rise in basal $[Ca^{2+}]_i$ that was significantly greater in day 22 compared with NP rats (142.8 ± 7.6%, n=5 vs 77.2 ± 14.4%, n=5, respectively). In 22 rats (n=3), nifedipine (1μM; an inhibitor of VOCC) reduced the amplitude of this basal $[Ca^{2+}]_i$ rise by 33.0 ± 4.4%, but SKF 96365 (50μM; an inhibitor of SOCE) inhibited almost all CPA-induced rise in basal $[Ca^{2+}]_i$. Preliminary data suggest that the effect of both nifedipine and SKF 96365 are very similar in NP rats.
These data suggest an increased contribution of the SR to quiescence towards the end of pregnancy and that SOCE contributes to its re-filling, which could be significant in labour as agonists induce store depletion causing Ca^{2+} entry and enhanced contraction.

Taggart MJ & Wray S (1998). *J Physiol* **511**, 133-144.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC48

Contractile properties of rat extra- and intrapulmonary arteries after 4 days of culture

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Organoid cultured explants from differentiated tissues have gained renewed interest in the undertaking of physiological and pharmacological studies. In the present work, we examined the pharmaco-mechanical properties of an *in vitro* model, consisting of organoid cultured rings derived from rat extra- and intrapulmonary arteries, over a period of 4 days in culture.
Male Wistar rats were humanely killed. The heart-lung block was removed, extra- and intrapulmonary arteries of the first and second order were dissected. Mechanical changes were quantified using isometric tension measurements on both fresh and cultured pulmonary arterial tissues, in the presence or absence of 10% fetal calf serum. Serum-free culture medium was supplemented with 1% insulin–transferrine–selenium (ITS). Conventional histochemical and immunofluorescent stainings were also performed to assess tissue structure integrity while the TUNEL method was used to detect apoptosis. Results are expressed as the mean ± S.E.M. with n indicating the number of rings used. Statistical analyses were performed using unpaired Student's t tests as well as ANOVA for global comparisons of the curves. Values of $P < 0.05$ were considered significant.
The explants developed spontaneous rhythmic contractions (SRC) in approximately half of the vessels. SRC amplitude and time course were significantly modified by conditions and agents acting on membrane potential, namely high potassium solutions or levcromakalim (0.1–1 μM), a potassium channel opener (n = 5–10). Nitrendipine, an L-type calcium channel blocker, suppressed SRC (n = 4–9). Cultured explants also developed a hyperreactivity to high potassium challenges of 10–40 mM (n = 9–19). Whereas contraction to serotonin (5-HT) was enhanced in intrapulmonary arteries (n = 7–16), contraction to endothelin-1 remained unchanged after 4 days of culture (n = 10–18). Serum did not alter contractile properties during the culture period. Endothelial-dependent relaxation was maintained in response to A23187 (500 μM; n = 7–10) but was abolished in response to carbamylcholine (10 μM; n = 16–22). Histological analyses revealed the absence of hypertrophied vascular wall while the TUNEL technique attested to the absence of apoptosis.
In conclusion, contractile phenotype and tissue structure integrity are well preserved during short-term culture of organoid explants making this model quite suitable for pathophysiological studies, such as the use of small interference RNA to study the functional role of specific proteins. Moreover, this organ culture model could be relevant in addressing the effect of chronic hypoxia *in vitro*.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC49

Modulation of myogenic reactivity of rat middle cerebral arteries by over-expression and dominant-negative suppression of voltage-gated Kv1 channels

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The myogenic response of cerebral arteries is critical for regulation of blood flow to the brain, maintaining constant flow during variations in systemic blood pressure via graded changes in diameter. Precisely controlled changes in membrane potential are involved, but the underlying ionic mechanisms are not completely defined. A role for voltage-gated K^+ (Kv) channels was indicated using 4-aminopyridine (Knot & Nelson, 1995) and Kv1 family subunits were shown to be expressed by cerebral arteries and arterioles (Cheong et al. 2001; Albarwani et al. 2003).
This study employed a dominant-negative, myc-tagged rabbit Kv1.5 (Kv1.5DN) pore-mutant subunit to assess the contribution of Kv1 channels to the myogenic response of rat middle cerebral arteries. The Kv1.5DN construct was first shown to reduce whole-cell currents due to wild-type rabbit Kv1.5 (Kv1.5WT) and Kv1.2, but not Kv2 channels expressed in HEK293 cells. Expression and co-assembly of Kv1.5DN with wild-type Kv1 was confirmed by co-immunoprecipitation.
Myogenic reactivity of freshly isolated arteries, as well as arteries cultured for 48 hrs after transfection (Opazo-Saez et al.,2004) with empty plasmid (pcDNA3), Kv1.5DN or Kv1.5WT was assessed by pressure myography (Knot & Nelson, 1995). Rats were humanely killed. Expression of Kv1.5DN enhanced, whereas Kv1.5WT suppressed myogenic reactivity compared to arteries transfected with empty plasmid. The myogenic responses of empty plasmid, Kv1.5DN and Kv1.5WT vessels at 80 mmHg (i.e. difference in diameter in Ca^{2+}-containing vs. Ca^{2+}-free solutions) were 79.3±5.3 μm (mean±S.E.M.; n=11), 109.6±7.3 μm (n=9) and 56.9±4.0 μm (n=9), respectively ($P<0.05$ by ANOVA). There

was no difference between the response of freshly isolated (89.4±10.3 μm; n=5) and cultured, empty plasmid vessels. Message encoding rabbit Kv1.5DN or Kv1.5WT was identified using rabbit Kv1.5-specific primers in RNA samples of transfected, but not freshly isolated or empty plasmid rat arteries by RT-PCR. All amplicons were sequenced to confirm their identity.
This study provides the first molecular evidence for a contribution of Kv1 channels to the control of myogenic reactivity of resistance arteries. Kv1 channel activation in response to myogenic depolarization likely serves as a negative-feedback mechanism, providing for precise control over the extent of L-type Ca^{2+} channel activation and arterial constriction evoked by increased intraluminal pressure.

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Opazo-Saez A *et al.* (2004). *Am J Physiol* **286**, C433-C447.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC50

Downregulation of TRPC6 expression by pro-inflammatory cytokines in cultured human airway smooth muscle cells

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The transient receptor potential canonical (TRPC) family form a group of non-selective cation channels and are candidates for receptor-operated calcium channels (ROCs) and store-operated calcium channels (SOCs) in airway smooth muscle. TRPC1, 3, 4 and 6 have previously been shown to be expressed in cultured human airway smooth muscle (HASM) (Corteling *et al.* 2004) and are believed to contribute to the control of calcium homeostasis. However, little is known about how these channels are regulated and their role in disease. In this study, we investigated the regulation of TRPC mRNA expression in HASM cells by pro-inflammatory cytokines using quantitative real time PCR.
Primary HASM cells were isolated from human trachea explants (with ethical approval) and cultured *in vitro* with cytokines: TNF-α (10ng/ml), IL-13 (50ng/ml), IL-1β (10ng/ml) and IL-4 (10ng/ml). RNA was extracted following exposure to cytokines for either 4 or 24 h and reverse transcribed to cDNA. Quantitative, real time PCR was performed on the cDNA using specific primers and probe sets designed against the different TRPC genes (TRPC1, 3, 4 and 6). An 18s ribosomal RNA specific assay was used as a housekeeping control. Relative quantitation using the comparative (Ct) method was used to analyse changes in gene expression. Expression in each of the cytokine treated samples was compared relative to the level of expression in the untreated medium controls. All experiments were performed in triplicate for each donor: HASM isolated from 3 separate donors were used. All cytokines failed to alter TRPC1, 3 and 4 expression by more than 2-fold. However, substantial changes in TRPC6 expression were observed. Incubation of HASM cells with TNF-α, IL-13, IL-1β and IL-4 for 4 h, decreased TRPC6 expression to 22±6%*, 32±12%*, 26±6%*, 53±9%*, respectively, with the medium control set to 100%. This effect was further enhanced at 24 h (4±2%*, 20±4%*, 8±3%*, 45±5%* respectively). Data are expressed as mean values ± s.e.m., n=3 donors. Comparison between groups was achieved using one-way ANOVA followed by Dunnetts multiple comparison test: *$p<0.01$.
Cytokines such as TNF-α are thought to be important mediators of inflammatory airway disease. The current study has shown that a range of pro-inflammatory cytokines have the ability to selectively downregulate the expression of TRPC6 in HASM.

Corteling RL et al (2004). Am J Respir Cell Mol Biol 30, 145-154.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC51

Role of intracellular stores in the regulation of rhythmical calcium waves in interstitial cells of Cajal from rabbit portal vein

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Interstitial cells of Cajal (ICs) are believed to play an important role by generating and propagating electrical slow waves to gastrointestinal muscles and/or mediating signals from the enteric nervous system. It was shown recently that single ICs from the rabbit portal vein (RPV) can generate rhythmical Ca^{2+} waves associated with membrane depolarisation and therefore may act as pacemakers in this tissue.
Freshly isolated RPV ICs (freshly isolated from humanely killed rabbits) preloaded with the Ca^{2+} indicator fluo-3 and viewed by confocal microscope displayed rhythmical Ca^{2+} waves with a frequency from 1 to 6 waves/min. The frequency of waves correlated with the rate of rise of the fluorescence signal during the initial slow Ca^{2+} increase between Ca^{2+} waves, but not with their peak amplitude. This suggests that partial depletion of the endo/sarcoplasmic reticulum (E/SR) during each wave is not the factor determining the wave frequency and the initial slow Ca^{2+} increase is likely to reflect some pacemaking process, which modulates the wave frequency. Fast application of 5 mM caffeine during the declining phase of the slow Ca^{2+} wave evoked a Ca^{2+} transient of higher amplitude than the Ca^{2+} wave suggesting that intracellular stores are not completely depleted during Ca^{2+} wave generation.
Ca^{2+} waves in the RPV were resistant to the L-type Ca^{2+} channel blocker nicardipine (10 μM) (n=10), but were abolished by Ca^{2+}-free solution (n=2) and E/SR Ca^{2+}-ATPase inhibitors, cyclopiazonic acid (10 μM) (n=4) or thapsigargin (1μM) (n=4). This suggests that Ca^{2+} release channels on the Ca^{2+} stores such as ryanodine receptors (RyRs) and IP_3 receptors (IP_3Rs) could be responsible for Ca^{2+} waves during slow wave generation. Blockers of the IP_3-induced Ca^{2+} release, 2-APB (30 μM) (n=6)

and xestospongin C (10 μM) (n=4) abolished these waves suggesting involvement of IP_3Rs in the Ca^{2+} wave generation in RPV ICs.
Application of ryanodine (100 μM) (n=5) abolished Ca^{2+} waves in the RPV ICs. Moreover, after blocking of Ca^{2+} waves with ryanodine, application of 5 mM caffeine (n=4) evoked a Ca^{2+} transient confirming that ryanodine directly inhibited the waves but not through depletion of the Ca^{2+} stores.
Spatial distribution of S/ER and RyRs was visualised with a confocal z-sectioning protocol in ICs (n=20) freshly isolated from RPV loaded with the low affinity Ca^{2+} indicator fluo-3FF and stained with BODIPY TR-X ryanodine. A high degree of similarity between fluo-3FF and BODIPY TR-X ryanodine fluorescence suggested that all elements of intracellular calcium stores are enriched with RyRs. BODIPY TR-X ryanodine staining of ICs revealed RyRs irregularly distributed throughout the ICs with a continuous high-density region in the perinuclear E/SR. Simultaneous x-y confocal imaging of fluo-3 fluorescence revealed that slow Ca^{2+} waves originate locally within high-density RyR regions of the ICs.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC52

The role of the Na^+–Ca^{2+} exchanger in $[Ca^{2+}]_i$ signalling in phenotypically distinct myocytes from the rat pulmonary vein

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The Na^+–Ca^{2+} exchanger in smooth muscle from the pulmonary artery, is known to be involved in regulating $[Ca^{2+}]_i$ (Wang *et al.* 2000). However, the role of the Na^+–Ca^{2+} exchanger in regulating $[Ca^{2+}]_i$ in the pulmonary vein (PV) has received little attention. In the PV, two phenotypically distinct myocytes have been reported (see Michelakis *et al.* 2001). Thus, we have examined how $[Ca^{2+}]_i$ signalling in these two distinct myocyte phenotypes is affected by inhibition of the Na^+–Ca^{2+} exchanger.
Male Sprague-Dawley rats (200-300g) were humanely killed. Myocytes were isolated from intrapulmonary veins (< 400 μm), using a procedure similar to that described by Drummond & Tuft (1999) for the pulmonary artery, and thereafter incubated with 5 μM fura-2 AM. To achieve a Na^+-free extracellular solution, equimolar LiCl replaced NaCl. All experiments were carried out at room temperature. Mean data±S.E.M. are given and n is the number of cells. Statistical differences were tested for using Student's paired *t* test. $P < 0.05$ was considered to be significant.
In cardiac-like myocytes isolated from the vein, spontaneous oscillations in $[Ca^{2+}]_i$ were observed, with a frequency of 0.3 ±0.1 Hz. Replacing Na^+ in the bath solution with Li^+ resulted in an increase in basal $[Ca^{2+}]_i$ from 72±17 nM to 156 ±46 nM ($P< 0.05$, n=6), and the frequency of spontaneous oscillations also increased to 0.6±0.1 Hz ($P< 0.05$, n=6). A small but significant decrease in peak $[Ca^{2+}]_i$ during oscillations was observed in Na^+-free bath solution (132±3 nM to 124±4 nM, $P< 0.05$, n=6). Caffeine-induced $[Ca^{2+}]_i$ transients were significantly increased from 755±133 nM to 1150±230 nM (n=6, $P<0.05$) in Na^+-free bath solution, and the time for 90% recovery ($t_{90\%\ recovery}$) was increased from 56±8s to 132±16s ($P<0.05$). Spontaneous oscillations in $[Ca^{2+}]_i$ were absent in typical spindle shaped smooth muscle cells isolated from the vein. Application of caffeine (20 mM) or ATP (10 μM) transiently increased $[Ca^{2+}]_i$ to 759±24 nM and 556±32 nM, respectively. In Na^+-free solution, there was no change in basal Ca^{2+}. However, both caffeine and ATP-induced $[Ca^{2+}]_i$ transients were increased (13 ±3% and 36±5%, respectively). The $t_{90\%\ recovery}$ also increased from 9±2s to 15±5s for caffeine and from 12±3s to 26±4s for ATP ($P< 0.05$). In 60% of cells, the frequency of ATP (10 μM) induced $[Ca^{2+}]_i$ oscillations was increased in Na^+-free bath solution.
These results suggest an important role for the Na^+–Ca^{2+} exchanger in regulating $[Ca^{2+}]_i$ signalling in the two distinct phenotypes of myocytes found in the pulmonary vein.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC53

Effects of chronic hypoxia on Ca^{2+} handling in proliferative human smooth muscle cells

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Smooth muscle cells were obtained by explant culture from segments of human internal mammary artery taken (with approval from the local research ethics committee and informed patient consent) from patients undergoing routine coronary bypass surgery. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air. Experiments were conducted at passage 1. Chronic hypoxia was imposed 24 hours prior to experiments by switching cells to a gas mixture of 2.5% O_2, 5% CO_2 and balance N_2. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was monitored in fura-2-loaded cells as previously described (Smith *et al.* 2003). Results are given as % changes in the 340/380 nm fluorescence ratio. Values have been determined from measurements in at least 7 cells (and as many as 90) from between 3 and 12 patients. Data have been analysed by Student's unpaired t test or one-way ANOVA as appropriate.
The basal $[Ca^{2+}]_i$ measured (as the 340/380 nm fluorescence ratio) in human internal mammary artery smooth muscle cells (IMASMC) bathed in Ca^{2+}-containing (2.5mM) solution was not significantly altered by chronic hypoxia. However, on removal of external Ca^{2+} (solution contained 0mM $CaCl_2$ and 1mM EGTA), a notable drop in $[Ca^{2+}]$ was observed in the normoxic cells which was not seen in cells exposed to chronic hypoxia.
Perfusion of normoxic cells with a high K^+ (50mM) solution caused a rapid, sustained and reversible rise in $[Ca^{2+}]_i$. In cells

exposed to chronic hypoxia this high K^+-induced Ca^{2+} elevation was significantly reduced by 38% (compared to the normoxic cells; P<0.01). In both normoxic and hypoxic cells the high K^+-induced increase in $[Ca^{2+}]_i$ was abolished by removal of external Ca^{2+} or by blockade of both L-type and T-type voltage-operated Ca^{2+} channels (VOCC) with 2 μM nifedipine and 3 μM pimozide, respectively (P<0.01 for each condition). Use of nifedipine alone inhibited the K^+-induced rise in $[Ca^{2+}]_i$ by approximately 90% in normoxic cells (P<0.01) and 72% in hypoxic cells (P<0.01), while pimozide alone inhibited the response by approximately 27% in normoxic cells (although this decrease was not significant) and 47% in hypoxic cells (P<0.01). These data indicate that in proliferative human IMASMC, chronic hypoxia leads to changes in Ca^{2+} handling compared to normoxic controls. The lack of effect of removing external Ca^{2+} on the basal $[Ca^{2+}]_i$ of hypoxic cells is consistent with an inhibition of Ca^{2+} extrusion mechanisms under hypoxic conditions, although further experiments are required to confirm this. In addition, chronic hypoxia was found to decrease the size of the high K^+-induced increase in $[Ca^{2+}]_i$, consistent with hypoxic modulation of VOCC in these cells.

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

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC54

Physiological functions of ryanodine receptor type 2 (RyR2) at resting state and during excitation–contraction coupling in urinary bladder smooth muscle

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Ryanodine receptor type 2 (RyR2) is expressed in heart, smooth muscle and brain, and is considered to be the most general Ca^{2+}-induced Ca^{2+} release (CICR) channel in sarco/endo-plasmic reticulum. We previously reported that, in excitation–contraction (E–C) coupling in smooth muscle cells (SMCs), elevation of $[Ca^{2+}]$ occurs via CICR in discrete local sites in subcellular spaces as Ca^{2+} hot spots and that the Ca^{2+} signal subsequently spreads to other parts of the cell to induce a contraction (Imaizumi et al. 1998; Ohi et al. 2001). It is not yet fully clear how much CICR via RyRs contributes to E–C coupling in SMCs. The present study on cells from humanely killed mice was undertaken to elucidate the significance of RyR2 in E–C coupling in urinary bladder (UB) SMCs using RyR2 heterozygous KO mice ($RyR2^{+/-}$), since homozygotes were embryonic lethal. RyR2 mRNA expression in $RyR2^{+/-}$ was decreased by over 50% compared with wild-type mice ($RyR2^{+/+}$). RyR type3 mRNA expression was not changed in $RyR2^{+/-}$. The elevation of $[Ca^{2+}]$ in hot spots and whole-cell area at ~30 ms from the start of depolarization was significantly smaller in $RyR2^{+/-}$ than in $RyR2^{+/+}$. Moreover, the number of hot spots was also smaller in $RyR2^{+/-}$. Contractile force development by electrical stimulation under moderate conditions was examined in the presence of atropine and some other antagonists of transmitter receptors and was also found to be smaller in $RyR2^{+/-}$. The force development was substantially decreased by 100 μM ryanodine in both but the extent of decrease was significantly smaller in $RyR2^{+/-}$, suggesting smaller a contribution of CICR to E–C coupling in $RyR2^{+/-}$. It has been well established that Ca^{2+} sparks activate BK channels to elicit spontaneous transient outward currents (STOCs), and STOCs regulate resting membrane potential and muscle tone (Heppner et al. 2003). The frequency of STOCs in single cell and the membrane depolarization by paxilline, a specific BK channel blocker, in tissue preparation were significantly reduced in $RyR2^{+/-}$. BK channel expression in $RyR2^{+/-}$ was comparable to that in $RyR2^{+/+}$. These results strongly suggest that RyR2s play crucial roles in CICR for the regulation of E–C coupling and also in the generation of STOCs to regulate resting membrane potential and tone in UBSMC.

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PC55

The pharmacology of voltage-insensitive Ca^{2+} channels in rat retinal arteriolar smooth muscle

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Smooth muscle cells express a variety of voltage-independent Ca^{2+} channels but studies on the functional role of these has been hampered by their confused pharmacological susceptibilities. Previously, we showed that nifedipine in rabbit choroid arterioles, and mibefradil in rat retina appeared to block store-, receptor- and L-type Ca^{2+} channels at the same equally low concentrations. Here we used a range of classical Ca^{2+} channel blockers to identify agents which might be useful in delineating voltage-dependent and -independent Ca^{2+} channels in the same arteriolar smooth muscle cells.

Arteriolar fragments (15-35 μm outside diameter) were isolated by gentle trituration of the retinae removed immediately from humanely killed rats. After loading with fura-2 AM, cytosolic Ca^{2+} was measured in the smooth muscle layer by microfluorimetry. Ca^{2+} influx was measured as the rate of Ca^{2+} rise on adding 1 mM to the Ca^{2+}-free medium normally superfusing the microvessels. This was performed in the presence of either: (a) 70 mM KCl (to open voltage-dependent Ca^{2+} channels), (b) cyclopiazonic acid (10 Mμ CPA, to stimulate store-depletion operated Ca^{2+} channels), or (c) endothelin-1 (10nM Et-1, to activate receptor-controlled Ca^{2+} influx). Concentration-effect curves were constructed for the Ca^{2+} channel blockers and these used to read off ID_{50}s (Table 1).

The effects of SKF96365 and LOE908 were not consistent with their specific actions on either store or receptor induced Ca^{2+}

influxes. In this preparation store-operated channels were resistant to nifedipine in contrast to rabbit choroidal arterioles. The results with diltiazem and verapamil suggest that these cells have at least three voltage-independent Ca^{2+} influx pathways which can be separated pharmacologically. Two of these are store-depletion activated channels and can be separated by their differential sensitivity to diltiazem but both have poor sensitivity to verapamil. The receptor-operated component is highly sensitive to both drugs.

TRP channels are thought to underlay voltage-indendent Ca^{2+} influx pathways in smooth muscle and a similar screening approach of known Ca^{2+} channel blockers could be a useful strategy to find pharmacological tools which can be used to associate functions with particular molecular entities.

Table 1. ID50s as μmol/l for the 3 modes of Ca^{2+} entry were read from the averaged curves using 4-14 microvessels

	KCl	CPA	Et-1
SKF96365	0.48	1.3	0.064
LOE908	0.15	2.6	0.15
Nifedipine	0.017	52	0.068
Diltiazem	0.71	0.03(>100)**	0.045
Verapamil	0.012	>100	0.006

With CPA, diltiazem** produced a 45±8% (S.E.M.) inhibition at low concentrations and the remaining Ca^{2+} influx requiring much higher concentrations

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC56

Immunofluorescence microscopy of transient receptor potential channel proteins in the plasma membrane of bovine ciliary muscle cells

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In bovine ciliary muscle (BCM) cells, we have previously shown that stimulation of M_3-muscarinic receptor (MR_3) opens two types of receptor-operated non-selective cation channel (ROC) which serve as major pathways for Ca^{2+} entry during the tonic phase of contraction (Takai et al. 2004). We have also shown by RT-PCR that BCM (whole tissue) contains mRNA for four TRP channel homologues (TRPC 1, 3, 4 and 6), commonly regarded as molecular candidates for ROCs (see Inoue et al. 2003, for review). In the present experiments we tried to visually identify these TRPCs in the plasma membrane of BCM by immunofluorescence microscopy.

Smooth muscle cells were isolated by treatment with collagenase and papain from the ciliary body dissected out from bovine eyes obtained from a local slaughterhouse. A CCD fluorescence microimaging system (Olympus Co., Tokyo, Japan) was used. After 4-5 days culture of 150-300 BCM cells on the centre of a fibronectin-coated round glass plate (14 mm diameter) in serum-free HAM F12 media (Sigma), the body of the cells was removed by gentle sonication under hypotonic conditions. The plasma membrane remaining attached on the glass surface was treated with polyclonal primary antibodies (2.5-5 μg/ml; Chemicon Co., Temecula, CA, USA; catalogue codes: AB5446, AB5576, AB5812 and AB5914) against putative cytoplasmic segments of the four TRPCs and visualized with secondary antibodies labelled with an Alexa Fluor 546 fluorescent dye (Invitrogen; peak absorption and emission at 556 and 573 nm, respectively). The membrane preparations were also similarly immunostained with antibodies against MR_3 (Biogenesis Ltd, Poole, UK; cat. number: 6391-2150) and α-actin (Progen Biotechnik GmbH, Heidelberg, Germany; cat. number, 65001).

All the anti-TRPC antibodies used gave many microscopically visible spots of immunofluorescence (roughly 1 spot/μm^2) in the regions of the cell-free membrane preparation which were also positively stained with antibodies against MR_3 and α-actin. Essentially similar results were obtained in at least three replicated experiments for each of the four anti-TCPC antibodies. The existence of the four TRPC homologues in the plasma membrane of the BCM was thus clearly demonstrated. Much fewer fluorescent spots were detected in the membrane regions devoid of α-actin staining, indicating a relatively sparse distribution of the TRPC homologues in non-smooth muscle cells (such as fibroblasts) in the ciliary muscle tissue.

The present results encourage further study to examine the possible relationship between TRPCs and muscarinic receptor-operated cation channels.

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Inoue R, Hanno, T, Shi J, Mori Y & Ito Y (2003). J Pharmacol Sci 91, 271-276.

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PC57

Effects of polyamines on the muscarinic receptor-operated cation current in guinea-pig ileal smooth muscle myocytes

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The 60 pS channel, which mainly carries muscarinic receptor-gated cation current (mI_{CAT}) in ileal myocytes, could rarely be activated by carbachol in cell-attached patches (4 out of 77) although the channel activity more readily appears after patch excision with carbachol in the bath (19 out of 62). It thus appears that some native intracellular factors inhibit mI_{CAT} of which polyamines (PAs), the ubiquitous aliphatic polycations known to block various ion channels, seem the likely candidates. The effects of extra- and intracellular PAs (spermine and putrescine) on the mI_{CAT} evoked either by external carbachol or by internal $GTP_\gamma S$ were studied using patch-clamp techniques. Experiments

were performed on single collagenase-dispersed myocytes isolated from the small intestine of humanely killed adult guinea-pigs. External PAs rapidly and reversibly inhibited mI_{CAT} in a concentration- and voltage-dependent manner. Cation conductance activation curve was N-shaped both before and after PA application although membrane depolarization significantly relieved the inhibition. The concentration producing 50% current inhibition at -40 mV was 1.03 (n=16) and 4.17 mM (n=14) for spermine and putrescine, respectively. The mI_{CAT} values were similar for both carbachol- and $GTP_{\gamma}S$-evoked currents suggesting that the cationic channel rather than the muscarinic receptor was the primary site of the PAs action. External spermine and putrescine reversibly inhibited the activity of the 60 pS muscarinic cation channel by reducing both unitary conductance and open probability. In the perforated-patch configuration (0.1 mg ml^{-1} amphotericin B in the pipette) mICAT in response to 100 μM carbachol was reduced on average by 65% compared to the conventional whole-cell recordings. However, in the latter case adding spermine to the pipette solution at 0.3 μM (which corresponds to its physiological cytoplasmic concentration) inhibited mI_{CAT} by 39±3% (n=19) without any noticeable change of the conductance curve shape. Internal putrescine was less effective, inhibiting mI_{CAT} by 31±3% at 10 mM (n=18). It is concluded that endogenous PAs, spermine in particular, strongly inhibit mI_{CAT} and this effect can contribute to their well-known suppressing effect on the gastrointestinal tract excitability and contractility.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC58

Colocalisation between the large conductance calcium-activated potassium channel, L-type calcium channel, and β_2 adrenergic receptor, with caveolin in pregnant human myometrial cells

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Caveolae are membrane organizing centres that recruit proteins and lipids for participation in intracellular trafficking and signal transduction. Our recent findings have revealed both a structural (Chanrachakul *et al.* 2003a,b) and functional (Chanrachakul *et al.* 2004) associations between the large conductance calcium-activated potassium (BK_{Ca}) channel and the β_2 adrenergic receptor (AR) in mediating uterine relaxation. The aim of this study was to investigate whether the BK_{Ca} channel/ β_2 AR complex and L type calcium (Ca^{2+}) channels are localized together with caveolae of pregnant human myometrium in order to regulate uterine excitability.
This study was approved by Derbyshire Research Ethics Committee and written informed consent was obtained from each participant. Myometrial biopsies were taken from term pregnant women (n = 5) undergoing elective Caesarean section. Myometrial cells were enzymatically dispersed and grown in culture media. Cultured human myometrial cells were then fixed and indirect double immunofluorescence staining was performed. Mouse monoclonal anti-caveolin-1 and rabbit polyclonal anti-BK_{Ca} channel, β_2 AR and L type Ca^{2+} channel were used in this study. Confocal microscopy was used to examine the association between the BK_{Ca} channel, β_2 AR and the L type Ca^{2+} channel with caveolin-1 in isolated pregnant human myometrial cells.
Confocal immunofluoresecence demonstrated strong labelling of BK_{Ca} channels, β_2 AR, and L type Ca^{2+} channels in uterine myocytes. Caveolin-1 was located both on the membrane and intracellularly. Double staining immunofluorescence analysis indicated colocalization between BK_{Ca} channels, β_2 AR, and L type Ca^{2+} channels with caveolin-1 in primary cultures of human myometrial cells.
These results suggest that the L type Ca^{2+} channels might be linked with the BK_{Ca} channels/ β_2 AR complex and are localized together in caveolar microdomains of pregnant human myometrial cells. Further studies are underway to examine the role of this compartmentation in regulating uterine activity.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC59

Differential responses to growth factors and inhibitors in arterial and venous smooth muscle cells derived from bypass graft tissue

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Saphenous vein (SV) bypass grafts have poor long-term patency compared to internal mammary artery (IMA) grafts [1]. Smooth muscle cell (SMC) proliferation is a major component of intimal hyperplasia and vein graft disease. This study indicates that intrinsic differences in SMC proliferative capacity may contribute to the different patencies of arterial and venous grafts.
With informed consent and local research ethics committee approval, matched SV and IMA SMCs were isolated by explant culture from surplus graft tissue within six individual patients undergoing coronary artery bypass surgery. In the presence of 20% fetal calf serum (FCS), venous and arterial explant outgrowth rates were compared and population growth rates of extracted SMC at passage 4 were analysed over 8-10 days using a resazurin dye method. DNA synthesis experiments using ^{3}H-thymidine incorporation were performed on cells rendered quiescent in low serum before stimulation with platelet derived growth factor (PDGF, 30ng/ml) or basic fibroblast growth factor (bFGF, 20ng/ml). The effects of heparin, transforming growth factor β (TGF-β) and increased nitric

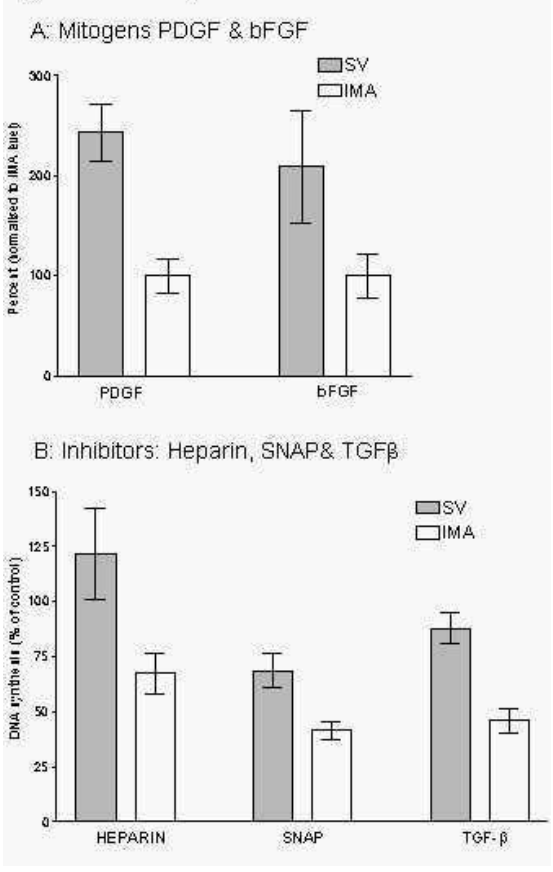
oxide produced by the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) were assessed on DNA synthesis induction by 5% FCS.

Data are presented as mean ± standard error of the mean. One-way ANOVA was used to determine differences between groups and treatments with a P value of <0.05 considered significant.

Although SMC proliferation rates varied considerably between patients, SV SMC populations increased significantly faster than IMA measured as outgrowth from explant (not shown); increase in cell number over 8 days (7860 ± 1300 vs 4514 ± 930) and DNA synthesis induction with PDGF (1143 ± 89.9 vs 594 ± 96.2) and bFGF (407 ± 138 vs 195 ± 66). In addition, heparin stimulated DNA synthesis in SV SMC (121.6 ± 20.41% of control) but significantly inhibited IMA SMC (67.3 ± 9.2%). SV SMCs were also less sensitive than IMA SMCs to the inhibitory effects of TGF-β (88.0 ± 6.8% vs 45.8 ± 5.5% of control) and SNAP (68.7 ± 8.0% vs 41.7 ± 4.1% of control).

Using paired SV and IMA SMC cultures we have demonstrated that, despite large variations between individuals, venous SMCs have a significant growth advantage over arterial counterparts. This is due to enhanced responses to growth factors and reduced sensitivity to endothelium-derived and other inhibitors of proliferation and implies an extra level of proliferation control in arterial SMCs.

Figure 1. DNA synthesis in SV and IMA SMCs.



Goldman S et al. (2004). J Am Coll Cardiol **44**, 2149-2156.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC60

Inhibition of non-cholinergic neurotransmission in guinea-pig isolated urinary bladder by P2X receptor antagonists

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Parasympathetic nerves provide the major excitatory innervation to the detrusor muscle of the urinary bladder, but it was shown as long ago as the 19th century that a substantial component of the neurotransmission is atropine-resistant (Langley & Anderson, 1895). Subsequently, desensitisation of $P2X_1$ receptors by α,β-meATP was found to abolish the non-cholinergic component, identifying ATP as a cotransmitter with acetylcholine (Kasakov & Burnstock, 1983). The aim of this study was to characterise the effects of P2X receptor antagonists on the non-cholinergic component of neurotransmission.

Adult, male Dunkin Hartley guinea-pigs were humanely killed and longitudinal strips (12 mm x 3 mm) of urinary bladder mounted under isometric conditions in 2 ml baths at 35°C. Intramural nerves were stimulated by electrical field stimulation (EFS) at 4 Hz, 0.15 ms pulse width, for 20 s at 10 min intervals, via platinum wire electrodes. Atropine (1 μM) and prazosin (100 nM) were present throughout. Data are expressed as mean ± s.e.m. or geometric mean with 95% confidence limits (95% cl), and were compared by Student's t test, or one-way ANOVA and Tukey's comparison as appropriate. Concentration-inhibition response curves were fitted to the data by logistic (Hill equation), non-linear regression analysis.

Atropine (1 μM) abolished contractions evoked by exogenous acetylcholine (10 μM) (n=4), but reduced the peak amplitude of responses to 4 Hz EFS by only 27.7 ± 7.9% (n=6). PPADS (0.1-100 μM) and suramin (1-300 μM) inhibited the atropine-resistant contractions with IC_{50} (95% cl) values of 6.9 μM (0.7-78.9 μM, n=5) and 13.8 μM (7.9-24.1 μM, n=6) respectively, but 30-40% of the response remained at the highest concentrations used. Tetrodotoxin (1 μM) abolished the remaining responses. PPADS and suramin also inhibited contractions elicited by exogenous ATP (300 μM) and α,β-meATP (1 μM), with potencies similar to those against 4Hz EFS (n=4-8). Whilst the responses to α,β-meATP were abolished, 30-40% of the peak response to ATP was not. The atropine-resistant neurogenic contractions were also only partially inhibited by 100 μM of the P2X antagonists NF279 (31.9 ± 3.7% decrease, n=7), MRS2159 (57.0 ± 4.4% decrease, n=5) and reactive blue 2 (32.8 ± 5.2% decrease, n=6), all of which abolished contractions evoked by α,β-meATP (1 μM). In contrast, desensitisation of $P2X_1$ receptors by administration of α,β-meATP (50 μM) abolished responses to 4 Hz EFS (n=12), α,β-meATP (1 μM, n=4) and ATP (300 μM, n=5).

Thus, whilst the non-cholinergic component of neurotransmission is abolished by desensitisation of the $P2X_1$ receptor by α,β-meATP, it is only partially inhibited by $P2X_1$ antagonists. At present the mechanism underlying the atropine- and $P2X_1$ receptor antagonist-resistant component of parasympathetic neurotransmission is unclear.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC61

Selective pulmonary vasoconstriction by KCNQ channel blockers

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Linopirdine is a putative cognition-enhancing agent. It acts by blocking M-current (I_{KM}), which is carried by heteromeric KCNQ2/3 channels (Wang et al. 1998). A non-inactivating potassium current identified in pulmonary artery smooth muscle cells has kinetic properties similar to I_{KM} (Evans et al. 1996). Moreover, KCNQ channel expression in murine portal vein (Ohya et al. 2003) raises the possibility of a role in the vasculature. Therefore, we investigated the expression of KCNQ subunits and the effects of the KCNQ channel blockers, linopirdine and XE991, in rat isolated intra-pulmonary artery.

Male Sprague-Dawley rats (250-300g) were humanely killed. Arteries (300-400 μm) were dissected and mounted on a wire myograph for isometric tension studies. An RNeasy Midi Kit was used to isolate mRNA from tissue preserved with RNAlater. It was reverse transcribed to cDNA, which was amplified using gene-specific primers for mouse KCNQ1, KCNQ2, KCNQ3, KCNQ4 and KCNQ5, following an established protocol (Osipenko et al. 2000). Data are expressed as mean±S.E.M. of n animals and compared using Student's t test with significance indicated by $p \leq 0.05$.

RT-PCR showed the presence of mRNA for KCNQ1, KCNQ4 and KCNQ5 subunits. Both linopirdine and XE991 produced pronounced constriction of pulmonary, but not mesenteric, arteries. The maximum response to linopirdine, seen at 10 μM, was 85 ± 12% (n=20) of the response to 50mM KCl in pulmonary arteries compared with 5 ±2% in mesenteric arteries. XE991 produced a maximum response at 1μM of 79 ± 8% (n=21) in pulmonary and 4±1% (n=6) in mesenteric arteries. The concentration producing 50% maximum response was 1.3 ± 0.5 μM for linopirdine (n=5) and 0.4 ± 0.3 μM (n=6) for XE991, close to the 50% blocking concentrations at recombinant KCNQ channels. Neither removal of the endothelium nor applying 10 μM phentolamine (to block effects of nerve released noradrenaline) significantly affected the contractile response to either drug. Constrictor responses were abolished in calcium-free medium. Nifedipine (1 μM, n=5) blocked contractions produced by linopirdine (10 μM) and XE991 (1 μM) by 99 ±0.01% and 98 ±1%, respectively, while levocromakalim (10 μM) reduced the responses to both drugs by 98 ± 1% (n=4).

The results indicate a functional role for KCNQ channels in pulmonary artery smooth muscle, to regulate Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and suggest that pulmonary vasoconstriction is a potential side-effect of cognition enhancers that block KCNQ channels.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC62

High-resolution Ca^{2+} imaging of neurotransmission in the rat anococcygeus

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Intracellular Ca^{2+} transients occur during neurotransmitter release from postganglionic autonomic nerve terminals, and during smooth muscle cell contraction, yet in both of these cases the pathways for generating these transients, and their relationship with function, are not clear. The anococcygeus muscle is a smooth muscle preparation that is currently little studied, which offers the opportunity to study noradrenergic and nitrergic neurotransmission, which in this tissue control contraction and relaxation respectively. Experiments were performed on anococcygeus muscles isolated from male Wistar rats (200-400 g). Rats were killed humanely. The anococcygeus was excised and incubated in the cell-permable acetylmethoxy ester (AM) form of the Ca^{2+} indicator Oregon Green BAPTA-1 for 70 min at 35°C, before being transferred to a superfused stage on a laser scanning confocal microscope (Leica DM-LFSA SP2). The indicator accumulated in smooth muscle cells and in structures resembling nerve bundles. Drugs were applied by addition to the perfusion reservoir. Electrical field stimulation (1-10 pulses, 1-10 Hz, pulse duration 0.05-0.2 ms, at supramaximal voltage) evoked Ca^{2+} transients in the nerve bundles, which preceded contraction. Trains of electrical pulses also elicited three distinct types of Ca^{2+} transients in smooth muscle cells: fast (usually < 500 ms), localised transients; whole cell transients of variable duration (often > 1000 ms); and propagating waves, which spread (at a mean speed of 49 ± 5 (S.E.M.) μm s^{-1}; n = 6 cells) along the length of the smooth muscle cells. Prazosin (100 nM), an α_1-adrenoceptor antagonist, did not affect electrically-evoked Ca^{2+} transients in the nerve bundles nor the fast localised transients but abolished waves and whole cell Ca^{2+} transients in the smooth muscle cells, and eliminated contraction (n = 9 preparations). Tetrodotoxin (1 μM), a voltage-gated Na^+ channel blocker, abolished electrically evoked Ca^{2+} transients in both nerve bundles and smooth muscle cells, and eliminated contraction (n = 5 preparations). α,β-methylene ATP (1 μM), a P2X receptor agonist, elicted localised Ca^{2+} transients in smooth muscle cells, and following continued exposure abolished the fast, localised transients produced during electrical stimulation (n = 3 preparations). These results indicate that neurotransmitter release in the anococcygeus elicits fast, focal purinergic Ca^{2+}

transients, as well as Ca^{2+} waves and whole cell transients initiated by noradrenaline.

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Relaxation of phenylephrine-contracted rat isolated mesenteric arteries by elevated potassium: The role of intracellular sodium and the Na,K-ATPase

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The proposal that potassium is an endothelial derived hyperpolarizing factor (EDHF) has focussed attention on the effects of elevated extracellular potassium ($[K^+]_o$) on arterial tone. Results from different laboratories show that rat isolated mesenteric arteries do not reliably relax to an increase in $[K^+]_o$ with no consensus for that discrepancy. The aim of this study was to define the experimental factors that determine the response of rat mesenteric artery to elevated $[K^+]_o$.

Male Wistar rats (200-250 g) were killed humanely and the superior mesenteric arterial tree removed. For isometric tension recording, sections of third order arteries were mounted in a Mulvany-type myograph containing bicarbonate buffered physiological salt solution (PSS) at 37 ºC. We have previously shown that relaxation of phenylephrine (PE) induced force by raised $[K^+]_o$ was dependent upon extracellular sodium, being attenuated in reduced sodium PSS and enhanced, sodium-dependently, by monensin (Brochet *et al.*, 2002). Relaxation was independent of the endothelium and sensitive to ouabain, with 100 μM being required for full inhibition (Brochet and Langton, 2003). In this study, we found that the concentration of PE had no effect on the relaxation of arteries to an increase in $[K^+]_o$. In addition, inhibition of BKCa by TEA (5 mM), charybdotoxin (100 nM) or iberiotoxin (100 nM) did not induce a relaxation when $[K^+]_o$ was increased from 5.9 to 13.8 mM. The hyperpolarization and relaxation to an increase in $[K^+]_o$ from 4.6 to 13.8 mM was enhanced by increasing the duration of exposure to PE. Moreover, the time dependence of the effect of PE was attenuated in the presence of diphenylboric acid 2-aminoethyl ester (2-APB; 75 μM), an IP_3 receptor inhibitor, and abolished by SKF96365 (10 μM), a store-operated cation channel (SOC) inhibitor. In contrast, 50 nM ryanodine, a concentration previously shown to induce the Ca^{2+} release from intracellular stores (Meissner, 1986), enhanced the relaxation to raised $[K^+]_o$.

These data suggest that accumulation of $[Na^+]_i$ following activation of SOCs by PE and the depression of Na,K-ATPase activity in low $[K^+]_o$ both serve to potentiate the upturn in the activity of the Na,K-ATPase when $[K^+]_o$ is subsequently increased. This results in hyperpolarization and relaxation of the arterial smooth muscle.

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PC64

Lack of additional effect of *E. coli* STa enterotoxin on fluid absorption *in vivo* in the prior absence of net fluid uptake: evidence against the epithelial cell chloride secretion model II

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E. coli (STa) enterotoxin reduces net fluid absorption but stimulation by STa of epithelial cell chloride ion and resultant fluid secretion could also contribute to diarrhoeal disease. Yet, contrary to expectations, net secretion does not occur *in vivo* when perfusate recovery methods are used (1) although inhibition of absorption does. Experiments were done with zero sodium ion perfusates and hence minimal fluid uptake, in the expectation that STa-stimulated secretion might then become evident. Fluid uptake from recirculated loops was measured in anaesthetised (70 mg/kg i.p. Sagatal) unfasted Sprague-Dawley rats using recovered perfusate to assess fluid absorption. Twenty five cm loops of proximal jejunum were perfused with buffers in which either choline or mannitol replaced sodium ion. At the end of the experiment, the animals were humanely killed. In zero sodium ion perfusates containing choline ion, net fluid absorption was negative 12.1 ± 3.3(9) μl/cm/h, further reduced ($p<0.05$) to negative 29.2 ± 4.2(8) μl/cm/h by STa. As previously (1), to determine whether the small effect of STa in the nominal absence of sodium ion depended on persisting sodium ion uptake, 0.1 mM of the NHE-3 inhibitor EIPA (ethyl-iso-propyl-amiloride) was added. In the zero sodium ion perfusates with EIPA, fluid movement of –10.8 ± 3.8(8) μl/cm/h did not differ from –14.6 ± 6.2(6) μl/cm/h with STa. These experiments indicate that when fluid uptake is inhibited by inhibiting sodium ion uptake, there is no subsequently detectable effect of STa on fluid movement, attributable to secretion. Similar results were obtained when mannitol substitution was used. Similarly, extending previous work with bumetanide (2), the chloride channel blocker NPPB did not restore STa-inhibited fluid absorption since fluid transport was 22.3 ± 4.2(6) μl/cm/h after STa exposure and 34.1 ± 8.0(6) μl/cm/h with STa + NPPB, although both were lower than control values of 80.0 ± 14.0(6) μl/cm/h. These observations further challenge the concept of chloride-ion-dependent epithelial fluid secretion as a basis for enterotoxin action.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC65

Protection against ischaemia/reperfusion injuries by vasostatin-1 in the isolated rat heart

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Chromogranin A (CGA) is present in various endocrine glands and in the central nervous system (Helle, 2004). In human plasma CGA concentration is nanomolar and increases in the presence of neuroendocrine tumors and heart failure (Ceconi et al. 2002). The CGA N-terminal 1-76 peptide has been named vasostatin-1 (VS-1) because of the inhibition of vasomotor tone. In isolated eel, frog and rat hearts, physiological concentrations of VS-1 depress contractility and counteract the effect b-adrenergic stimulation (Tota et al. 2004). In rat heart Langendorff preparations perfused at constant flow, VS-1-depending negative inotropic effect is abolished by the blockade of b-adrenergic receptors, Gi/o protein and NO-cGMP-PKG pathway (Angelone et al. 2004). On the basis of the above findings, a possible role of VS-1 in limiting the extent of the necrosis induced by ischaemia and reperfusion (I/R) was studied in the present investigation.

Hearts excised from humanely killed Wistar rats were attached to a perfusion apparatus, perfused at constant flow with oxygenated Krebs-Henseleit buffer, paced at 280 b.p.m. and kept at 37°C. Left ventricular pressure (LVP) and aortic pressure (AP) were measured. In the control AP was about 90 mmHg for a coronary flow of 20 ml/min. Developed LVP was taken as an index of contractility. Infarct size was determined with the nitro-blue tetrazolium technique. The release of lactic dehydrogenase (LDH) was determined on samples of effluent fluid taken at regular intervals during reperfusion. The hearts were divided into four groups. Group I (n=5) was used as control. Before ischaemia, ischaemic preconditioning was performed in Group II (n=10) and VS-1 at 80 nM concentration for 19 min was given to Group III (n=12). Finally a 10 μM solution of the NO-synthase inhibitor L-N-nitroarginine (L-NNA) was infused into the hearts of Group IV (n=4), 5 min before, during and 5 min after the end of the infusion of VS-1.

In Group I, necrosis affected 66±16% of left ventricle and was reduced to 34±18% ($p<0.002$) in preconditioned Group II. In VS-1-pretreated Group III, infarction was limited to 32±10% of the ventricle ($p<0.001$ vs. Group I and n.s. vs. Group II). In Group IV the protection by VS-1 was completely suppressed by L-NNA. During reperfusion LVP recovery was proportional to the extent of protection. The release of LDH, measured to confirm the extent of the damage by I/R, was 1813±670 U.L. in Group I, 780±226 in Group III ($p<0.01$) and 1129±322 in Group IV (n.s.). The results show that VS-1 limits the damage caused by I/R and that the protective activity is mediated by NO signalling.

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The effects of insulin treatment on heart rhythm, body temperature and physical activity in streptozotocin-induced diabetic rat

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Defects in contraction and heart rhythm have been frequently observed in streptozotocin (STZ)-induced diabetic cardiomyopathy. A telemetry system (Data Sciences Int., USA) was used to monitor the effects of insulin treatment on heart rhythm, physical activity and body temperature in STZ-induced diabetic rats. The system comprised transmitter devices (TA11CTA-F40), receivers (RPC-1), a data exchange matrix (20CH) and a personal computer for system configuration, control and data storage. Transmitters were surgically implanted in 5 male Wistar rats (225-230g) under general anaesthesia (sodium pentobarbitone, 45 mg/kg i.p.). The transmitter devices were inserted in the peritoneal cavity and electrodes from the transmitter were arranged in Einthoven bipolar Lead II configuration. After recovery from surgery baseline data was collected for a period of 10 days. All 5 rats then received a single intraperitoneal injection of STZ (60 mg/kg bodyweight) dissolved in citrate buffer. Twenty days later rats received daily subcutaneous injections of Ultratard insulin (Novo Nordisk) for a period of 30 days; after completion of the experiments the rats were humanely killed. The dose of insulin ranged from 4-9 units per day dependent on levels of blood glucose. Biotelemetry data were recorded for 5 min of every hour, 24 h per day for the duration of the study. Statistical comparisons were made using paired t test and P values less than 0.05 were considered significant. Typical blood glucose values before STZ treatment were 63±1, and 20 days after STZ were 317±13 and during the period of insulin treatment were 64±13 and 165±12 mg/dl measured at 8 and 24 h after insulin, respectively. Heart rate (HR) during the baseline period was 361±7 b.p.m. and was significantly reduced after STZ treatment (266±12 b.p.m.). There was a significant recovery of HR towards pre-STZ levels during insulin treatment (303±14 b.p.m.). Heart rate variability (HRV) during the baseline period was 25±3 b.p.m., during STZ was 16±2 and was not significantly altered by insulin treatment (17±1 b.p.m.). Activity during the baseline period was 1.4±0.2 counts per minute (c.p.m.), after STZ was 0.8±0.2 c.p.m. and during insulin treatment was 0.9±0.1 c.p.m.. Body temperature during the baseline period was 37.5±0.1°C and was signi-

ficantly reduced after STZ treatment (37.2±0.2°C). There was a significant recovery of body temperature towards pre-STZ levels during insulin treatment (37.5±0.1°C). Treatment with insulin produced a significant recovery in HR and body temperature but had little effect on HRV and physical activity in STZ-induced diabetic rats.

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Antioxidant enzyme activity in cultured human arterial and venous endothelial cells

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In coronary artery bypass grafting (CABG), arterial grafts demonstrate superior long term patency rates than their venous counterparts. Evidence supports a role for oxidant stress in endothelial dysfunction and it is this dysfunction that precedes the development of atherosclerosis and, more relevant to bypass grafting, intimal hyperplasia. Our aim was to determine whether human arterial endothelial cells (ECs) have a greater ability to protect themselves from oxidant stress than venous graft ECs.
ECs were isolated from sections of human radial artery (RA) and human saphenous vein (SV) obtained surplus to CABG, with informed patient consent and ethical committee approval. Following culture as described previously (Conant et al. 2000), cells were treated with 20nM TNFα 24 hours prior to cell extraction and compared with untreated controls. The enzyme activities and total glutathione (GSH) were determined in cell lysates by established methods. Superoxide dismutase (SOD) was determined by the method of McCord & Fridovich (1969). Catalase activity was measured as described by Beers & Sizer (1952) and GSH levels as described by Tietze (1969). Protein levels were determined by MicroBCA (Pierce, USA). Data are presented as mean±s.e.m.
The activity of SOD (U/mg protein) was 3.2±0.6 in SVECs (n=6) and 3.9±1.3 in RAECs (n=4). Extracellular SOD, measured as the amount of activity removed by washing cells with excess heparin prior to isolation, was only measurable in RAECs. Cu/ZnSOD or MnSOD activity, measured as the component of activity sensitive or insensitive to 2mM NaCN, respectively, showed no significant difference between the two groups. In SVECs and RAECs TNFα increased total SOD activity 1.6±0.2 times and 1.5±0.1 times, respectively, solely by increases in MnSOD activity. Catalase activity (U/mg protein) was determined as 10.2±1.6 (n=5) in SVECs and 9.7±2.8 (n=4) in RAECs. GSH levels (nmoles/mg protein) were 21.4±4.7 in SVECs (n=5) and 11.1±5.8 (n=4) in RAECs. TNFα did not increase catalase activity or GSH levels in either cell type.
In conclusion, extracellular SOD was only found in measurable amounts in RAEC. Neither SVEC nor RAEC showed significant differences in either total SOD or catalase activity or GSH levels. TNFα stimulation increased only MnSOD activity. Extracellular SOD was previously thought to only be produced by vascular smooth muscle (Fukai et al. 2002). The production of extracellular SOD by arterial ECs may confer a first line of defense against extracellular oxidants not available to SVEC.

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