C01 and PC01

NAADP and two-pore channels are important for the contraction of airway smooth muscle cells

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Three second messengers that activate intracellular Ca^{2+} release are established so far: IP_3, cADPR, and NAADP. Of these, NAADP is the most recently identified and the most potent. It has been shown to act on acidic organelles, but there is also evidence that it might act on the RyR. We have recently identified a member of the two-pore channel (TPC) family as a major component of the NAADP-receptor complex, and as a NAADP-gated Ca^{2+}-channel (Calcraft et al., 2009; Pitt et al., 2010). A role for NAADP and TPCs has been shown to be important for Ca^{2+} release and/or contraction in various smooth muscle cells systems (Durlu-Kandilci et al., 2010, Aley et al., 2010, Kinnear et al., 2004). Since TPCs and NAADP-mediated Ca^{2+}-signalling has been shown to be of importance in smooth muscle cells, we were interested whether it could be involved in asthma.

Here we present data showing the intracellular localization of TPCs on the endolysosomal system using immunofluorescence staining (n=3). Using RT-PCR, we show the presence of TPC1 and TPC2 in mouse trachea.

To investigate whether TPCs and NAADP are important for the contraction of airway smooth muscle cells, we established dose-response curves to cholinergic stimulation using carbachol (CCh; 0.3 nM – 300 μM) in trachea from WT and TPC2 KO mice. In addition, we tested the effect of pre-incubation of the tissue with 10 μM Ned-19, a selective inhibitor of NAADP receptors. Pre-incubation with Ned-19 caused an increase in the EC_{50} of CCh-stimulated Ca^{2+} elevation from 0.3 ± 0.04 μM (n=3) to 1 ± 0.5 μM (n=4) in trachea from WT mice, and an increase from 0.2 ± 0.04 μM (n=4) to 0.5 ± 0.2 μM (n=3) in trachea from TPC2 KO mice. All values are given as mean ± S.E.M. Besides this change in the EC_{50}, we also found differences in the maximum contractility: trachea from TPC2 KO mice showed a significantly higher maximum contractility (11.9 ± 0.5 mN/mg tissue, n=4) compared to WT mice (5.2 ± 0.2 mN/mg, n=4; $p<0.0001$, T-test). Consistent with the finding in TPC2 KO mice, we also observed an increased maximum contractility after pre-incubation with Ned-19 in trachea from WT mice (9.1 ± 0.7 mN/mg, n=3).

These results indicate that Ca^{2+} release via NAADP and TPCs acts in an inhibitory manner on the contractile response of airway smooth muscle cells. One yet to be tested hypothesis to explain this inhibition is that NAADP causes localised Ca^{2+} release near the plasma membrane, thus activating BKCa channels and causing a hyperpolarisation of the plasma membrane. This hyperpolarization could in turn effectively dampen the CCh response. This inhibition is missing after pre-treatment

with Ned-19, or in the absence of TPC2, explaining the higher maximum contraction observed.

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Kinnear et al. (2004). J Biol Chem. **279 (52)**, 54319-54326

We thank Margarida Ruas for providing the TPC2 knockout mice. We also want to thank the Wellcome Trust and the Asthma Society UK for supporting this research.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C02 and PC02

Subcellular STIM1 re-localisation and Orai1-mediated calcium entry in vascular smooth muscle cells

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Activation of calcium-release activated calcium (CRAC) channels is suggested to require re-organisation and clustering of ER-localized STIM1 proteins prior to localisation with, and co-clustering of, plasma membrane Orai1 proteins, which form the CRAC channel pore. Vascular smooth muscle cells were cultured from discarded segments of human saphenous vein, which were obtained with ethical approval. Fluorescence was used to detect intracellular calcium and subcellular dynamics of eYFP-STIM1 and mCherry-Orai1. In the presence of physiological extracellular calcium, platelet-derived growth factor elicited transient followed by sustained elevations of intracellular calcium. As previously reported (Li et al 2011 Br J Pharmacol 164, 382-393), the sustained elevation of calcium was suppressed by Orai1 siRNA or a CRAC channel blocker, showing that CRAC channels were activated. Platelet-derived growth factor did not evoke clustering of eYFP-STIM1 or mCherry-Orai1 unless there was CRAC channel blockade, after which there was re-organisation and co-clustering of eYFP-STIM1 and mCherry-Orai1 in response to platelet-derived growth factor. In the absence of CRAC channel blockade, clustering was routinely observed in response to thapsigargin, the inhibitor of sarco-endoplasmic reticular calcium ATPase (SERCA). The data suggest that physiological STIM1 clustering is suppressed by calcium influx through Orai1 channels. Supported by the Wellcome Trust.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C03 and PC03

Airway smooth muscle contraction is regulated by Ca^{2+} oscillation frequency and Ca^{2+} sensitivity

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Airway hyper-responsiveness (AHR) is a characteristic of asthma and COPD and results from the excessive contraction of airway smooth muscle cells (ASMCs). The exact cause of this excessive contraction is unknown but may involve changes in ASMC Ca^{2+} signaling or ASMC sensitivity to Ca^{2+}. We have investigated the underlying mechanisms of Ca^{2+} signaling and Ca^{2+} sensitivity in order to better define mechanisms of AHR.

We have investigated these processes in ASMCs surrounding small airways in lung slices made from the lungs of mouse, rats and humans. Isolated lungs are inflated with warm liquid agarose. When solidified by cooling, the agarose stiffens the lung tissue to allow slices, 250 um thick, to be cut. These slices are examined by phase-contrast microscopy to follow airway contraction as well as by confocal microscopy to observe ASMC Ca^{2+} signaling.

We have found that methylcholine (from 100 – 1000 nM) induces Ca^{2+} oscillations in ASMC with frequencies ranging from 8 min-1 in humans to 20 min-1 in mice (at 1000 nM). In all cases, the extent of airway contraction increased in proportion to the increase the frequency of the Ca^{2+} oscillations. In human lung tissue, histamine induced similar changes (500 nm histamine induce a decrease in area to 63.98% +/- 7.88 and Ca^{2+} oscillations with a frequency of 5.73+/- 0.66 per min). These Ca^{2+} oscillations appeared to be primarily mediated by inositol-trisphosphate receptors (IP3Rs) because the addition of ryanodine, an antagonist of the ryanodine receptor, had no effect on agonist-induced Ca^{2+} oscillation frequency.

Interestingly, the relationship of airway contraction with respect to the Ca^{2+} oscillation frequency was different between the 3 species. We developed an assay to evaluate Ca^{2+} sensitivity of ASMCs using caffeine and ryanodine to empty Ca^{2+} internal store and “clamp” internal Ca^{2+}. With this preparation, we found that mouse ASMCs have a low Ca^{2+} sensitivity as compared to rat and human airways. In conclusion, ASMC contraction is a function of the frequency of Ca^{2+} oscillations, driven by the IP3R, and the level of Ca^{2+} sensitivity.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C04 and PC04

Mechanisms of O_2 sensing and Ca^{2+} homeostasis during hypoxic pulmonary vasoconstriction in pulmonary arteries from rats

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Hypoxic pulmonary vasoconstriction (HPV), the process by which hypoxia constricts pulmonary arteries (PA), requires that PA smooth muscle cells contain both oxygen sensing and downstream effector mechanisms causing contraction. Here, we characterised the roles of the electron transport chain and NADPH oxidase in oxygen sensing, and of effector mechanisms involving Ca^{2+} release. HPV in isolated PA and perfused lung is of low amplitude, and is therefore generally studied in the presence of agonist-induced prestimulation ('pretone'). This approach magnifies the response, making it easier to study, but may also distort its properties. We therefore recorded HPV in the absence of pretone in second and third order PA branches from young (~250 g) Wistar rats, using conventional myography. Arteries were exposed to severe hypoxia (95% N_2, 5% CO_2) over three 40 minute periods, each separated by ~30 min, with the second and third HPVs being used as the control and intervention responses, respectively (*eg.* drugs were applied 20 min before and during the third response, which was compared to the second using paired T-tests). HPV typically exhibited a transient peak (phase 1), after which tension fell slightly and then rose gradually to a plateau (phase 2). Phase 1 was abolished but phase 2 was unaffected by pretreatment with the mitochondrial complex I inhibitor rotenone (1 μM, n = 7), whereas both phases of HPV were abolished by the complex IV inhibitor cyanide (10 μM, n = 5). Pretreatment of PA with superoxide dismutase (200 units/ml), catalase (200 units/ml), the combination of these enzymes, or with the putative NADPH oxidase inhibitor VAS 2870 (10 μM) had no effect on either phase of HPV (n = 7,7,10 and 8, respectively). Conversely, the superoxide scavenger TEMPOL (3 mM) reduced phase 1 from 0.48 ± 0.10 mN to 0.28 ± 0.06 mN ($p < 0.05$) and abolished phase 2 HPV (n=5). The putative two pore channel antagonist NED-19 had no effect on either phase of HPV at concentrations of either 1 or 10 μM (n = 12 and 5, respectively). Pretreatment with 100 μM ryanodine to block the ryanodine receptor reduced phase 1 from 0.51 ± 0.07 mN to 0.16 ± 0.07 mN, and phase 2 from 0.56 mN ± 0.15 mN to 0.20 ± 0.08 mN (n=6, $p<0.05$ for both phases), whereas pretreatment with a combination of ryanodine (10 μM) and caffeine (10 mM) to deplete SR Ca^{2+} abolished both phases. Application of Ca^{2+} -free solution containing 200 μM EGTA ablated phase 1 HPV but had no significant effect on phase 2, and re-addition of Ca^{2+} to the solution during phase 2 did not increase contraction. These data indicate that the oxygen sensing mechanisms for phase 1 and 2 may differ, but in neither case involve NADPH oxidase. Phase 2 HPV seems to be fully accounted for by the release of Ca^{2+} from the SR.

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C05 and PC05

The effect of hypoxia and perivascular fat on potassium channels in mice mesenteric arteries

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Previously studies demonstrated that perivascular adipose tissue (PVAT) releases adipoycyte-derived relaxing factors (ADRF) which reduces vascular contractility (1). The study explores the potential influences of hypoxia on PVAT and potassium channels in isolated mice mesenteric arteries.

Male and female, $^{-/-}BK\beta1$ (large-conductance Ca^{2+} activated potassium (BK_{Ca}) channel knockout), C57BL/6 (Wild type) mice (12-18 week old ~25g weight) were killed by stunning and cervical dislocation. Mesenteric arteries with or without PVAT were mounted on a wire myograph for isometric tension recording. Cumulative concentration responses (10^{-9}-10^{-5}M) to norepinephrine (NE) were performed before and after 2.5 hours hypoxia (95%N_2, 5%CO_2), in the presence or absence of ATP-sensitive potassium channel (K_{ATP}) blocker glibenclamide (10μM). Responses are expressed as mean (±SEM) % of KPSS constriction and analysed using 2-way ANOVA.

NE produced a concentration dependent constriction of arteries. PVAT significantly ($P<0.01$) reduced this constriction in wild-type vessels (Figure A). Hypoxia significantly enhanced ($P<0.001$) vasorelaxation effect of adipose tissue (n=4, 54.4±11.4%vs21.1±8.9%), while no vasorelaxation effect was observed in the arteries without adipose tissue (n=4, 85.3±12.4%vs81.3±18.5%). In the $^{-/-}BK\beta1$ group hypoxia had no significant effect on the contractility between the non-PVAT and PVAT vessels (n=5, 58.23±11.5%vs46.85±22.5%).

Glibenclamide significantly ($p<0.01$) impaired the hypoxic vasorelaxation effect of PVAT in wild-type arteries (n=4, 72.1±21.3%vs138.0±55.4%). Glibenclamide does not affect the contractile responses of vessels in normoxic conditions (n=6, 84.0±26.1%vs51.4±15.2%). In arteries from $^{-/-}BK\beta1$ mice, glibenclamide does not significantly affect the contractile response of PVAT vessels to NE compared with the corresponding control responses irrespective of hypoxic (n=4, 46.9±22.5%vs33.6±13.7%) and normoxic (n=5, 37.7±11.6%vs40.3±16.0%) conditions.

Our findings indicate that hypoxia has a vasorelaxation effect on mice mesenteric arteries and it is dependent on the presence of intact PVAT. In addition, activation of BK_{Ca} and K_{ATP} channels plays an important role in this response.

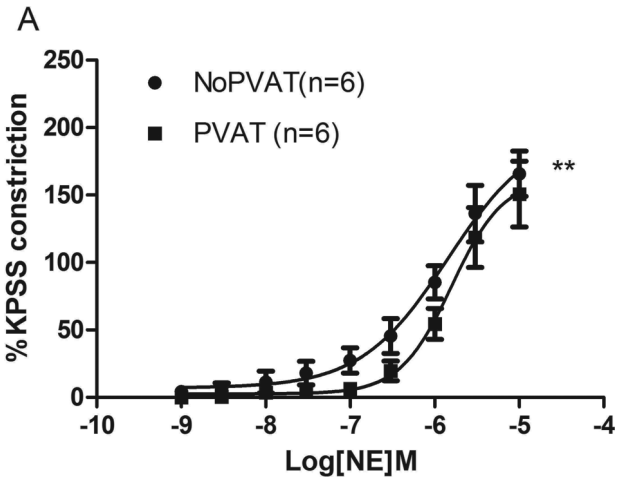

Figure A. Response of mesenteric arteries from C57BL/6 mice in presence and absence of PVAT to cumulative concentration of NE. Healthy fat caused a reduction in contractility to NE compared with arteries without fat (** P<0.01).

Lohn, M., G. Dubrovska, et al. (2002). FASEB J 16(9): 1057-1063.

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C06 and PC06

Ablation and acute inhibition of Plasma Membrane Calcium ATPase 4 (PMCA4) with a novel inhibitor have different effects on isolated mouse mesenteric resistance arterial contractility

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PMCA4 is a calcium extrusion pump which may also modulate Ca2+ -triggered signal transduction pathways. Previous studies postulate that PMCA4 modulates signalling via an interaction with neuronal nitric oxide synthase (nNOS) in localised plasmalemmal microdomains [1,2,3]. Although PMCA4 is the most abundant isoform in vascular smooth muscle its role is somewhat unclear [1,2]. Here, PMCA4 ablated mice (PMCA4KO) and a novel specific PMCA4 inhibitor (termed AP2) [4] were used to study the role of PMCA4 in resistance artery contractility.
Contractility of isolated, pressurised (60mmHg), 3rd order mesenteric arteries from male PMCA4KO and wild type, (WT) mice in response to 100mM K+ depolarisation & noradrenaline (NA) (Log[NA] -9.0 to-5.0M) were determined. Passive lumen diameter and left & right wall thicknesses of arteries from PMAC4KO and WT mice were

taken at transmural pressures of 5-140mmHg. Effects of acute PMCA4 inhibition with AP2 (10μM), nitric oxide synthase (NOS) inhibition with LNNA (100μM) & specific nNOS inhibition with Vinyl-L-Nio (10μM) were also investigated. Change in diameter is expressed as % of resting diameter. Data is expressed as mean+SEM.
PMCA4 ablation had no effect on the magnitude of constrictions to 100mM K+ (PMCA4KO 53.2 ± 3.5% (n=11), WT 56.3 ± 3.6% (n=14)) or to noradrenaline (maximum constriction: PMCA4KO 59.5 ± 4.7% (n=11), WT 58.2 ± 2.5% (n=14)). Ablation of PMCA4 did not modulate the passive intra-lumen diameter, wall thickness, wall to lumen diameter or cross sectional area (CSA) of mesenteric arteries across the intravascular pressure range studied. A leftwards shift in the stress to strain relationship and significantly increased beta elastic modulus (β) were exhibited by arteries from PMCA4 KO mice compared to those from WT mice (β= PMCA4KO 10.03 ± 2.0 (n=19), WT 5.75 ± 0.64 (n=21)) suggesting ablation of PMCA4 increases mesenteric arterial stiffness.
Acute PMCA4 inhibition with AP2, significantly reduced constriction to noradrenaline in arteries from wild type mice (maximum constriction: controls 70.6 ± 3.4% (n=8) and 63.8 ± 2.6% (n=10) in the absence and presence of AP2 respectively) but had no effect on arteries from PMCA4KO mice. Inhibitory effects of AP2 were reversed in arteries by NOS inhibition after LNNA incubation (AP2 61.8 ± 4.0% (n=8) and AP2+LNNA 69.2 ± 1.7% (n=10)) and also by nNOS inhibition with Vinly-L-Nio (AP2 61.8 ± 4.0% (n=8) and AP2+Vinyl-L-Nio 75.2 ± 1.3% (n=5)). Hence, PMCA4 inhibition with AP2 reduces vascular constriction by a nNOS-dependent mechanism. In conclusion, ablation and acute inhibition of PMCA4 with AP2 have different effects on mouse mesenteric resistance arterial contractility owing to the interaction with nNOS in localised plasmalemmal microdomains.

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To the BHF and University of Manchester

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C07 and PC07

Adiponectin induces perivascular adipose tissue anticontractility in mouse mesenteric small arteries via activation of BK_{Ca} channels

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Perivascular adipose tissue (PVAT) exerts an anticontractile effect on vascular tone which may provide protection against the development of hypertension. The factors responsible for this effect remain to be identified in this vascular bed. Adiponectin has been identified as responsible for anticontractility in human vessels, therefore the aim of this study was to determine if and how it is acting in the mouse mesenteric bed[1].

Male and female, C57BL/6 (Wild type (WT)), BK$\beta1^{-/-}$ mice, $Slo^{+/+}$ (WT) and $slo^{-/-}$ mice, (12-18 week old ~25g weight) were killed by stunning and cervical dislocation. Mesenteric arteries were mounted on a wire myograph. Cumulative concentration responses (10^{-9}–10^{-5}M) to norepinephrine (NE) were performed. Solution transfer experiments were performed. Solution was transferred from baths containing preconstricted (10^{-5}M NE) WT PVAT arteries to WT and KO arteries lacking PVAT. In a second series of experiments cumulative concentration responses NE were performed before and after 45 minute incubation with adiponectin blocking peptide for PAb to adiponectin Receptor 1 (BPR1) (5μg/mL) (Enzo Life Sciences). Transfer of bath solution from preconstricted PVAT arteries to arteries without PVAT was also performed. Responses are expressed as mean (±SEM)% of KPSS constriction for cumulative concentration response curves and analysed using 2-way ANOVA or as mean change in tension(±SEM) and analysed using student's t-test for transfer experiments. Responses to exogenous adiponectin (3μg/mL) were also assessed. Transfer of bath solution from preconstricted WT PVAT arteries to preconstricted (0.65±0.1mN/mm) WT arteries -PVAT (n=6) significantly reduced ($P<0.05$) tension (0.43±01mN/mm). However, transfer of solution from preconstricted WT PVAT arteries (n=6) to preconstricted (0.48±0.2mN/mm) BK$\beta1^{-/-}$ arteries -PVAT did not significantly alter tension (0.56±0.3mN/mm). Transferral of solution from preconstricted WT PVAT arteries to preconstricted (0.25±0.1mN/mm) $Slo^{+/+}$ arteries -PVAT (n=6) did not significantly alter tension (0.26±0.1mN/mm). PVAT (n=12) significantly ($P<0.05$) reduced tension (126.6±14.5%) produced by norepinephrine (10-6M) compared to that produced (98.4±7.7%) in the absence of PVAT (n=11). The presence of BPR1 abolished the anticontractile effect of PVAT; tension produced (113.6±12.9%) by PVAT arteries (n=8) was not significantly different from that produced (127.1±21.3%) by arteries without PVAT (n=5). Adiponectin produced significant ($P<0.05$) vasodilatation of preconstricted arteries (12.3±4.6%).

These data suggest that adiponectin is the mediator of PVAT induced anticontractility and that it acts via AdipoR1. This acts via the BKCa channel.

Greenstein AS, et al (2009). Circulation. 119(12)1661-1670.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C08 and PC08

Differential effects of cupric ion on TRPC and TRPM2 cation channels

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Copper is an essential element required for normal physiological function of human body. Disruption of copper homeostasis has been associated with the development of cardiovascular diseases. As a redox-active metal, the over-accumulation or deficiency of copper in the body may lead to increased oxidative stress and cause dysfunction of many copper-dependent enzymes. Although a number of copper-binding proteins have been recognised, how this metal regulates the cardiovascular function is still largely unknown.

Here we tested the effects of cupric ion (Cu^{2+}) on the canonical (TRPC) and melastatin transient receptor potential 2 (TRPM2) channels. Patch-clamp technique was used to record the currents in HEK293 cells overexpressing TRPC3/4/5/6 and TRPM2 channels. We found that Cu^{2+} potently activated TRPC4 and TRPC5 channels at micromolar level, but had no stimulatory effect on TRPC3, TRPC6 and TRPM2 channels. In contrast, Cu^{2+} showed dramatic inhibition on TRPM2 channels activated by intracellular ADP-ribose. The microdomains responsible for the activation of TRPC4/5 or inhibition of TRPM2 channels by Cu^{2+} were found to be localised at the cell surface, since intracellular application of Cu^{2+} had no influence on the channel activities. However, the mechanism for the stimulation of TRPC4/5 channels by Cu^{2+} seems different from that by lanthanides, because the TRPC4 mutant with negatively charged glutamic acid residues replaced by neutral glutamines in the outer pore region can still be activated by Cu^{2+}, but not by gadolinium (Gd^{3+}).

We conclude that copper plays an important role in cellular Ca^{2+} signaling by targeting to multiple cation channels and differentially regulating their activities.

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C09 and PC09

Hormonal state changes the mRNA profile of calcium signaling proteins in uterine smooth muscle

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Calcium plays a key role in the physiology of uterine smooth muscle. Transmembrane calcium fluxes contribute to action potential shape and intracellular calcium mediates excitation contraction coupling. The electrical activity of the uterus is significantly altered throughout the estrous cycle, as well as in pregnancy. Uterine muscle is contractile, but the degree of coordinated contraction changes significantly during the estrous cycle and pregnancy. Changes in uterine contraction reflect changes in coordination of excitation due to electrical connections through gap junctions and excitatory currents, but other cellular changes may also occur during the course of pregnancy. For many ion channels, mRNA expression is altered by hormonal regulation. Therefore, the molecular basis of excitation-contraction coupling is also likely to be significantly altered depending on the state of uterine muscle. To test the hypothesis that there is substantial remodeling of cellular uterine excitation contraction coupling during different stages of estrous and during pregnancy, we undertook quantitative analysis of mRNA expression of calcium channels and other channels and related genes which affect calcium dynamics, during the estrous cycle and pregnancy.
Uteri were removed from anaesthetized (inhaled isoflurane) mice (C57BL) which were regularly cycling (n=15), ovariectomized (OVX, n=10), or pregnant (not in labor, n=10). Mice were euthanized by cervical dislocation following removal of organs. mRNA was extracted (Trizol, Invitrogen and RNAeasy, Qiagen) and converted to cDNA (First strand synthesis, SABiosicences). We used quantitative real time PCR (SYBR Green in an iQ icycler, Biorad) to determine changes in relative mRNA expression. mRNA Expression was normalized to 5 housekeeping genes (beta Glucuronidase, beta, Hypoxanthine guanine phosphoribosyl transferase 1, Heat shock protein 90 alpha (cytosolic), class B member 1, Glyceraldehyde-3-phosphate dehydrogenase, and beta actin). Data were analyzed using ANOVA ($p<0.05$ was regarded as significant).
There were significant changes in relative mRNA expression including Cacna1a (P/Q type, alpha 1A subunit, Cav2.1), Cacna1c (L type, alpha 1C subunit, Cav1.2), Cacna1g (T type, alpha 1G subunit, Cav3.1d), Cacna1h (T type, alpha 1H subunit, Cav3.2). There were also significant differences in the mRNA expression of the ancillary subunits cacnb1 (beta 1 subunit) and Cacnb4 (beta 4 subunit). Cacna1c, Cacna1g, Cacna1h all had lowest expression in pregnancy (not in labor), which is consistent with reduced excitation-contraction during this period. Conversely, inward rectifier currents, which can suppress excitation, were upregulated in pregnancy.

Our observed changes in gene expression suggest that down regulation of calcium channels and up regulation of inward rectifier potassium channels help maintain uterine smooth muscle in a relaxed state in pregnancy prior to the onset of labor.

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C10 and PC10

Effect of lipoxin A4 on acute inflammation in primary equine airway smooth muscle cells

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INTRODUCTION Recurrent airway obstruction (RAO) is a condition that may occur in mature horses and has similarities to human asthma. Permanent airway remodelling occurs in long term cases. Airway remodelling features an increase in airway smooth muscle (ASM) mass. Chronic respiratory inflammation has been postulated as a factor that contributed to such remodelling. Airway inflammation in both RAO and human asthma features an increase in the expression of Toll-like receptor 4, the inducible enzymes inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2), and the release of mediators including tumour necrosis factor alpha (TNF-α) and interleukin-1 β (IL-1β).Lipoxin A4 (LXA4) is one of a group of arachidonic acid metabolites that modulate acute inflammation through action on specific lipoxin receptor (ALX) in a number of species. LXA4 has been shown to have anti-inflammatory properties in a number of models of acute inflammation. Dysfunction of the mechanisms that result in LXA4 synthesis and expression of the ALX receptor may contribute to the chronic inflammatory response seen in asthma. Abnormal LXA4 and/or ALX expression may also be present in horses with RAO thereby promoting ASM remodelling. AIMS OF THE STUDY To investigate the effect of endogenous LXA4 on acute LPS-induced inflammation in primary equine ASM cells. METHODS Primary ASM cells were cultured from explants of equine trachealis muscle. Primary cells were identified by positive staining for α-smooth muscle actin. Near-confluent ASM cells were treated with:100μM/ml endogenous LXA4, 0.1μg/ml lipolysaccharide (LPS) or a combination of LXA4/LPS. Cells were harvested after 24 and 72 hrs. Real-time PCR quantified mRNA expression of β-actin (reference gene),ALX, TLR-4, TNF- α, IL-1β, iNOS and COX-2. Protein expression of COX-2 and ALX were additionally examined using Western Blot.RESULTS After 24hrs, expression of mRNA coding for ALX peaked in the LXA4 treated cells but was decreased in other treatment groups. Expression of mRNA coding for iNOS mRNA was also upregulated in the LXA4/LPS treated cells at 24hrs. At 72hrs post treatment, mRNA for TLR-4, TNF-α, IL-1β and COX-2 was increased in LPS-treated cells. Incubation with LXA4 decreased mRNA expression for TLR-4, TNF-α, IL-1β, iNOS and COX-2 at 72hrs.

Similarly, reduced expression of mRNA for TNF-α, IL-1β, iNOS and COX-2 was evident in LXA4/LPS treated cells at 72hrs. Western Blotting showed that protein for ALX was present at 24 and 72hrs in control and treated cells. COX-2 was not expressed at 72hrs in the LXA4 and LXA4/LPS treated cells. CONCLUSION These findings suggest that treatment of equine ASM cells with endogenous LXA4 causes a decrease in LPS-induced release of inflammatory mediators after 72hrs of incubation. However, an increase in expression of TLR-4 mRNA at 72hrs indicates that this effect may not be sustained at later time points

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C11 and PC11

The role of cGMP dependent protein kinase (PKG) in mediating the anticontractile function of perivascular fat

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INTRODUCTION: Obesity leads to a loss of adiponectin-mediated anticontractile function of perivascular fat (PVAT), thereby providing a link between obesity and subsequent hypertension. cGMP dependent protein kinase 1 (PKG) regulates smooth muscle relaxation and may be important in this vascular pathology, as high fat feeding is associated with downregulation of its expression in mice; furthermore overexpression of constitutively active PKG protects female mice from the effects of diet induced obesity. We aim to investigate the role of PKG in mediating the anticontractile property of PVAT.
METHODS: PKG knockout mice (KO) and wildtype littermates (WT) were investigated for contractility and adiponectin expression. Arteries +/- PVAT were studied by wire myography under normoxia and experimental hypoxia +/- ANP (100nM). Contractile responses to noradrenaline (NA) were calculated as a percentage of KCl constriction. Solution transfer experiments were performed on arteries constricted with 10-5M NA, change in tension was measured following transfer to determine the role of PKG in the release of the anticontractile factor(s) or in mediating the downstream effects. Adiponectin expression in mesenteric fat samples was assessed using immunohistochemistry. Data are expressed as mean ± SEM and analysed by 2-way ANOVA or student's t-test where appropriate. $P<0.05$ was deemed significant.
RESULTS: PVAT conferred an anticontractile effect on WT arteries in normoxic conditions (n=7, $P<0.001$). PKG KO arteries + PVAT demonstrated increased contractility compared with WT littermates (n=5, $P<0.001$). Hypoxia increased contractility of WT arteries + PVAT alone but not KO arteries (WT normoxia vs. WT hypoxia: n=5, $P<0.05$; KO normoxia vs. KO hypoxia: n=5, P=NS). Incubation of arteries with ANP during hypoxia rescued the loss of PVAT function in WT arteries only (n=4, $P<0.05$) and was associated with an increase in adiponectin expression, although

no effect was observed in arteries from KO mice (P=NS, n=4). Transfer of solution from WT + PVAT arteries to KO – PVAT and KO + PVAT to WT – PVAT caused a relaxation (Δ tension 0.21±0.03mN/mm and 0.17±0.03mM/mm respectively, n=4), although significantly less than that observed between WT + PVAT to WT – PVAT arteries (0.3±mN/mm, n=4).
CONCLUSION: PKG mediates the effects of healthy PVAT and is involved in the release and mediation of the downstream effect of factor(s) secreted by PVAT. ANP restores the anticontractile capacity of PVAT which is compromised following experimental hypoxia; this is mediated, at least in part, through a PKG dependent pathway as ANP does not have the same rescuing capacity in the absence of PKG.
Furthermore, ANP increases the expression of adiponectin in these samples. The regulation of adiponectin by PKG remains to be fully understood.

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C12 and PC12

Zinc deficiency results in apoptosis of vascular smooth muscle cells *in vivo*

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Dietary zinc deficiency, which affects one third of the world's population, has been associated with a role in the development of cardiovascular diseases although the causes of this are unclear. The aim of this study is to elucidate the effect of a zinc deficient environment on vascular smooth muscle cell (VSMC) function. Male Lister hooded rats (8 weeks old) were maintained for 2 weeks on either zinc adequate diets (35 mg/kg/day) or zinc deficient diets (3 mg/kg/day). Plasma was obtained intravenously following terminal anaesthesia with sodium pentobarbital. There was a significant reduction in plasma zinc levels in rats fed a zinc deficient diet compared to the zinc adequate diet as assessed by atomic absorption spectrophotometry (35 mg/kg/day- 158 ± 4 μg/ml, 3 mg/kg/day- 80 ± 8 μg/ml, n=7, mean ± s.e.m., ANOVA $p<0.05$). Aortae were dissected from zinc adequate and zinc deficient rats and a Tunel assay to detect apoptosis was performed on fixed sections. There was a significant increase in the number apoptotic VSMC in aortic sections from zinc deficient rats compared to the zinc adequate rats (35 mg/kg/day- 3 ± 2 apoptotic cells; 3 mg/kg/day- 39±3 apoptotic cells, n=3, $p<0.05$). A protein closely associated with apoptosis is the Bcl-2-associated death promoter (BAD) and over expression of this protein can lead to apoptosis. BAD was over-expressed in the VSMC of aorta from rats on a 2 week zinc deficient diet compared to a zinc adequate diet (n=3 individual aortae) as assessed by immunolocalization with confocal microscopy. In parallel *in vitro* experiments, primary cultured rat aortic VSMC were maintained for 24 hours in 10% plasma recovered from rats on either a 2 week

zinc adequate or 2 week zinc deficient diet. Similar to *in vivo* experiments, there was a significant increase in the number of apoptotic cells in cultured aortic VSMC maintained in plasma from zinc deficient rats compared to the control (35 mg/kg/day plasma- 2 ± 1 apoptotic cells, 3 mg/kg/day plasma- 32 ± 9 apoptotic cells, n=3, $p<0.05$). Similar to aorta *in vivo*, over-expression of BAD was observed *in vitro* on cultured rat primary VSMC treated with zinc deficient plasma (n=3). In further experiments, cultured aortic VSMC were incubated for 24 hours in medium with 10% foetal bovine serum (FBS) which had reduced zinc (zinc concentration 1 μM) compared to normal FBS (Zinc 12 μM). Cells incubated with zinc-reduced FBS revealed similar levels of apoptosis and BAD expression compared to cells incubated in normal FBS.
In conclusion, a zinc deficient diet *in vivo* induces apoptosis in VSMC from blood vessels probably partly via an increase in the pro-apoptotic protein BAD. This pro-apoptotic effect can be induced by the plasma from zinc-deficient animals however is not a direct effect of the zinc deficiency itself. Such mechanisms may be important in the development of cardiovascular disease in dietary zinc deficiency.

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C13 and PC13

Arteriovenous fistula failure: vascular smooth muscle cell proliferation and the role of inflammation

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An arteriovenous fistula (AVF) is a vein graft which is created to permit access to the bloodstream allowing haemodialysis to be performed in renal failure patients. An AVF is considered the best option in clinical practice; however a significant failure rate is still reported, with as little as 50% remaining patent at 6 months (Field et al., 2008). Failure of these grafts is largely due to vascular smooth muscle cell (VSMC) hyper-proliferation, leading to the development of neointima which invades the lumen causing stenosis and impaired blood flow (Lee and Roy-Chaudhury, 2009). Inflammation is known to play a key role in the development of vascular stenosis; although the exact mechanisms and triggers are not fully known. The aims of this study were to 1) evaluate the changes undergone in VSMC present within human failed AVF vs. non-stenosed controls, and 2) investigate the inflammatory processes upregulated in human failed AVF vs. non-stenosed controls.
Histology of failed human AVF stained with H & E confirmed a significant increase in media: lumen ratio increasing from 2.2 ± 0.6 in control veins to 18.8 ± 6.9 in AVF vein sections ($p<0.005$, Control vs. AVF; n=8), with the majority of these cells pos-

itive for α-SMA. Toluidine blue staining of the vein wall for mast cell infiltration highlighted a 5 fold increase in mast cells within AVF vein tissues ($p<0.005$ Control vs. AVF; n=9, 7), with VCAM-1 also found to be expressed within the vein wall by immunohistochemistry (IHC). The percentage of cells within the stenosed vein segments undergoing proliferation, as measured by expression of PCNA using IHC, was 33.6 ± 4.9% in the AVF vs. 3.5 ± 1.5% in the control ($p<0.005$ AVF vs. Control; n=5, 8). VSMC were explanted from both groups of patients, and cell proliferative capacity was measured by 3H thymidine incorporation. At maximal stimulation with 10% FCS the fold increase in thymidine uptake within the AVF-derived cells was 32 times that of the non-stimulated cells, compared to a 15 fold increase within the control-derived cells ($p<0.005$ AVF vs. Control; n=4). The cell cycles of these explants were also investigated using propidium iodide and flow cytometry. AVF-derived explants showed a trend towards an increase in the number of cells undergoing the G2/M stages of cell cycle.
These results highlight significant venous hypertrophic remodelling in human AVF-vein sections. The remodelling can be largely attributed to vascular smooth muscle cell accumulation with inflammatory cell infiltrates. In VSMC studies we have demonstrated that cells derived from the AVF have an increased capacity to proliferate.

Field, M. et al. (2008). J. Vasc. Access 9, 45-50.

Lee, T. & Roy-Chaudhury, P. (2009). Adv. Chronic Kidney Dis. 16, 329-338.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C14 and PC14

Vein graft neointima formation: chronic recruitment of circulating cells by dysfunctional endothelium in addition to smooth muscle cell proliferation

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Background: Neointima hyperplasia has generally been considered to originate primarily from smooth muscle cell proliferation, and is the most important pathology in development of vein graft occlusion as well as other types of vascular disease. However, the dynamics of neointimal cell proliferation and vascular remodelling is not fully understood.
Methods: To perform the vein graft surgery, a donor mouse was sacrificed by CO_2 and the thoracic inferior vena cava harvested. The recipient mouse was anaesthetised by intra-peritoneal injection of 60mg/kg sodium pentobarbital. The vena cava from the donor mousewas then inserted into the common carotid artery of the recipient mouse. Buprenorphine (0.1mg/kg) was given by subcutaneous injection right after the surgery. 4 weeks after implantation, the recipient mouse was sacrificed by CO_2. The vein graft was harvested and fixed with 4% formaldehyde for morphology or immunohistochemistry.

Results: Neointima thickening developed progressively over 4 weeks following vein grafting. The smooth cell markers alpha-actin and smooth muscle myosin heavy chain were exclusively expressed in the neointima by 4 weeks; however cell proliferation (stained by anti-PCNA antibody) was observed throughout the whole vein graft wall (neoadventitia plus neointima). Interestingly, the most proliferative region was usually co-localised with the most inflamed neointimal or neoadventitial area, with less or no smooth muscle markers expression. Proliferating cells negative for smooth muscle cell markers were also observed located peripherally to mature neointima, which may contribute to the second wave of neointima thickening. Leukocytes or monocular cells originated from the blood stream were often observed rolling or firmly adherent to the regenerated endothelium. The neointima are usually less developed but more inflamed in the middle part of the vein graft, compared to the anastomosis area. The poorly developed neointimal area is often associated with poor endothelial regeneration and severe adventitial inflammation, which were of very high cell density with intensive proliferation and cell death.
Summary: Our observations were consistent with published reports that a large portion of the neoinitma cells may originated from the circulation. The non-smooth muscle cell recruitment and proliferation may contribute to the vein graft chronic inflammation and neointima thickening even at a chronic remodelling stage. The inflamed neointimal area and regenerated dysfunctional/immature endothelium may attract continuous cell recruitment leading to accelerated atherosclerosis.

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C15 and PC15

Effects of Kv7 potassium channel modulators on human intra-pulmonary artery

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Pulmonary arterial hypertension (PAH) is a progressive disease with increasingly debilitating symptoms and survival of only a few years (McLaughlin et al, 2004). Kv7 potassium channels were recently implicated in the regulation of membrane potential in rodent pulmonary artery smooth muscle cells (PASMC) and pulmonary artery constriction (Joshi et al 2006; Joshi et al 2009. Presumably as a consequence of this role, the Kv7 channel opener flupirtine was beneficial in two independent mouse models of PAH (Morecroft et al, 2009). To determine if Kv7 channel activation could be beneficial in humans, we have now investigated the effects of Kv7 modulators on distal pulmonary arteries isolated from human lung samples.
Intra-pulmonary arteries (iPA, <1mm diameter) were dissected from normal lung tissue collected during tumour resection from lung cancer patients. RT-PCR was performed on RNA isolated from iPAs from separate patients, using primers directed against each of the five KCNQ genes that encode Kv7 channels. RT-PCR products

were confirmed by sequencing. Myography experiments were conducted to measure the effects on isometric tension of the Kv7 blockers, linopirdine and XE991, and the activators, retigabine and flupirtine. pEC50 values were measured by fitting the Hill equation to concentration-effect data obtained for each vessel. All data is expressed as the mean ± S.E.M with concentrations in molar unless stated. The dependence of constrictor effects on voltage-gated calcium influx was investigated using the L-type calcium channel blocker, nifedipine.

RT-PCR detected KCNQ subunits 1,3, 4 and 5, although there was variability among individual patients. The Kv7 blockers produced concentration-dependent constriction of human iPAs, with pEC50 values of 6.3 ± 0.3 (N=9) for linopirdine and 5.3 ± 0.2 (N=13) for XE991. The Kv7 openers flupirtine and retigabine both produced concentration-dependent dilation of agonist pre-constricted vessels, with pEC50 values of 4.5 ± 0.4 (N=4) and 5.3 ± 0.2 (N=4), respectively. Preliminary experiments show that 100nM nifedipine suppressed the vasoconstrictor actions of 1μM XE991 and 5μM linopirdine.

We conclude that some Kv7 channel subunits are expressed at the mRNA level in human iPAs. Functional Kv7 channels are likely to be expressed in human PASMCs, because known modulators of Kv7 channel activity were able to alter the tone of the vessels in the predicted manner and constrictor effects depended on voltage-gated calcium entry into PASMC. The data therefore supports the idea of Kv7 channels as a drug target in the treatment of PAH.

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Morecroft, I., Murray, A., Nilsen, M., Gurney, A M & MacLean, M.R. (2009) Treatment with the Kv7 potassium channel activator flupirtine is beneficial in two independent mouse models of pulmonary hypertension. Brit J Pharm 157: 1241-9.

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C16 and PC16

Impaired vascular Kv7 function in animal models of hypertension

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Background: Voltage-gated potassium (K+) channels encoded by KCNQ genes (Kv7 channels) are present functionally in vascular and non-vascular smooth muscle cells (Yeung et al., 2007; Greenwood and Ohya, 2009). However, nothing is known about the functional impact of these channels in vascular disease. The aim of the present study was to compare the effect of structurally different activators of Kv7.2-7.5 channels: S-1, retigabine and BMS-204352, on blood vessels from normotensive and hypertensive animals.

Methods and Results: Isometric tension recordings were performed on segments of mesenteric artery and thoracic aorta and the coronary blood flow was studied using the Langendorff heart preparation, in which rats were anaesthetised with i.p. injection of 50 mg/kg sodium pentobarbitone according to the Danish guidelines for animal experiments. Blood vessel segments from normotensive rats were relaxed by all three Kv7 activators with potencies of BMS-204352= S-1 >retigabine. In the Landendorff isolated-heart BMS-204352 and S-1 dose-dependently increased coronary perfusion at concentrations between 0.1-10 μM whereas retigabine was effective at 1-10 μM. The ability of these agents to relax precontracted vessels and increase coronary flow was considerably impaired in tissues isolated from spontaneously hypertensive rats (SHRs). For example, segments of mesenteric artery from normotensive rats were relaxed by S-1 with an EC50 of 2.5 μM but in segments from SHRs S-1 had an EC50 of 47.2 μM. Of the 5 KCNQ gene isoforms the expression of KCNQ4 was reduced (~3.7 fold) in the SHR aorta. Kv7.4 protein levels were ~50 % lower in aortae and mesenteric arteries from SHRs compared to the normotensive vessels. A similar attenuated response to S-1 and decreased Kv7.4 abundance was observed in mesenteric arteries from mice made hypertensive by angiotensin II infusion compared to normotensive controls.

Conclusions: In two different models of hypertension the functional impact of Kv7 channels is dramatically down-regulated.

Yeung et al. (2007) Br. J. Pharmacol., 151:758-770

Greenwood and Ohya (2009) Br. J. Pharmacol., 156:1196-203.

The staff at St. George's Biological Research Facility.

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C17 and PC17

The AGE-breaker ALT-711 restores high-blood-flow-dependent remodeling in mesenteric resistance arteries in type 2 diabetic rats

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Objective: Advanced glycation end products (AGEs) are generated by glucose in diabetes. Flow-mediated remodeling of resistance arteries (RA) is essential for revascularization in ischemic diseases, but this is impaired in diabetes. We hypothesized that breaking AGE crosslinks could improve flow-dependent remodeling in mesenteric RA in Zucker Diabetic Fatty (ZDF) rats.
Research Design and Methods: The animals were anesthetized (Isoflurane, 2.5%) and mesenteric resistance arteries were exposed to high (HF) or normal blood flow (NF) after alternate arterial ligation in vivo and were compared to age-matched lean Zucker (LZ) rats. All animals were treated with buprenorphine (Temgesic®; 0.1 mg/kg, s.c.) before and after surgery. Half of the rats were simultaneously treated by i.p. injection with ALT-711 (3 mg/kg per day).
Results: In LZ rats, HF artery diameter was larger than for NF vessels, but this was not the case in ZDF rats. Furthermore, endothelium-mediated dilation in ZDF rats, which was lower than in LZ rats, was further decreased in HF arteries. Treatment of rats with the AGE-breaker ALT-711 (3 mg/kg per day; 3 weeks) reversed the diabetes-induced impairment of HF-dependent remodeling, as shown by an increased arterial diameter. Breaking AGE crosslinks also improved cross-sectional compliance and endothelium-dependent relaxation in mesenteric RA. Additionally, contribution of the major endothelium-derived relaxing factor, nitric oxide, increased in acetylcholine-induced relaxation in NF and HF arteries in ZDF rats treated with ALT-711. Accumulation of AGEs and the receptor for AGE expression was reduced by ALT-711 in arteries in ZDF rats. Finally, ALT-711 increased eNOS protein expression in both HF and NF arteries.
Conclusions: AGE crosslinks oppose outward remodeling of RA in response to chronic increases in blood flow. Breaking AGE crosslinks provides a therapeutic potential for overcoming the microvascular complications of ischemic diseases in long-term diabetes.

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C18 and PC18

Induction of calcium-sensing receptor expression in human vascular smooth muscle cells by mechanical strain prevents calcification

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Vascular smooth muscle cells (SMC) play a crucial role in the development of medial arterial calcification, contributing to premature cardiovascular mortality in chronic kidney disease patients. We have previously shown that human aortic SMC (HAoSMC) express a functional calcium-sensing receptor (CaSR) and we have demonstrated a correlation between CaSR expression and calcification[(1)]. This study investigated the effect of mechanical strain on CaSR expression, SMC phenotype and the development of vascular calcification. HAoSMC were cultured under static or cyclic strain conditions using Flexcell apparatus (7% stretch, 30 cycles/min) for up to 14 days. HAoSMC phenotype was quantified by expression of smooth muscle α-actin (α-SM-actin; western blotting), alkaline phosphatase (ALP) activity and osteocalcin (OC) secretion. Calcification was assessed by alizarin red staining. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests; data are presented as mean ± S.D. (n=3). Culture of HAoSMC in static conditions resulted in a gradual decline in α-SM-actin expression, which decreased by 35% ($p<0.05$) after 2 weeks and >60% ($p<0.01$) after 4 weeks of culture. This was accompanied by a reduction in CaSR protein expression ($p<0.05$). Culture under cyclic strain prevented these changes and resulted in up-regulation of α-SM-actin expression by days 7 and 10 (23%, $p<0.05$) and a 45% increase in CaSR expression ($p<0.05$) compared to static cultures. Alizarin red staining of day 7 and 14 cultures revealed significantly smaller areas of calcification in strained cells compared to control cultures ($p<0.05$). To assess the role of the CaSR, cells were treated with CaSR agonists. Treatment of static HAoSMC with 2mM and 5mM Ca^{2+}, 50μM Gd^{3+} alone or in combination for 7 days induced a significant down-regulation ($p<0.01$) of CaSR expression and a dramatic up-regulation of HAoSMC calcification ($p<0.01$). ALP and OC were increased in cells treated with both CaSR agonists ($p<0.05$). In HAoSMC cultured under cyclic strain, the Ca^{2+} and Gd^{3+}-induced down-regulation of CaSR expression and increased calcification were significantly attenuated ($p<0.05$ to $p<0.001$). In addition, cyclic strain induced a significant down-regulation of ALP and OC production in both control and CaSR agonist-treated cells ($p<0.01$). CaSR knockdown with CaSR siRNA resulted in a further increase in calcification in Ca^{2+} and Gd^{3+}-treated cells compared to untransfected cells ($p<0.05$). Importantly, this was accompanied by up-regulation of ALP and OC production in control and agonist-treated CaSR knockdown cells ($p<0.01$). Our findings indicate that maintaining expression of a functional CaSR may serve to prevent a shift towards calcifying SMC phenotype and protect against calcification.

Molostvov G *et al*. (2007) *A. J. Physiol (renal)* **293**, F946-955.

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C19 and PC19

Expression of 25-hydroxyvitamin D_3-1α-hydroxylase and functional vitamin D signalling in human vascular smooth muscle cells

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Progressive medial arterial calcification is a major cause of cardiovascular mortality in patients with chronic kidney disease. It is known that vascular smooth muscle cells (VSMC) play a key role in this process. They are capable of phenotypic transformation into osteoblast-like cells and they promote the deposition of calcium. Recent studies have suggested a protective role for vitamin D. The exact mechanism for this process is unclear and vitamin D may have several actions. Some of these may be due to circulating levels of 1,25-dihydroxvitamin D_3 ($1,25D_3$), but it is also apparent that local synthesis of $1,25D_3$ is important, where it may act in a paracrine/autocrine manner. Synthesis of $1,25D_3$ from the major circulating form, 25 hydroxyvitamin D_3 ($25D_3$), is catalysed by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase, CYP27B1). $1,25D_3$ is metabolised by 24-hydroxylase (24-OHase, CYP24A1). This study investigated the expression and regulation of 1α-OHase, 24-OHase and the vitamin D receptor (VDR) in arterial tissue from healthy patients and primary cultures of human aortic smooth muscle cells (HAoSMC). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests; data are presented as mean ± S.E.M. (n=3). RT-PCR analyses demonstrated that HAoSMC expressed 1α-OHase and 24-OHase mRNA. The transcripts were similar to that seen in HKC-8 cells (a kidney cell line, which expresses both hydroxylases). VDR mRNA is also expressed. Western blot analyses identified single protein bands in HAoSMC and normal arteries demonstrating expression of both 1α-OHase and 24-OHase. The bands correspond to the size of the 1α-OHase and 24-OHase proteins (approximately 56kDa) and were identical to those seen in HKC-8 cells and human kidney tissue. VDR protein was detected as a double band of approximately 54 and 56kDa in all samples. These results were confirmed by immunohistochemistry of arteries. 24-OHase mRNA was significantly increased by $1,25D_3$ (10nM; 6 hours; $p<0.05$. Conversely, 1α-OHase protein was significantly decreased by $1,25D_3$ (1nM; 6 hours; $p<0.05$). Importantly 24-OHase mRNA was also increased and 1α-OHase protein decreased by $25D_3$ (100nM & 10nM respectively; 6 hours; $p<0.05$) indicating 1α-OHase activity in the HAoSMC. This data clearly demonstrates that arteries and VSMC express 1α-OHase and functional vitamin D signalling. We believe we are the first to demonstrate the presence and localization of 24-OHase in human arteries and VSMC. We have also shown that VSMC are able to synthesize sufficient $1,25D_3$ to stimulate a local response. Therefore

we would postulate that alterations in the local production and metabolism of $1,25D_3$ modulates the function of VSMC and may play a role in the development and progression of the vascular calcification seen in kidney disease.

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C20 and PC20

Perivascular adipocyte induced anticontractility and adiponectin levels in type 1 diabetic rats

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Vascular diseases such as microangiopathy represent the main cause of death in Type-1 diabetes. Although traditionally this is thought to be due in part to endothelial dysfunction, the role of perivascular adipose tissue (PVAT) has yet to be investigated. PVAT functions as an endocrine organ in its own right and provides an anticontractile effect in health to arteries due to release of various adipokines, including adiponectin. Although we have shown PVAT function is compromised in the metabolic syndrome, the effect in Type-1 diabetes however is unclear[1]. This study aims to examine arterial function in the presence and absence of PVAT in a rat model of type 1 diabetes and assess adiponectin levels in this model.

Diabetes was induced in male Wistar rats (Charles River, UK) via intraperitoneal injection of freshly dissolved STZ (55mg/kg in sterile saline serum). Rats were humanely killed by cervical dislocation at 12 weeks. Age- and weight-matched rats were used as non-diabetic controls (group 1). A second control group of normal chow fed rats was also studied. Second order mesenteric arteries were dissected ± PVAT and studied using wire myography. Cumulative concentration responses (10^{-9}–10^{-5}M) to norepinephrine (NE) were performed. Responses are expressed as mean (±SEM)% of KPSS constriction (60mM) and analysed using 2-way ANOVA. Three different tissue samples were taken from each rat: adipose tissue (F), aorta (A), and mesenteric artery (M) for immunohistological analysis of adiponectin using a mouse anti-rat adiponectin antibody.

Starting body weight of control animals was 358.4 ± 8.1g and of diabetic animals 357.3 ± 5.7g. At the end of the study control animals (group 1) significantly increased ($P<0.05$) body weight to 580.3 ± 15.8g compared with diabetic rats (382.2 ± 22.3g). Blood glucose of control groups was <6 mmol/L which was significantly less ($P<0.05$ t test) than that of the diabetic group (<27.4 mmol/L). PVAT induced an anticontractile effect (elicited by 10^{-6}M NE) in normal chow fed controls (PVAT: 18 ± 3.7 % vs. no PVAT: 75.6 ± 8.0%; (n=36); $P<0.05$). The anticontractile effect was also present in weight-matched controls (PVAT: 8.9 ± 3.6% vs. no PVAT: 60.1 ± 13.9%; (n=6); $P<0.05$). However there was no significant difference in responses in the presence

(42.3 ± 14.8%) or absence of PVAT (46.1 ± 10.8%) from arteries from diabetic rats (n=6). Immunohistochemistry indicated an increase in the expression of adiponectin in all three tissue samples (F, A and M) from diabetic rats compared with controls. These data demonstrate that diabetes inhibits the anticontractile effect of PVAT. This is accompanied with an increase in adiponectin levels. This is indicative of adiponectin resistance in these arteries and points to a pathological mechanism different to that seen in type 2 diabetes.

Greenstein et al (2009) Circulation **119**(12):1661-1670.

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C21 and PC21

Use of a novel *in vitro* technique to investigate needle–induced vascular injury

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The arteriovenous fistula (AVF) undergoes a structural adaptation which in the long term does not favour patency (Ross, 2005). AVF failure is attributed primarily to luminal occlusion due to neointimal hyperplasia (Maya et al, 2004). The arterial wall response to injury and the relationship between inflammation and the cardiovascular system has not been considered in this type of vascular disease. To date there is no clear mechanistic understanding of the neointimal hyperplasia that develops in the AVF. Specifically there is an absence of data of the cumulative effect of needle cannulation injury and its possible association with neointimal growth and consequent AVF failure. Thus the aim of this study was to identify the effect of repeated needle cannulation on vascular structure in our established in-vitro organ culture model.

Aorta were dissected from Male, Sprague-Dawley rats, (12 weeks, 250-300g) under tissue culture conditions and cultured in growth medium 50:50 (V:V) mix of Ham's nutrient mix F12 (Gibco), Waymouths MB 752/1 (Gibco), 10% fetal bovine serum (FBS) (Biosera) and 1% pen-strep (Invitrogen). Intact aorta was cultured for 28 days under the following conditions:- cultured non-injured (control), cultured-injured and cultured-injured-drug treated (flurbiprofen; 10uM). Tissues were needle cannulated through the side wall following 24 hours culture and thereafter every 72 hours over the 28 day culture period. Vascular smooth muscle (VSM) cells were isolated and cultured for cell proliferation and western blot analysis.

Planimetry of wax embedded and sectioned tissues using haematoxylin and eosin staining: Needle injury produced significant hyper-proliferative structural remodelling. Media: lumen ratios were 0.47 ± 0.033 and 0.64 ± 0.047 in the control and needle injured aortas respectively (n=6; $p<0.05$). The cyclooxgenase inhibitor (flurbiprofen) reduced needle injury-dependent hypertrophy by 18 ± 3.2% when com-

pared with cultured non-injured tissues n=6, $p<0.05$). VSM cell proliferation assay confirmed flurbiprofen's (10 uM)anti-proliferative effect on VSM cells reducing proliferation by 8-fold when compared with control (n=8; $p<0.05$). Western blot analysis, established that flurbiprofen (10 uM) reduces the phosphorylation of the MAP Kinase Erk-1/2.

Presently there is no prophylactic intervention to postpone or reduce occlusive failure in the forearm AVF graft. We have identified for the first time that needle injury can evoke hyperproliferative adaption in vascular tissues. Moreover we have identified that cyclooxygenase inhibition can reduce this hyperproliferative response and this may provide insight to a potential therapeutic advance in promoting the functional survival of the AVF in patients undergoing haemodialysis.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC22

Mechanisms of attenuation of P2X-mediated $[Ca^{2+}]_i$ transients in renal microvascular myocytes in primary hypertension

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Rise of $[Ca^{2+}]_i$ in renal vascular smooth muscle cells (RVSMCs) following P2X receptor (P2XR) activation results from Ca^{2+} entry via P2XRs and voltage-gated Ca^{2+} channels (VGCCs), and ryanodine receptor (RyR)- and inositol 1,4,5-trisphosphate receptor (IP_3R)-mediated Ca^{2+} release from the sarcoplasmic reticulum (SR) [1]. Here we analysed how this Ca^{2+} signalling system is altered in primary hypertension by comparison of: (i) expression of genes encoding P2XRs, VGCCs, RyRs and IP_3Rs using real-time PCR analysis, (ii) P2XR-mediated currents (I_{P2X}) using perforated-patch tight-seal recording, (iii) $\alpha\beta$-meATP-induced $[Ca^{2+}]_i$ transients in fluo-3-loaded myocytes using confocal imaging, (iv) the SR calcium load using caffeine as Ca^{2+}-liberating agent, in RVSMCs from spontaneously hypertensive rats (SHR) and their normotensive control, Wistar Kyoto (WKY) rats. Ca^{2+} signal calibration was performed using the method modified from Kao et al. [2]. Data are presented as mean ± S.E.M. and compared using unpaired Student's t-test. We found that in SHR RVSMCs P2rx1 was downregulated 17.9±5.5-fold ($p<0.001$), Cacna1C and Ryr2 were upregulated 30.3±13.8- and 24.5±8.6-fold, respectively ($p<0.01$), while there was no significant difference in P2rx4 and Itpr1 expression ($p=0.368$ and $p=0.165$, respectively). The peak of $\alpha\beta$-meATP-induced $[Ca^{2+}]_i$ transients was reduced in SHR RVSMCs ($p<0.001$) from 746±58 nM (WKY, n=76) to 270±23 nM (SHR, n=55). The peak density of I_{P2X} was reduced in SHR RVSMCs ($p<0.01$) from 101±12 pA/pF (WKY, n=25) to 57±7 pA/pF (SHR, n=18). Ca^{2+} entry via P2XRs, estimated from the fraction of the fluo-3 response remaining after depletion of Ca^{2+} stores and block of VGCCs, was reduced in SHR RVSMCs ($p<0.01$) from 29±3 % (WKY, n=20) to 15±2 % (SHR, n=11). Ca^{2+} entry via VGCCs, estimated from nicardipine-sensitive fraction of the fluo-3 response to $\alpha\beta$-meATP in RVSMCs with depleted SR, was increased in SHR RVSMCs ($p<0.01$) from 35±5 % (WKY, n=7) to 62±4 % (SHR, n=6). Ryanodine-sensitive fraction of the fluo-3 response to $\alpha\beta$-meATP increased in SHR RVSMCs ($p<0.01$) from 19±4 % (WKY, n=7) to 42±5 % (SHR, n=8). The peak of caffeine-induced $[Ca^{2+}]_i$ transients was reduced in SHR RVSMCs ($p<0.001$) from 688±32 nM (WKY, n=47) to 246±19 nM (SHR, n=21). Thus, both, reduced Ca^{2+} entry via downregulated P2X1 and decreased Ca^{2+} release from the SR with lower Ca^{2+} load (resulting from an enhanced RyR-mediated Ca^{2+} leak) contribute to attenuation of $[Ca^{2+}]_i$ transients in SHR RVSMCs and may underlie impairment of sympatheti-

cally driven and autoregulatory responses in renal vasculature [3] leading to glomerular damage in hypertension.

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PC23

The vascular smooth muscle T-type calcium channel: an anti-proliferative target for heme oxygenase-1?

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Excess proliferation of vascular smooth muscle cells (VSMC) is a central feature of vascular disorders such as hypertension, restenosis and atherosclerosis (1). T-type calcium channels provide an important route for calcium entry in disease-associated VSMC proliferation, and under these conditions VSMC also increase expression of heme oxygenase-1 (HO-1) (1) (2). HO-1 catabolises free heme to produce biliverdin, iron, and carbon monoxide (CO), and exhibits anti-proliferative effects, possibly through the production of CO (2). We aimed to investigate the effects and possible interactions of HO-1, CO, and T-type calcium channel inhibition on VSMC proliferation.

VSMC were explanted from fragments of saphenous vein (SV) of patients undergoing coronary bypass and propagated in primary culture. Proliferation assays were performed over 4 days in the absence or presence of the T-type channel inhibitor mibefradil, an inducer of HO-1, CoPPIX, or the CO donor compound, CORM-3. Viable cells were counted using trypan blue and a hemocytometer. Statistical analysis was performed using two-tailed paired T-test and one-way ANOVA with Bonferroni's multiple comparison test, as appropriate.

Proliferation was reduced by 39.7 ±8.8%, $p<0.05$, n=3, in the presence of 3μM mibefradil, and by 37.7 ±2.8%, $p<0.05$, n=3, following HO-1 induction by 3μM CoPPIX (induction verified by western blotting, n=2). CORM-3 application (3-30μM) produced a dose dependent decrease in proliferation, a reduction of 36.0 ±4.4%, $P<0.01$, n=3, was observed in the presence of 3μM CORM-3. The application of 3μM mibefradil and 3μM CoPPIX simultaneously reduced proliferation by 57.7 ±8.1%, $P<0.05$, n=3.

These preliminary data support the idea that CO is antiproliferative through inhibition of VSMC T-type calcium channels. This signalling pathway may therefore be

a novel target for pharmacological intervention in vascular disorders involving excess SMC proliferation.

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PC24

Vasorelaxant and hypotensive effects of Allium cepa peel hydroalcoholic extract in rat

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The aim of present study was to investigate the effect of onion (Allium cepa) peel hydroalcoholic extract (OPE) on rat hypertension induced by high-fructose diet and aorta contractility. The OPE was prepared by maceration method using 70% ethanol. In *in vitro* study, animals were anaesthetized by ether and exsanguinated. The thoracic aorta from male adult rat (Wistar) was dissected and suspended in Krebs-Henseleit solution under 1 g resting tension. Tissue preparation was contracted by KCl (80 mM) or phenylephrine (Phe, 1 μ M) and then the extract was applied cumulatively (0.0625-2 mg mL-1). Hypertension was induced in negative control and three groups of rats by adding fructose (10% W/V) in drinking water for 6 weeks but control group received tap water. Hypertensive groups received saline or OPE at 200, 400 and 800 mg kg-1 daily for last 3 weeks by gavage. Systolic arterial pressure in conscious and restrained rat was recorded weekly by using tail cuff (Powerlab, UK). Heart rate was also recorded by using the same instrument. Results showed that OPE reduces aorta contractions induced by KCl or Phe in a concentration-dependent manner ($p<0.001$). Removing aorta endothelium did not attenuate the OPE activity. Inhibition of nitric oxide, cGMP and prostaglandin synthesis by L-NAME (100 μM), methylene blue (10 μM) and indomethacin (10 μM), respectively, did not attenuate OPE activity. Atropine abolished ACh-induced relaxation

in Phe precontracted aorta but not the OPE-induced relaxation. Although the extract did not change heart rate but after 3 weeks reduced the hypertension induced by fructose ($p<0.001$). Present results indicated that OPE reduces aortic contractions possibly via inhibition of calcium influx but without involving NO, cGMP, endothelium and prostaglandins. The OPE hypotensive effect could be due to extract quercetin content, antioxidant activity and inhibiting vascular smooth muscle cells Ca2+ influx.

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PC25

ATP inhibits IP_3-mediated Ca^{2+} release in smooth muscle via $P2Y_1$ receptors

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Introduction The inhibitory neurotransmitter, ATP, is pivotal in regulating smooth muscle activity. The underlying mechanism(s) by which ATP evokes relaxation remain unclear, but have been proposed to involve phospholipase C (PLC)-mediated IP_3 production, to evoke Ca^{2+} release from the internal store and stimulation of Ca^{2+}-activated potassium (K_{Ca}) channels to cause membrane hyperpolarization. ATP-induced Ca^{2+} rises have been reported in smooth muscle, but the frequency with which they are observed are often varied. Indeed, Ca^{2+} rises to ATP may be either limited or not observed at all. To investigate the role of Ca^{2+} release in ATP-evoked smooth muscle relaxation, the effect of ATP on IP_3-mediated Ca^{2+} release from the store was examined. *Methods* Single voltage-clamped myocytes were dissociated [1] from the colon of guinea-pigs (sacrificed by intraperitoneal

injection of euthatal (200 mg/kg) followed by exsanguination). $[Ca^{2+}]_i$ was measured as fluorescence using fluo-3. Intracellular Ca^{2+} release was evoked either by activation of IP_3 receptors (IP_3R; by carbachol or photolysis of caged IP_3) or ryanodine receptors (by caffeine). *Results* ATP transiently increased $[Ca^{2+}]_i$ in only 10% of voltage-clamped single smooth muscle cells. Interestingly, ATP inhibited IP_3R-mediated Ca^{2+} release in cells that did not show a Ca^{2+} rise in response to purinergic stimulation (1mM, n=5). The reduction in IP_3R-mediated Ca^{2+} release was mimicked by its metabolite, ADP (1mM, n=4), but not by adenosine (1mM, n=4). Furthermore, the inhibitory response to ATP was blocked by the $P2Y_1R$ antagonist, MRS2179 (10μM, n=5), and the G-protein antagonist, GDPβS (1mM, n=8), but not by the PLC inhibitor, edelfosine (10μM, n=4). *Discussion* Since ATP did not release Ca^{2+}, neither activation of K_{Ca} channels nor depletion of the store of Ca^{2+} can explain the inhibitory response to ATP. Neither can the inhibitory response be attributed to breakdown of ATP to adenosine since the nucleoside was without effect on IP_3R-mediated Ca^{2+} release. ADP, however, inhibited Ca^{2+} release, indicating that the reduction is mediated by P2YR. The inhibitory response to ATP was blocked by MRS2179 and by GDPβS, confirming a role of G-protein coupled $P2Y_1R$ in this response. ATP remained effective in inhibiting IP_3R-mediated Ca^{2+} release in the presence of edelfosine, thus excluding a role of PLC. The study reveals, for the first time, an inhibitory effect of $P2Y_1R$ activation on IP_3R-mediated Ca^{2+} release, such that purinergic stimulation acts to prevent the excitatory influence of IP_3 on smooth muscle and promote relaxation. The observed inhibitory response to ATP appears to be independent of PLC.

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PC26

Testosterone promotes vascular hypertrophic effect of a high salt diet in male Sprague-Dawley rats

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Salt sensitivity exhibits sexual dimorphism which is higher in males when compared with females (1). This has been attributed to the effect of androgens on blood vessels in males (2). Sodium overload, which is present in high salt diet (HSD) has been reported to change the shear stress and geometric modifications of the blood vessels (3) and haemodynamic factors are very important in the regulation of the structure of the artery (4). Therefore, experiments were designed to study the vascular

smooth muscle histomorphometry in orchidectomy-induced (under ketamine and xylazine: 90mg and 10mg/Kg/body weight i.m respectively (5) androgen deficient rats placed on a normal (0.3%) or high (8%) NaCl diet for 6 weeks, with or without testosterone replacement (10mg/kg bodyweight sustanon 250® i.m once in 3 weeks). Histological examination of thoracic aorta and mesenteric artery were carried out with specific dyes; haematoxylin and eosin stain for the cytoplasm and nucleus and Verhoeff – Van Geison and Picro-sirius red stains for elastin and collagen content estimation respectively. Histomorphometric analysis was done using a programmed software IMAGE-PRO 3DS 6.1. Tunica media thickness and cross sectional area, elastin and collagen contents were all significantly elevated ($P < 0.05$) in the rats placed on HSD while orchidectomy significantly reduced the values of the parameters in HSD group but concomitant administration of testosterone restored them to the levels observed in intact rats. Orchidectomy ameliorated vascular hypertrophic effect of a HSD by reducing vascular smooth muscle proliferation and decreasing extracellular matrix protein deposition induced by HSD. Testosterone replacement following orchidectomy accentuated vascular hypertrophic effect of a HSD.

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PC27

Inhibition of Rho Kinase by 5-HT in 5-HT-induced constriction of isolated Bovine pulmonary arteries

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The Rho-A/Rho-kinase pathway plays an important role in agonist –induced constriction of vascular smooth muscle. Indeed inhibition of Rho-kinase has been promoted as a putative management therapy in pulmonary hypertension (Abe et al, 2004). Rho-kinase can be activated by agonist receptors that couple to G12/13 (Fukata et al, 2001) and/or by elevated intracellular calcium (Ayeman et al, 2003). In bovine pulmonary arteries 5-HT induced constriction is mediated by the 5-HT2A

and 5-HT1B receptors (McKenzie et al, 2010). The present study investigated the role of Rho-kinase in 5-HT-induce constriction of bovine pulmonary arteries.
Bovine lungs from animals under twenty months were obtained fresh from the local abattoir. Ring segments (0.3-0.5cm in diameter), dissected from the 5th. Arterial generation were mounted in 5 ml organ baths suspended between stainless steel hooks in Krebs-Henseleit buffer (37oC) under a tension of 2 g and gassed with a mixture of O2:CO2 95%/5% v/v. Tissues were allowed to equilibrate for 1 hour before the addition of drugs. All tissues were first contracted with 60 mM KCl. After washing, cumulative concentration response curves (CRCs) to 5HT or a single constriction to the 5-HT2A agonists DOI (10nM) or TCB (1μM) were constructed in the absence or presence of the Rho kinase inhibitors Y27632 (30μM) or H1152 (10μM). CRC to 5-HT were further examined in presence of the 5-HT1B (SB216641, 3μM) and 5-HT1D (BRL15572, 10nM) receptor antagonists or the selective serotonin reuptake inhibitor paroxetine (10nM) alone or in the presence of Y27632.
The concentration response curve for 5-HT-induced constriction was unaffected by the Rho-kinase inhibitors Y27632 or H1152. In contrast constriction induced by the 5-HT2A agonists DOI was reduced by 58% (from 95.28±6.62 to 39.88±4.02, n=4, values expressed as percentage of 60mM KCL contraction) by Y27632. The 5-HT1B receptor antagonist SB216641 shifted the CRC for 5-HT to the right (control EC50=-5.915±0.096, SB216641 EC50=-4.771±0.102, n=7) and reduced the maximum response by 20% (from 188.2±7.90 to 149.8±9.83, n=7) and this response was unaffected by the further addition of Y27632. The presence of the 5-HT1D receptor antagonist (BRL15572) produced a 26% increase in tone (from 190.4±9.44 to 239±14.76, n=7), the selective serotonin re-uptake inhibitor (paroxetine) produced a similar potentiation of 24% to the maximal contraction of the 5-HT CRC (from 174.4±3.79 to 217.1±8.6, n=4). The further addition of Y27632 reduced the maximum response by approximately 36-42% (from 239±14.76 to 153±9.77, n=7, in the presence of BRL15572 and from 217.1±8.6 to 125.1±7.45, n=4, in the presence of paroxetine).
These observations suggest that 5-HT acting through the 5-HT1D receptor and/or the selective serotonin reuptake mechanism may inhibit the involvement of Rho-kinase in 5-HT-induced constriction of BPA.

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PC28

Investigating the role of α2-adrenoceptors in tail artery of wild type mice and mice lacking α1-adrenoceptors (α1-null)

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In rat tail artery, perivascular nerve stimulation leads to vessel contraction through stimulation of α1-, α2-adrenoceptors (AR) and P2X receptors. The ensuing contraction has an initial fast depolarisation (P2X receptor activation) followed by a sustained response from α1- and α2-AR activation (Sneddon and Burnstock, 1984; Rummery and Brock, 2011). Generation of mice lacking all three α1-AR subtypes has allowed the α2-ARs to be fully investigated.

Male Wild Type (C57Bl) and α1-null mice (aged 4-6 months) were killed by carbon dioxide asphyxiation. 2mm ring segments were prepared free of fat from the tail artery and mounted on a wire myograph suspended in freshly gassed PSS at 37oC. In both strains of mice, responses to 1μM 5-HT were recorded and frequency response curves (FRCs) were constructed in the absence and presence of 100nM rauwolscine (α2-AR antagonist). Vessels were stimulated with the following parameters: 0.5Hz-8Hz; 0.3ms pulse width; 20pulses; 20v.

The response to 5-HT (1μM) was significantly smaller in the α1-null mice (0.51g ± 0.20) than in the WT (0.69g ± 0.24) ($P<0.05$; unpaired t test; WT – n=13, α1-null – n=8). Nerve stimulation revealed no significant difference in size of contraction between the strains at 0.5Hz and 1Hz. However at 2Hz, 4Hz and 8Hz the WT response was significantly greater than in the α1-null strain ($P<0.05$; unpaired t test; WT – n=12, α1-null – n=6). For example, at 8Hz the WT response was 0.53g (± 0.21) and the α1-null response was 0.30g (± 0.16).

In the presence of rauwolscine, both strains produced responses at all frequencies that were significantly lower than control responses ($P<0.05$; unpaired t test; WT – n=6, α1-null – n=5) with a greater percentage reduction at the lower frequencies. In the WT at 0.5Hz and 1Hz there was an 87±9% and 83±5% reduction respectively. As the frequency increases the percentage reduction in response decreases so that at 8Hz there was a 56±5% decrease. In the α1-null mice the percentage decrease in response after rauwolscine incubation was similar across the range of frequencies (68%–74%).

In conclusion, the α1-null vessels showed a modest reduction in contractility to the test agonist 5-HT but demonstrated adrenergic nerve responses equivalent to those in WT mice at low frequencies. At higher frequencies the WT had greater responses that can be attributed to α1-ARs, which were lost in the α1-null mice and survived α2-AR blockade by rauwolscine; however they had a substantial rauwolscine-sensitive, α2-AR-mediated component. In the α1-null vessels throughout the frequency range there was a substantial reduction in response by rauwolscine, indicating a dominant α2-AR response to nerve activation that can be studied without the complication of drug action overlapping with α1-ARs.

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PC29

Reduction of vasomotion in thoracic aorta of immature rats using distilled water

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Rhythmical contraction known as vasomotion occurs in blood vessels. This phenomenon is observed under pathological conditions, and sometimes under physiological conditions. Vasomotion had been observed mainly in low resistance microcirculation vessels (Nilsson et al., 2003). Particular attention has been focused on cyclic variations of the aorta diameter in immature rats, in part because it may contribute to the regulation of blood flow (Haddock et al., 2005).
The aim of the current study was to observe if immature rats have vasomotion in thoracic aorta and to determine the effect of tap water (control rats; 1-month-old) and distilled water (treated rats; 1-month-old) on vasomotion. In some experiments, immature Sprague-Dawley rats (1-month-old; 100 to 150 g) were compared with mature rats (4-month-old; 300 to 350 g). Animals were sacrificed by cervical dislocation. All groups drank tap water and distilled water from the time of weaning. In each experiment, we studied 6 to 8 adjacent aortic rings from the same animal, using a known method for isometric tension measurements (Palacios et al., 2006). The mechanical oscillations (vasomotion) observed in the aorta of the control group was influenced significantly by the concentration of phenylephrine (10^{-7} M PE; 136±4 mg maxima amplitude) and of KCl (20 mM; 110±4 mg maxima amplitude); this effect was diminished after 4 months (10^{-7} M PE; 31±8 mg maxima amplitude). The treated group did not show vasomotion in aorta (10^{-7} M PE; 5±2 mg maxima amplitude). Interestingly, the preincubation with EGTA of the control group eliminated the vasomotion phenomenon (10^{-7} M PE; 20±4 mg maxima amplitude). The aortic vasomotion and PE-induced tension were dependent on extracellular calcium concentration (0 to 1 mM Ca^{2+}). It is possible that endothelial nitric oxide (NO) could be involved in vasomotion of immature rats; however, the oscillations were initiated only after the reduction in tension by acetylcholine (10^{-7} M) or NO (10^{-8} M). Therefore, the increase in vasomotion seems to be always associated with a reduction in tension to an intermediate level of the vasomotor tone. Vascular

relaxation to ACh reduced in treated rats compared to control rats. We conclude that vasomotion in immature rats is associated with the calcium in drinking water, among other factors.

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PC30

Store-operate Ca^{2+} entry is over-expressed in endothelial colony forming cells isolated from patients suffering of Renal Cellular Carcinoma

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OBJECTIVE: Formation of new blood vessels plays a key role in tumor growth and metastasis and may be supported by bone marrow-derived endothelial progenitor cells (EPCs). Different studies have demonstrated that the engagement of EPCs is fundamental for the progression of avascular micrometastatic tumors to lethal macrometastatic ones. We have recently shown that store-operated Ca^{2+} entry (SOCE) controls cell proliferation in endothelial colony forming cells (ECFCs). SOCE is contributed by the Ca^{2+}-permeable channel, Orai1, and the ER Ca^{2+}-sensor, Stim1. The search for novel molecular targets to exploit in the fight against cancer prompted us to investigate the expression and role served by SOCE in ECFCs isolated from patients suffering from renal cellular carcinoma (RCC).
METHODS: ECFCs were isolated from the peripheral blood of healthy donors or patients suffering from RCC as described elsewhere. Changes in $[Ca^{2+}]_i$ were monitored loading the cells with Fura-2/AM (4 μM, 30 min) and with the aid of a CCD camera.
RESULTS: SOCE was activated by exposing cells to the classic "Ca^{2+} add-back" protocol in presence of either cyclopiazonic acid (CPA), a selective inhibitor of the Sar-

coEndoplasmic Reticulum Ca^{2+}-ATPase (SERCA), or the physiological agonist, ATP. In both cases we found that SOCE was significantly higher in RCC-ECFCs as compared to control cells, while the intracellular Ca^{2+} release was smaller. SOCE was selectively inhibited by BTP-2, as previously reported in control cells, whereas carboxyamidotriazole (CAI), an orally active anti-angiogenic drug which is in phase II clinical trials of metastatic RCC, was less specific and affected also Ca^{2+} release. The higher amplitude of SOCE was associated to the over-expression, at both mRNA and protein level, of Stim1 and Orai1 in RCC-ECFCs, while Stim2, Orai2 and Orai3 were not differently expressed in these cells. Finally, pre-incubation with BAPTA, an intracellular Ca^{2+} buffer, BTP-2, and CAI dramatically affected RCC-ECFC proliferation and tubulogenesis.
CONCLUSIONS: These data indicate that SOCE is over-expressed in ECFCs isolated from patients suffering of RCC due to the selective up-regulation of Stim1 and Orai1. SOCE controls RCC-ECFC proliferation and tubulogenesis and might, therefore, be regarded as a novel molecular target to device alternative anti-cancer treatments.

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PC31

Characterisation of transcriptional and post transcriptional properties of vascular interstitial and cultured smooth muscle cells

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Various *in vitro* models are used for studying mechanisms of phenotypic modulation of vascular smooth muscle cells (VSMCs) and the established culture of VSMCs (cVSMCs) is often used for this purpose. Vascular interstitial cell (VICs), recently found in wall of various blood vessels, likely represent resident phenotypically modulated VSMCs under normal physiological conditions[1]. In this study we compared the expression of proteins and corresponding genes for VSMCs-specific markers in freshly dispersed contractile VSMCs, VICs and cVSMCs from rat aorta using immunocytochemistry (30 cells for each protein, 3 animals) and comparative real-time RT-PCR from separately collected cells (~250 cells of each type, 3 animals). The data is presented as mean±S.E.M. ANOVA test for three variables was used for statistical analysis and differences of $p<0.05$ were considered significant. We observed that VICs, similarly to cVSMCs, display the presence of sparse αSM-actin-enriched

stress fibres, while in VSMCs this protein is more tightly packed with higher density in superficial region of the cell. We found that compared to contractile VSMCs both VICs and cVSMCs show a decrease in gene and protein expression for smoothelin (0.30±0.06 in VICs and 0.34±0.15 in cVSMCs for gene and 0.39±0.05 in VICs and 0.53±0.04 in cVSMCs for immunofluorescence, all $p<0.05$), myosin light chain kinase (MLCK) (0.20±0.03 in VICs and 0.21±0.12 in cVSMCs for gene and 0.17±0.02 in VICs and 0.20±0.02 in cVSMCs for immunofluorescence, all $p<0.05$) and SM22α (0.22±0.00 in VICs and 0.20±0.08 in cVSMCs for gene and 0.18±0.03 in VICs and 0.40±0.02 in cVSMCs for immunofluorescence, all $p<0.05$). The expression of αSM-actin and smooth muscle myosin heavy chain (SM-MHC) was significantly decreased in cVSMCs compared to contractile VSMCs (αSM-actin 0.49±0.06 for gene, 0.27±0.03 for immunofluorescence; SM-MHC, 0.51±0.03 for gene, 0.41±0.06 for immunofluorescence, all $p<0.05$), however, compared to VSMCs it was not decreased in VICs (αSM-actin, 0.87±0.06 for gene ($p>0.05$), 1.42±0.11 for protein ($p<0.05$) and in SM-MHC (0.99±0.13 for gene and 1.03±0.06 for protein, both $p>0.05$). Our study demonstrates that both VICs and cVSMCs display the features of the putative phenotypically modulated VSMCs such as the presence of actin-enriched stress fibers and decrease in expression of contractile VSMCs markers such as smoothelin, MLCK and SM22α compared to contractile VSMCs. However, it was observed that the expression of two major contractile proteins SM-MHC and αSM-actin was decreased only in cVSMCs while it was not decreased in VICs compared VSMCs. These results suggest that mechanism of governing the phenotypic modulation may be different or altered in the cultured VSMCs comparing to the native phenotypically modulated VSMCs.

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PC32

Sphingosine 1-phosphate-induced release of TIMP-2 from vascular smooth muscle cells inhibits angiogenesis in coronary artery endothelial cells

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Following myocardial infarction resulting from thrombus formation, angiogenesis occurs and permits reperfusion of damaged myocardium. Sphingosine 1-phosphate (S1P) is a naturally occurring lipid mediator released from platelets and is found at sites of thrombosis. S1P could be involved in regulating angiogenesis following myocardial infarction and may therefore influence reperfusion. The aims of this study was to determine the effects of S1P in human coronary artery endothelial cell (EC) angiogenesis and delineate the subsequent mechanisms. An *in vitro* model

of angiogenesis was developed using a co-cultures of human coronary artery ECs, human coronary smooth muscle cells (SMCs) and human fibroblasts. After 14 days, endothelial tubule formation was visualized by immunofluorescence labelling with anti-CD146 antibody. Fluorescent images were analysed using ImageJ software. S1P (1 μM) significantly inhibited tubule formation in these co-cultures by 80 ± 7% (mean ± s.e.m., n=7, $p<0.05$ Student's t-test). When SMCs were omitted from the co-culture (ECs and fibroblasts only) EC tubules were still observed as previously, but S1P had no significant effect on tubule formation. In order to determine if this inhibitory effect of S1P was due to a soluble factor released from SMCs, conditioned medium from SMCs incubated with S1P was used on co-cultures of ECs and fibroblasts. S1P-treated conditioned medium from SMCs also inhibited endothelial tubule formation compared to untreated conditioned medium (64 ± 7%, n=4, $p<0.05$). As tissue inhibitor of metalloproteinases (TIMPs) are known inhibitors of angiogenesis and released by several different cell types, we examined TIMP-2 activity in conditioned medium from SMCs. In conditioned medium from SMCs treated with 1 μM S1P there was a significant increase in TIMP-2 activity as determined by reverse zymography compared to untreated conditioned medium (1.35 ± 0.07 fold increase, n=4, $p<0.05$). In co-cultures of ECs, SMCs and fibroblasts containing anti-TIMP-2 blocking antibody, S1P had no effect on tubule formation (n=4). Confocal microscopy of co-cultured treated with S1P revealed that vascular endothelial (VE)-cadherin localization to endothelial-endothelial cell junctions was disrupted thereby inhibiting tubule formation. This S1P-inhibitory effect was reversed by inclusion of TIMP-2 blocking antibody in the medium. In conclusion, S1P-induced inhibition of angiogenesis in human artery endothelial cells is mediated by a release of TIMP-2 from SMCs. This reduces the integrity of intercellular junctions between nascent endothelial cells by inhibiting VE-cadherin localization. S1P may therefore inhibit the angiogenic response following myocardial infarction.

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PC33

Resident phenotypically modulated myocytes in human arteries under normal physiological conditions

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It is generally accepted that contractile vascular smooth muscle cells (VSMCs) are not terminally differentiated and may change their phenotype in response to environmental cues such as injury or disease. On the other hand, vascular interstitial cells (VICs), which were recently described in various blood vessels of rodents under

normal physiological conditions, display some features of the phenotypically modulated VSMC. The objective of this study was to identify and characterise VICs in human arteries. The samples of healthy adipose tissue containing small fragments of human gastro-omental arteries were obtained during abdominal surgery and blood vessels were dissected and used in experiments. VICs were identified in the wall of resistance-sized gastro-omental arteries following enzymatic cell dispersal using transmission and scanning electron microscopy. Freshly dispersed VICs showed the ability to form new and elongate existing filopodia and actively change body shape, while contractile VSMCs did not display significant changes of their body during the same period of observation. RT-PCR analysis performed on separately collected contractile VSMCs and VICs (~200 cell of each type) showed that both cell types expressed the gene for smooth muscle myosin heavy chain (SM-MHC), thus confirming that VICs represent phenotypically modulated or de-differentiated VSMCs. Immunocytochemical staining showed that both VSMCs and VICs had similar fluorescence for SM-MHC and αSM-actin, VICs, however, had significantly lower fluorescence for smoothelin, myosin light chain kinase, and SM22α. It was also found that VICs do not have a cytoskeleton as rigid as in contractile VSMCs. In addition VICs displayed the presence of sparse individual stress fibers enriched in f-actin and aSM-actin. In VSMCs these two proteins were more densely packed so it was impossible to identify individual strands at the same resolution of the confocal microscope. Our results indicate that VICs from human arteries display multiple features of the putative phenotypically modulated VSMCs. VICs therefore represent resident phenotypically modulated VSMCs that are constitutively present in human arteries under normal physiological conditions.

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PC34

Vascular smooth muscle proliferation is regulated by an increase in mitochondrial motility

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Mitochondrial function is critical for multiple facets of cellular function including ATP production and Ca^{2+} handling. Subcellular location of the organelle is important for function. In smooth muscle, mitochondria modulate Ca^{2+} signals arising from InsP_3 receptors, even localised Ca^{2+} puffs, revealing a close physical relationship between the sarcoplasmic reticulum and mitochondria. This seems incompatible with the free movement of mitochondria observed in several cell types.

An *in vitro* study of intact cerebral resistance arteries at physiological pressure, individual mitochondria within smooth muscle were largely stationary (0.00159 ± 0.00004 moving mitochondria $\mu m^{-2}min^{-1}$, *n*=23); however in a small number of cells mitochondria displayed large-scale directed movements (0.00104 ± 0.00003 cells containing moving mitochondria $\mu m^{-2}min^{-1}$, *n*=23). When arteries were maintained in organ culture for up to 4 days to promote proliferation, an increased number of cells showing moving mitochondria (0.00341 ± 0.00008 cells $\mu m^{-2}min^{-1}$; *n*=16, p<0.01) and an increase in the extent of movement occurred (0.01043 ± 0.00027 moving mitochondria $\mu m^{-2}min^{-1}$, *n*=16, p<0.01). Expression of the mitochondrial tether mitofusin-2 decreased and the proliferative marker proliferating cell nuclear antigen (PCNA) increased, by western blotting and immunocytochemistry of fixed, pressurised arteries (*n*≥3 for each). Maintenance of arteries with the small molecule inhibitor of mitochondrial fission Mdivi-1 (50 μM) for the duration of the 4 days organ culture restricted mitochondrial dynamics (0.00228 ± 0.00024 mitochondria moving $\mu m^{-2}min^{-1}$ in 0.00145 ± 0.00015 cells $\mu m^{-2}min^{-1}$; *n*=6, p<0.01 for each, compared to untreated arteries maintained in culture for 4 days) and reduced the extent of proliferation as shown by PCNA staining (7.69 ± 2.87 compared to 15.70 ± 2.23 RFU/pixel corrected for non-specific staining of secondary antibody only, *n*=7, p<0.05), ^{3}H-thymidine incorporation and fluorescence-activated cell sorting by propidium iodide incorporation (*n*≥3 for each).
Thus, when smooth muscle is allowed to proliferate, mitochondria switch from being static to highly mobile, which may contribute to the altered Ca^{2+} signalling observed in proliferative smooth muscle. Inhibiting mitochondrial mobility decreases proliferation and may be a novel target for the prevention of proliferative vascular disease. Values are expressed as mean ± S.E.M. for *n* cells or regions of intact artery, significance calculated using Student's t-tests.

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PC35

VEGF-induced Ca^{2+} oscillations in umbilical cord blood-derived endothelial colony forming cells

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Objective
Endothelial colony forming cells (ECFCs) are endothelial progenitor cells capable of acquiring a mature endothelial phenotype. ECFCs are mobilized from bone-mar-

row to promote vascularization and represent a suitable tool for cell-based therapy (CBT). We have shown that VEGF stimulates ECFC proliferation and tubulogenesis by causing an intracellular Ca^{2+} oscillations. VEGF-induced Ca^{2+} spikes are driven by the interplay between inositol-1,4,5-trisphosphate (InsP3)-dependent Ca^{2+} release and store-operated Ca^{2+} entry (SOCE). Similar to peripheral ECFCs (PB-ECFCs), the therapeutic potential of umbilical cord blood–derived ECFCs (UCB-ECFCs) has recently been shown. Interestingly VEGF-induced proliferation and expansion of UCB-ECFCs are faster compared to their peripheral counterpart. Unlike PB-ECFCs, UCB-ECFCs express canonical transient receptor potential channel-3 (TRPC3), that mediated diacylglycerol (DAG)-dependent Ca^{2+} entry and promotes angiogenesis in endothelial cells. This study aimed at investigating whether the higher proliferative potential of UCB-ECFCs was associated to any difference in the molecular underpinnings of their Ca^{2+} response to VEGF.

Results

VEGF induced intracellular Ca^{2+} oscillations in UCB-ECFCs which did not occur in the absence of external Ca^{2+}. Therefore, Ca^{2+} entry triggers the Ca^{2+} response to VEGF in these cells. To assess whether TRPC3 contributes to the onset of VEGF-elicited Ca^{2+} spikes, we exploited the membrane permeable DAG analogue, OAG. Similar to VEGF, OAG elicited a Ca^{2+} transient in presence, but not in absence, of extracellular Ca^{2+}. This response was abolished by the TRPC3 blockers, flufenamic acid (FFA) and Pyr3. Ca^{2+} oscillations were inhibited by U73122, which prevent both InsP3 and DAG production. Consistently both FFA and Pyr3 abrogated VEGF-induced Ca^{2+} signals. Moreover, depletion of the InsP3-sensitive Ca^{2+} pools with cyclopiazonic acid (CPA), switched the oscillatory response to VEGF into a monotonic Ca^{2+} increase. Finally BTP-2, a selective inhibitor of SOCE, shortened the Ca^{2+} signal. Interfering with the Ca^{2+} response to VEGF by utilizing FFA, BAPTA, an intracellular Ca^{2+} buffer, and BTP-2 prevented UCB-ECFC proliferation and tubulogenesis.

Conclusion

These data indicates that VEGF induces Ca^{2+} oscillations in UCB-ECFCs which depend on TRPC3 recruitment by PLCγ-mediated production of DAG. The following Ca^{2+} entry elicits the oscillatory signal by promoting the interplay between InsP3Rs and SOCE: the repetitive Ca^{2+} spikes, in turn, drive cell proliferation and tubulogenesis. This signaling pathway is different to that observed in PB-ECFCs owing to the involvement of TRPC3. Future studies will have to outline whether TRPC3 overexpression in PB-ECFCs augments their proliferative rate *in vitro* and their regenerative potential *in vivo*.

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PC36

The effects of transient multiple episodes of hypoxia on spontaneous rat uterine contractility: does hypoxic preconditioning occur?

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During labour the myometrium experiences transient hypoxic episodes as contractions occlude blood vessels, yet labour progresses and contractions increase. We have therefore investigated the effect of short multiple episodes of hypoxia on uterine contractility.
Methods: Longitudinal uterine strips from pregnant and non pregnant rats were dissected and mounted in organ bath, bubbled with Hepes buffered, oxygenated physiological saline (2 mM Ca, pH 7.4), for isometric force recording. The effect of repeated 5 min hypoxia was induced by replacing the oxygen for nitrogen. In other protocols uterine preparations from pregnant rats were subjected to5 min episodes of repeated extracellular acidosis(from pH 7.4 to 6.9) or to hypoxia and 0-Ca (EGTA) to determine their role in the effects produced by hypoxia.
Result: In stable, spontaneously active preparations, the effect of a single episode of hypoxia was to significantly decrease or abolish the contractions in both pregnant and non pregnant rats (n=12 and 6 respectively). In pregnant rats, there was a gradual increase in force during and after each hypoxic episode. By the 5th hypoxic episode the amplitude of contractions upon re-oxygenation was significantly higher (141+/- 8.7% compared to 100% control) than that of the control period and was unchanged in time matched controls. There was also increased contractile activity during the hypoxic period. In contrast, in non pregnant rats hypoxic episodes always abolished contractions and although there was recovery with re-oxygenation, activity never exceeded that seen at the start of the experiments. Repeated 5 min episodes of 0-Ca in the presence of Oxygen abolished force during the episode and force rapidly recovered upon changing back to normal solution and although there was a transient increase in force amplitude activity never exceeded control (99+/-0.08% compared to 100% control, n= 7). However with repeated 0-Ca and hypoxia simultaneously we found a sustained gradual increase in force upon reoxygenation which was significantly different from that found in presence of Ca (159+/-9%,compared to 100% control, n= 10). We also found a gradual increase in force amplitude after each acidotic episode (after return to normal pH) (139+/-5% compared to 100% control, n= 8).
CONCLUSIONS: We concluded that transient, repeated hypoxic episodes significantly increase the amplitude of force produced by pregnant rat uterus. This suggests that hypoxic preconditioning may be present in the uterus. In non pregnant rats, the phenomenon was not seen, suggesting that this could be part of a mechanism switched on in preparation for labour. We also concluded that the extracellular acidosis associated with hypoxia could be a part of mechanism involved in hypoxic preconditioning and Ca entry may not be involved.

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PC37

Characterisation of signalling mechanisms that underlie P2Y receptor-mediated vasoconstriction of rat intrapulmonary artery

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P2Y receptors are a family of G protein-coupled receptors that are activated by the endogenous nucleotides UDP and UTP. In arteries, endothelial P2Y receptors mediate vasodilation, whilst P2Y receptors on arterial smooth muscle cells mediate vasoconstriction (Chootip et al., 2002). We have shown that Ca2+ influx via Cav1.2 ion channels (Mitchell et al., 2007), Ca2+-sensitisation via rho kinase and protein kinase C (PKC) (Tengah & Kennedy, 2007) all contribute to nucleotide-evoked contractions of rat intrapulmonary arteries (IPA). The aim of this study was to determine the relative roles of these signalling components and Ca2+-dependent Cl- channels (ICl,Ca).

5mm rings of IPA were dissected from male Sprague-Dawley rats (200-250g), which were killed by cervical dislocation. The endothelium removed by gentle rubbing of the intima and the rings mounted under isometric conditions in 1ml baths at 37°C and a resting tension of 0.5g. Tension was recorded by Grass FT03 transducers connected to a Powerlab/4e system. Data were analysed using Student's paired t test or 1-way ANOVA as appropriate.

UDP and UTP (both 300μM) evoked slowly developing contractions that reached a peak within 2-3 min. Preincubation for 15 min with the ICl,Ca blocker, niflumic acid (1μM) had no effect on contractions to KCl (40mM, n=5), showing that this concentration does not block Cav1.2 ion channels or interact directly with the myofilaments to depress smooth muscle contractility. The peak amplitude of responses to UDP ($P<0.05$, n=5) and UTP ($P<0.01$, n=5) was, however, reduced significantly by approximately 40-55%. Nifedipine (1μM) also significantly inhibits the peak response to UDP and UTP by about 40-55% (Mitchell et al., 2007) and coadministration of niflumic acid (1μM) plus nifedipine (1μM) for 15 min inhibited contractions to UDP and UTP to the same extent as either agent alone (n=4 each). Pre-incubation for 15 min with the rho kinase inhibitor Y27632 (10μM) reduced significantly the peak responses to UDP ($P<0.01$, n=6) and UTP ($P<0.01$, n=5) by around 20-30%, whilst the PKC inhibitor, GF109203X (10μM) depressed the response to UDP by about 50% ($P<0.01$, n=6) and that of UTP ($P<0.01$, n=5) by about 20%. Coapplication of both inhibitors produced significantly greater inhibition than either alone (UDP: $P<0.001$, n=6; UTP: $P<0.01$, n=7). Additional co-application of nifedipine (1μM) with Y27632 (10μM) and GF109203X (10μM) caused no further inhibi-

tion of the contractions to UDP (n=5), but further depressed the response to UTP ($P<0.05$, n=5).
These results indicate that ICl,Ca induces Ca2+ influx via Cav1.2 ion channels, which mediates about half of the peak contraction amplitude of rat IPA evoked by UDP and UTP. The remainder of the response is due to Ca2+-sensitisation via rho kinase and to PKC acting at an as yet unidentified site.

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PC38

Voltage-gated calcium channel antagonists block Nicotinic acid adenine dinucleotide phosphate-induced calcium signals via Two Pore Segment Channel subtype 2

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Our recent proposal that nicotinic acid adenine dinucleotide phosphate (NAADP) mobilises acidic Ca^{2+} stores via two pore segment channels (TPCs, TPCN for gene name) has received significant support from subsequent studies (1, 2). TPCs represent evolutionary intermediates between the Voltage-Gated Ca^{2+} Channels and Transient Receptor Potential Channel families (3). Using methods described previously (1), we assessed the effect of two different voltage-gated Ca^{2+} channel antagonists on NAADP-dependent Ca^{2+} signalling via TPC2, namely a dihydropyridine, Nifedipine, and a phenylalkylamine, Verapamil. All procedures accorded with current UK legislation.
When applied by intracellular dialysis from a patch-pipette (voltage-clamp mode, -40 mV holding potential), 10 nM NAADP evoked robust Ca^{2+} transients in both acutely isolated rat pulmonary arterial smooth muscle cells (PASMCs) and in HEK293 cells that stably over-expressed human (h) TPC2. The Fura-2 fluorescence ratio (F340/F380) increased from 0.65 ± 0.04 to 1.85 ± 0.14 (n=20) in acutely isolated rat PASMCs and from 0.31±0.02 to 1.11±0.06 (n=21) in hTPC2 expressing HEK293 cells, respectively. In both cell types, NAADP-evoked Ca^{2+} transients were markedly attenuated by thapsigargin (1μM), the F340/F380 ratio increasing from 0.55 ± 0.04 to 0.90 ± 0.03 (n=8) in PASMCs and from 0.25 ± 0.01 to 0.58 ± 0.03 (n=9) in hTPC2 expressing HEK293 cells, respectively. By contrast, evoked Ca^{2+} transients were abolished by bafilomycin (1μM), the F340/F380 increasing from 0.72 ± 0.04 to 0.87

± 0.06 (n=7) in PASMCs and from 0.43 ± 0.05 to 0.45 ± 0.05 (n=3) in hTPC2 expressing HEK293 cells, respectively. Consistent with the effects of bafilomycin, but not thapsigargin, 10μM Nifedipine and 10μM Verapamil abolished Ca^{2+} transients in response to 10 nM NAADP in both cell types. In the presence of Nifedipine, the F340/F380 ratio increased from 0.35 ± 0.06 to 0.39 ± 0.06 (n=6) in PASMCs and from 0.43 ± 0.03 to 0.51 ± 0.04 (n=5) in hTPC2 expressing HEK293 cells, respectively. With Verapamil, F340/F380 increased from 0.32 ± 0.04 to 0.35 ± 0.06 (n=5) in PASMCs and from 0.4 ± 0.08 to 0.47 ± 0.08 (n=4) in hTPC2 expressing HEK293 cells, respectively.
Using deconvolution microscopy we found that mcherry tagged hTPC2 was bound by extracellularly applied bodipy dihydrohypyridine (n=3). We conclude that both voltage-gated Ca^{2+} channel antagonists bind to and are effective blockers of hTPC2.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC39

Nicotinic acid adenine dinucleotide phosphate evokes global calcium signals in mouse pulmonary arterial smooth muscle cells by activating Two Pore Segment Channel 2

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Increasing evidence provides strong support for our proposal that the Two Pore Segment Channels (TPCs, TPCN for gene name) (TPCs) mediate intracellular calcium (Ca^{2+}) release from endolysosomal stores in response to nicotinic acid adenine dinucleotide phosphate (NAADP)(1). Studies have identified and characterized 3 subtypes of this channel family, with TPC2 targeted to lysosomes and TPC1 and TPC3 targeted to endosomes (2). In this study, we investigated the effects of TPC2 knockout on lysosomal distribution and Ca^{2+} signaling in pulmonary arterial smooth muscle cells (PASMCs) derived by explant culture. All procedures were in accordance with animal handling and procedure regulations of the United Kingdom.
Consistent with our previous findings on acutely isolated rat pulmonary arterial smooth muscle cells (3), intracellular dialysis from a patch pipette (voltage-clamp mode, -40 mV holding potential) of 10 nM NAADP induced a global Ca^{2+} transient

in PASMCs developed from pulmonary artery explants of wild type (WT) mice on day 4 of culture. The Fura-2 fluorescence ratio (F340/F380) increased from 0.36 ± 0.05 to 1.38 ± 0.18 (n=10). Evoked transients were markedly attenuated by pre-incubation of cells with thapsigargin (1μM) and abolished by pre-incubation with bafilomycin (1μM). In marked contrast, 10 nM NAADP failed to induce a Ca^{2+} transient in PASMCs developed by 4 day culture of artery explants from TPCN2 knockout mice; TPC2 knockout being confirmed by genotyping and by quantitative RT-PCR. Surprisingly, however, when the concentration of applied NAADP was raised to 1μM, small and spatially restricted Ca^{2+} transients were evoked in PASMCs (Day 4) from TPCN2 knockout mice; F340/F380 increasing from 0.32 ± 0.02 to a peak of 0.75 ± 0.04 (n=8). Semi-quantitative analysis of confocal images suggests that TPC2 knockout was not accompanied by an identifiable change in the size of Lysotracker labeled acidic organelles (WT day 4 = 1.3 ± 0.06μm^3; KO Day 4 = 1.4 ± 0.1μm^3). Nor did we see any change in the organization of Lysotracker labeled acidic organelles, which remained largely perinuclear in both WT and knockout PASMCs. We conclude that NAADP mediates global Ca^{2+} transients in PASMCs by activating the lysosome targeted TPC2.

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PC40

Lipid droplets in gravid myometrium from normal and obese BMI women

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Pregnancy in the presence of maternal obesity is associated with several undesirable outcomes including a reduction in the incidence of spontaneous term labour, and increases in the need for labour induction and caesarean sections (1). This implies maternal obesity may have an inhibitory effect on myometrial function.
In obesity there is an excess of lipid droplets (LDs) (2). LDs are intracellular accumulations of lipid surrounded by a phospholipid bilayer (3). In non-adipocyte cells, LDs function in intracellular lipid homeostasis and other diverse cellular functions. Our hypothesis states that increased BMI may be associated with an increase in LDs in the myometrium and this may adversely affect contractility of these cells.
Nile Red was used to stain LDs; this is a fluorescent dye with an emission wavelength that depends on the nature of the environment. In neutral lipids (eg triglyc-

erides found in LDs) it fluoresces yellow-gold, in polar lipids (eg phospholipids) it fluoresces red, and in aqueous media it is quenched.
Biopsies of human myometrium were obtained at elective caesarean section performed in the third trimester in obese (BMI 30.6-38.3, n=14) and normal (20.3-24.7, n=15) women. All biopsies were performed with local ethical committee approval. Biopsies were fixed, cryoprotected, frozen and sectioned. Sections were stained with 20μg/ml Nile red solution. Z-stacks were collected with an Olympus Fluoview confocal microscope. 2 slides were stained per biopsy and 2 z-stacks were imaged for each slide. Volocity Improvision Software was used to estimate volumes of LDs and total tissue from which volume fractions were calculated. Statistical analysis was carried out with Minitab Software using log transformed data which was normally distributed and had equal variance.
The mean volume fraction ± SEM of LDs in tissue was (0.00041 ±0.000073) for normal and (0.00073 ±0.000165) for obese groups. A 2-sample T-test was performed, no significant difference was found between obese (BMI >30, n= 14) and normal (BMI < 25, n=15) groups (p –value 0.082). There was also no significant difference observed when a morbidly obese subgroup (BMI >35, n=4) was compared to the normal group (p-value 0.079).
The distribution of LD volumes was also considered. The droplets were assumed to be spherical and volumes were calculated from the x and y dimensions. Volumes of individual droplets were distributed into 19 size categories and the number of droplets in each bin was considered. A Chi square test was performed which showed no significant difference in the distribution of LD volumes between normal and obese groups (p-value 0.995). Despite the apparently different functional characteristics of human myometrium in obese BMI women, in comparison to normal BMI women, the volume and size distribution of LDs does not vary in the myometrium from both of these groups.

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PC41

Modulatory effects of K+ channel blockers on contractile actions of P2X1-purinoceptor agonists in human vas deferens

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Contractions of human vas deferens circular muscle to nerve stimulation and noradrenaline are modulated by intermediate (IKCa) but not large (BKCa) conductance Ca2+-activated K+ channels (Medina et al., 2010). This finding raises issues on the

roles of BKCa and delayed rectifier (KV) K+ channels identified in electrophysiological studies of smooth muscle cells isolated from human vas deferens (Park et al., 2004). The current study investigated the effects of K+ channel blockers on the contractile actions of α,β-methylene ATP (α,β-meATP) and ATP in muscle types of human vas deferens in order to clarify the role of K+ channels in modulating P2X1-receptors-induced contractility and excitability of the tissues.

Human vas deferens specimens obtained after elective vasectomies (with patients' consent and College Ethical approval) were cut into longitudinal muscle strips or rings of circular muscle. These were set up for tension recording and superfused with Krebs medium (36o C; pH 7.4). The effects of K+ channel blockers were determined on α,β-meATP-induced contractions.

α,β-meATP (3-100 μM) evoked transient contractions of longitudinal but rarely of circular muscle. The contractions were blocked by suramin (300 μM). Both muscle types were unresponsive to ADP-βS (100 μM), a P2Y-receptor agonist. ATP (0.1-3 mM) activated only longitudinal muscle even in ARL 67156 (100 μM), an ecto-ATPase inhibitor. Longitudinal muscle contractility to α,β-meATP were enhanced by FPL 64176 (1 μM), an L-type Ca2+ agonist; 338-368%), TEA (0.3-1 mM), non-specific K+ channel blocker, 4-aminopyridine (0.1-0.3 μM), selective KV channel blocker and iberiotoxin (0.1 μM, $P<0.01$), selective BKCa channel blocker. Notably, quiescent circular muscles responded to α,β-meATP in FPL 64176 (1 μM) and robustly in iberiotoxin (0.1 μM). Apamin (0.1 μM), an SKCa channel blocker was ineffective in both muscle types.

The results indicate that stimulation of P2X1-receptors in human vas deferens elicits excitatory effects that (a) lead to longitudinal muscle contraction and secondarily to activation of KV and BKCa channels and (b) is sub-contractile in circular muscle primarily due to ancillary activation of BKCa channels. The results provide an experimental basis for resolving not only conflicting reports on the involvement of purinergic activation in human vas deferens (Banks et al., 2006; Medina et al., 2010; Hedlund et al., 1985; Anton and McGrath, 1977) but also how the modulatory effects of specific K+ channels may be pivotal for purinergic contribution to overall contractile function of the tissue, which also involves action by co-released neurotransmitter, noradrenaline.

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PC42

New insights into the mechanism of ATP sensitivity of cloned smooth muscle ATP-sensitive potassium channels

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ATP-sensitive K+ (K_{ATP}) channels are hetero-octameric complexes of two subunits: four pore-forming K_{ir}6.x and four regulatory SURx subunits. In vascular and non-vascular smooth muscle (SM), K_{ATP} channels contribute to the resting membrane potential and modulate the muscle tone. K_{ATP} channels in these tissues are comprised of the same auxiliary subunit (SUR2B) but different pore-forming subunits. K_{ir}6.1 is predominantly found in vascular SM while K_{ir}6.2 is found in non-vascular SM. In spite of the fact that K_{ir}6.1 and K_{ir}6.2 share ~70% sequence identity their sensitivity to ATP differs by more than 30-fold. These observations suggest that in vascular SM cells K_{ATP} channels are active under resting metabolic condition while in non-vascular SM the contribution of these channels to the membrane potential will become more relevant under conditions of high metabolic demand. Here we investigate the molecular basis for the differential response to ATP of K_{ir}6.1 and K_{ir}6.2-containing K_{ATP} channels. We performed systematic mutations in K_{ir}6.2 exchanging residues with the reciprocal residues in K_{ir}6.1. The putative ATP and PIP_2 binding sites, “slide helix” and critical residues at the interface between subunits were investigated.

Inside-out patch-clamp recordings from HEK293T cells heterologously expressing wild type and mutant K_{ir}6.x co-expressed with SUR2B were used in this study. The extracellular solution contained (in mM): 140 KCl, 10 HEPES, 1.2 $MgCl_2$ and 2.6 $CaCl_2$; pH 7.4 with KOH. The intracellular solution contained (in mM): 115 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 25 KOH; pH 7.2 with KOH. Mg-ATP was included as necessary and the pH was readjusted to 7.2 with KOH. All numerical data are presented as mean ± SEM.

Mutations within the ATP binding site, slide helix and subunit interaction domains of K_{ir}6.2 had no effect on ATP sensitivity. However, simultaneous mutation of two residues in K_{ir}6.2 within the PIP_2 binding site, K39S and N41A, caused an increase in IC_{50} for ATP from 101 ± 22μM (n=17), in wild-type channels, to 264 ± 55μM (n=16), bringing it towards that of K_{ir}6.1. This is a small shift (~2.5-fold) compared to the difference observed between wild-type K_{ir}6.1 and K_{ir}6.2 (>30-fold). The effect of this mutation is not secondary to a change in channel gating as the single-channel open probability (P_o) remained unchanged (0.44 ± 0.02, (n=6, K_{ir}6.2-K39S-N41A) vs 0.38 ± 0.07, (n=6, K_{ir}6.2)). Furthermore, maximal activation by PIP_2 and the effect of repeated PIP_2 application on ATP inhibition were unaffected. These data suggest the difference in ATP sensitivity between cloned vascular and non-vascular K_{ATP} channels is not due to distinct ATP-binding affinities/efficacies or the sensitivity to PIP_2 and that other regions of the channels than those tested here must be involved in determining the difference in ATP sensitivity.

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PC43

Effect of fenamates and their new synthetic analogues on TRPC4 and TRPC5 channels

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Fenamates are non-steroidal anti-inflammatory agents (NSAIDs). Flufenamic acid (FFA) activates TRPA1 and TRPC6, but inhibits TRPM2/4/5, TRPC3 and TRPC7 (Eisfeld and Lückhoff, 2007; Hu *et al*., 2010). However, the effect of fenamates on TRPC4 and TRPC5 is still unknown. To understand the structure-activity of fenamic compounds on TRPC channels, we synthesised a series of fenamic analogues and investigated their effect on TRPC4 and TRPC5 channels.

Human TRPC4 and TRPC5 cDNAs in tetracycline-regulated (tet-on) pcDNA4/TO vectors were stably transfected into T-REx HEK293 cells. The expression was induced by 1 μM tetracycline and the whole cell current of TRPC4 or TRPC5 was recorded by patch clamp. Calcium imaging was used for the measurement of Ca^{2+} influx and Ca^{2+} release in the cells.

We found that FFA, mefenamic acid (MFA), niflumic acid (NFA) and diclofenac sodium (DFS) inhibited TRPC4 and TRPC5 channels in concentration-dependent manner. The potency was FFA>MFA>NFA>DFS. Modification of the 2-phenylamino ring by substituting the three fluorine atoms in FFA with -F, -CH_3, -OCH_3, -OCH_2CH_3, -COOH and -NO_2 led to changes in the blocking activity. Selective COX1-3 inhibitors (aspirin, celecoxib, acetaminophen and indomethacin) had no effect on the channel activity. However, longer perfusion (>5 min) with FFA (100 μM) and MFA (100 μM) caused a gradual increase in TRPC4 and TRPC5 currents after their initial inhibitory effects. Our results suggest that fenamates and their new analogues are TRPC4 and TRPC5 channel blockers. The polarity and confirmation of the 2-phenylamino ring could alter their blocking activity on the channels. No effect of non-fenamate inhibitors on COX1-3 indicated that the channel blocking effect is not mediated by the COX inhibition. These findings give a clue that the direct effect on TRPC channel activity or Ca^{2+} influx could be one of the mechanisms of fenamates.

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SA01

Biological Nanospaces

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The beauty of biological systems is that cells as diverse as human muscle cells and growing plants cells are composed of the same basic set of macromolecules such as proteins, lipids and DNA. Common cellular macromolecules form membrane-bound organelle structures found in all cell types and the concept that a closed organelle system contains specialized biochemical functions is well accepted. An emerging and more revolutionary concept is that areas of the cell that are between organelles, as a consequence of their nanostructure, are also structurally specialized regions of distinct and important functions.
Conceptually, the specific three dimensional architecture of the nanospace allows strategically positioned selected transport molecules to convert random thermal motion into directed flow, enabling reactions that would otherwise be energetically unfavourable. Biological nanospaces vary in width from a few to about 50 nanometres and are situated between apposing membranes within the cell, but are contiguous with the cytosol, which differentiates them from membrane delimited organelles.
This introduction will address the importance of cytoplasmic nanospaces in Ca^{2+} transport and site and function specific signalling in smooth muscle. It will begin with a historical perspective on the study of the junctional space between the plasma membrane and the peripheral sarcoplasmic reticulum (SR), including experimental evidence for its role in the generation of asynchronous cytoplasmic Ca^{2+} waves. Subsequently a number of other critically important nanospaces, which are located between the SR, caveolae, mitochondria, lysosomes and other endosomes will be discussed. Finally it will be considered how loss of nanospace ultrastructure may lead to smooth muscle dysfunction and disease.

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SA02

Two-pore channels in integrative calcium signaling

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Recent studies have revealed the importance of two-pore segment channels (TPCs) in mediating NAADP-evoked Ca2+ release from acidic organelles. The Ca2+ signal initiated from the acidic stores can recruit additional Ca2+ release from endoplasmic reticulum (ER) via Ca2+-induced Ca2+ release (CICR). Both humans and mice express two TPCs, TPC1 and TPC2 whereas many other vertebrates express also TPC3. We have shown that each TPC subtype may be targeted to different acidic organelles, with TPC1 predominantly localized to a subpopulation of endosomes while TPC2 almost exclusively expressed in lysosomes. Consistent with these expression patterns and the fact that endosomes are smaller and less clustered than lysosomes, TPC1 mediated Ca2+ release in response to 10 nM NAADP in a manner that was spatially restricted, insensitive to thapsigargin, and unable to trigger global Ca2+ transient. By contrast, Overexpression of TPC2 resulted in a biphasic Ca2+ response to NAADP with a pacemaking phase followed by a large secondary and global Ca2+ transient and the latter response was largely attenuated by ER store depletion with thapsigargin. Therefore, only TPC2 appears to couple to the ER by CICR. Interestingly, the expression of chicken and rabbit TPC3 in HEK293 cells yielded distinct subcellular localizations and functional data are consistent with differential organelle targeting. Our data suggest that cross-talk between acidic stores and ER is governed by the capacity of local Ca2+ signals from acidic Ca2+ stores to summate, and in a manner that may determine whether the threshold for CICR from ER is breached.

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SA03

Calcium signaling in pulmonary arterial smooth muscle: are nanojunctions required to support the locks and the keys

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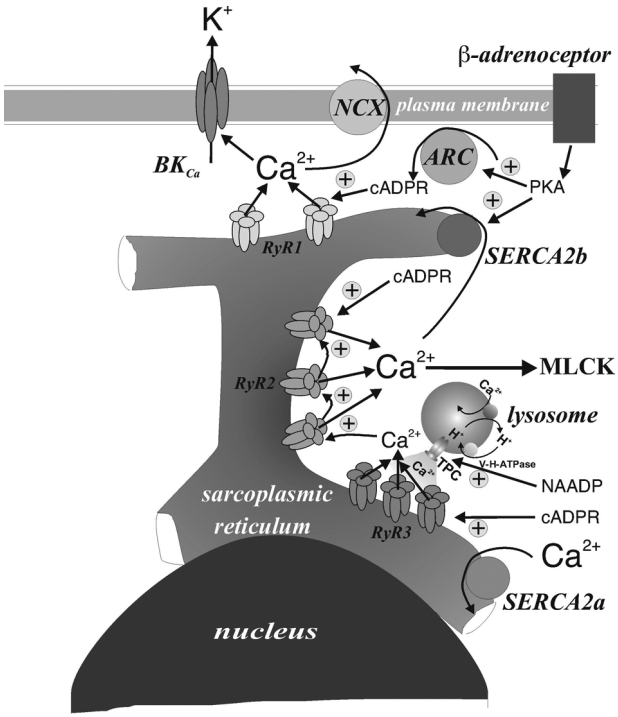
In acutely isolated pulmonary arterial smooth muscle cells (PASMCs) the Ca^{2+} mobilizing messenger nicotinic acid adenine dinucleaotide phosphate (NAADP) triggers bursts of Ca^{2+} release from lysosomes (Boittin et al., 2002; Kinnear et al., 2004) by activating the Two Pore Domain Channel subtype 2 (TPC2;Calcraft et al., 2009). These Ca^{2+} bursts initiate a propagating wave by subsequently triggering Ca^{2+}-induced Ca^{2+} release (CICR) from the SR via ryanodine receptors (RyRs). A large proportion of lysosomes form tight clusters within the perinuclear region of PASMCs and in a manner consistent with the spatially restricted nature of NAADP-dependent Ca^{2+} bursts. Moreover, these lysosomal clusters are closely associated with a sub-population of RyRs (Kinnear et al., 2004). In fact, lysosomes may be separated from closely apposed RyRs by a narrow junction or cleft (<100 nm) that is beyond the resolution of light microscopy. Thus, lysosome-sarcoplasmic reticulum (SR) junctions may form a highly organised "trigger zone" for Ca^{2+} signaling in response to NAADP. This may constitute a novel nanodomain with unique functional characteristics, in that the presence of such a junction offers an explanation for the fact that NAADP-dependent Ca^{2+} bursts induce propagating Ca^{2+} waves by triggering CICR from the SR via RyRs in an all-or-none manner, and as such these proposed junctions may provide a "margin of safety" with respect to the initiation of vasoconstriction by NAADP.

The precise mechanisms involved appear yet more complex, because all three RyR sub-types are expressed in arterial smooth muscle and some of these lysosome clusters may selectively couple to RyR3 (Kinnear et al., 2008). Determining factors in this respect could be the relative sensitivity of each RyR sub-type to CICR, the maximum gain in response to Ca^{2+} and the relative sensitivity of each receptor subtype to inactivation by Ca^{2+}. RyR3 would provide for a higher "margin of safety" with respect to the all-or-none amplification of Ca^{2+} bursts at the putative lysosome-SR junction. Inclusion of RyR3 alone in this model cannot, however, explain the subsequent generation of a propagating Ca^{2+} wave as RyR3 labeling declines markedly outside the perinuclear region of PASMCs. However, labeling for RyR2 increases markedly as we move from the perinuclear to the extraperinuclear region. This suggests that RyR2 may function to receive Ca^{2+} from RyR3 at the interface of lysosome–SR junctions and thereby allow for further propagation of the Ca^{2+} signal by CICR. Importantly, our recent studies have also shown that SERCA2a is almost entirely restricted to the perinuclear SR of PASMCs (Clark et al., 2010) and

thus sits proximal to RyR3. Therefore we have the necessary transporter to support Ca^{2+} uptake into the SR within the region of lysosome-SR junctions.
In marked contrast to SERCA2a, however, SERCA2b is targeted to the SR proximal to the plasma membrane (PM). Moreover it is this region of the SR to which RyR1 may be preferentially targeted. The combination of limited support for signal propagation by RyR1 with the removal of Ca^{2+} from the cytoplasm by SERCA2b may effectively segregate the PM-SR space in these cells (Clark et al., 2010). That this may be the case is supported by functional data. Thus, adenylyl cyclase coupled receptors have been shown to elicit Ca^{2+} release proximal to the PM via RyRs and thereby open Ca^{2+}-activated K^+ channels in the plasma membrane. This initiates membrane hyperpolarization which will facilitate Ca^{2+} removal from the proposed junctional space between the SR and the plasma membrane, leading ultimately to vasodilation.
In this respect kinetic information may be used to formulate hypotheses, given that SERCA2a and SERCA2b exhibit quite different kinetics. SERCA2b has a higher affinity for Ca^{2+} but lower Vmax than SERCA2a. SERCA2b may therefore be dominant at rest and function to maintain resting levels of Ca^{2+} in the vicinity of the contractile apparatus. However, its low Vmax may lead to saturation of this SERCA with Ca^{2+} upon release of Ca^{2+} from the bulk SR, via RyR3 and RyR2, in response to stimuli that elicit vasoconstriction. Thereby, SERCA2b would allow the Ca^{2+} concentration to rise in the vicinity of the contractile apparatus, until such time as vasodilation is promoted by, for example, activation of adenylyl cyclase coupled receptors that may: (1) increase the activity of SERCA2b by PKA-dependent phosphorylation and facilitate the removal of Ca^{2+} from the bulk cytoplasm, and (2) trigger PKA-dependent Ca^{2+} release from the peripheral SR store via RyR1, to promote PM hyperpolarization and Ca^{2+} removal from the proposed PM-SR nanojunction (Clark et al. 2010).
It seems unlikely that such co-ordinated regulation of opposing cellular functions could occur without the presence of membrane-membrane junctions, as these are required to support the locks and the keys required for the initiation of Ca^{2+} signals with the desired spatiotemporal pattern.
All procedures accorded with current UK legislation



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SA04

Imaging the activity and localization of single calcium channels in intact cells

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Cytosolic Ca2+ ions play a central role as a universal intracellular messenger, controlling many aspects of physiology such as gene expression, electrical excitability, secretion and contraction. The rich diversity in information encoded by Ca2+ arises through the mechanisms by which Ca2+ signals are generated and transmitted to act over very different spatial and temporal scales. In turn, the spatio-temporal patterning and transmission of Ca2+ signals involve the inherent properties of the Ca2+ channels themselves, their spatial organization, and how these channels interact via diffusion of Ca2+ both with themselves and with effector proteins. This is exemplified by the inositol trisphosphate receptor/channel (IP3R), the opening of which requires binding of IP3 together with Ca2+ to cytosolic receptor sites (Foskett et al., 2007). Gating by Ca2+ is biphasic, creating both positive and negative feedback loops, whereby Ca2+ liberated through one channel may modulate its own opening and that of neighboring channels. Channel-channel interactions are delimited by the spatial arrangement of channels, and IP3Rs are organized into tight clusters on the ER membrane. This leads to a hierarchy of Ca2+ signals of differing magnitudes, kinetics and spatial extent (Callamaras et al., 1998): 'Fundamental' signals (Ca2+ blips) representing Ca2+ flux through individual channels; 'elementary' Ca2+ transients (Ca2+ puffs) generated by openings of multiple IP3R within a cluster, orchestrated by Ca2+-induced Ca2+ release (CICR); and propagating global waves of Ca2+ resulting from progressive recruitment of multiple clusters by successive cycles of Ca2+ diffusion and CICR.

Electrophysiological patch-clamp recording - the gold standard for studying single channels under tightly controlled ionic and electrical conditions - is thus poorly suited to studies of the coordinated behavior of multiple, clustered Ca2+ channels in the intact cellular environment. Instead, our knowledge of Ca2+ signaling at the cellular and sub-cellular levels derives largely from optical imaging in intact cells employing fluorescent Ca2+ indicator dyes. Nevertheless, limitations of imaging technology have, until recently, precluded detailed study of single-channels and of channel-channel interactions within clusters which are too small to resolve by conventional light microscopy. This has now changed. Developments in optical imaging, particularly the application of total internal reflection fluorescence (TIRF) microscopy, enable the recording of single Ca2+ channel activity within live cells (Smith & Parker, 2009). Moreover, the wavelength of light no longer limits resolution: optical microscopy has become nanoscopy Patterson et al., 2010). We can

localize Ca2+ channels at nanometer scales and study how their function is influenced by their spatial distribution.
I will describe our use of these innovative techniques to take studies of Ca2+ signaling in cultured cell lines down to the truly single-molecule level, and discuss their application to studies in smooth muscle cells.

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SA05

Calcium channels of vascular remodelling

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Vascular remodelling in physiology and disease is substantially dependent on vascular smooth muscle cells and endothelial cells that switch to phenotypes characterised by increased proliferation, migration etc. We are seeking to understand ion channels of these processes, particularly with reference to diseased human tissues. Data are consistent with specific potassium channel types, Kca3.1 and Kv1.3, causing membrane hyperpolarisation that drives calcium entry through non-voltage-gated calcium channels. A mixture of calcium-selective and calcium-permeable non-selective cationic channels is involved. An apparently special player is the TRPC1 channel subunit, which is up-regulated in vascular injury and promotes hyperplasia of smooth muscle cells in human saphenous vein (Kumar et al 2006 Circ Res 98, 557-). TRPC1 forms heteromultimeric assemblies with other TRP channel subunits that include TRPC5 (Xu et al 2006 Circ Res 98, 1381-). Endogenous vascular TRPC1-TRPC5 channels are stimulated by a range of factors, but particularly important are likely to be specific oxidized phospholipids, acting through Gi/o-protein signalling without calcium-release (AL-Shawaf et al 2010, ATVB 30, 1453-). Such lipids are suggested to be pivotal players in innate immunity and atherosclerosis. Other types of calcium channel subunit and regulator also contribute in vascular remodelling: One of them is STIM1, which shows dominant plasma membrane localisation in vascular smooth muscle cells and co-localises with a pool of TRPC1 (Li et al 2008 Circ Res 103, e97-). STIM1 is a single-pass membrane protein with promiscuity in its function, in part because it also plays a role in calcium-release activated calcium (CRAC) channels, which are formed by Orai1 and underlie cal-

cium entry evoked by the vascular smooth muscle cell recruiter, PDGF (Li et al BJP 164, 382-). The key stimulator of endothelial cells and angiogenesis, VEGF, also signals through such CRAC channels (Li et al 2011, Circ Res 108, 1190-). Potent small molecule inhibitors of these channels are starting to emerge and may provide valuable foundations for novel therapeutics (Li et al BJP 164, 382-). Our understanding of the molecular complexities of the different calcium channels and their inter-relationships is, however, in its infancy and should be further advanced.

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SA06

Stochastic three dimensional modelling of ionic transport in cytoplasmic nanospaces

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Experimental data of vascular smooth muscle (VSM) cell Ca^{2+} signals obtained by means of radioactive labelling, optical and electron microscopy, as well as molecular biological methods, have long hinted that healthy signalling depends not on passive diffusion of messenger species within the cytosol, but rather on carefully orchestrated communication between organelles.
In particular, it seems necessary that organellar membrane domains of a few hundred nanometres in extension be separated by a distance of only a few tens of nanometres in order for efficient molecular transport to occur. These membranous juxtapositions, the transporters localized therein, the intervening cytosol, and molecular species form an environment we refer to as cytoplasmic nanospaces. Due to their restricted volume, they take advantage of the intrinsic variability, or stochasticity, of quantities like concentration, channel open probability, transporter activity, and thermal motion to ensure proper cellular function.
By their very nature, our experimental observations lack the quantitative character that would permit full understanding of processes underlying Ca^{2+} signalling at these scales. Realistic quantitative modelling is ultimately necessary to aid our understanding and is becoming an essential tool to accompany experimental investigation in the biophysics and physiology of VSM.
Based on firm experimental foundations, we have developed accurate stochastic models, using state-of-the-art tools for microphysiological stochastic simulations. These combine the ability to reconstruct three dimensional nanospace geometrical features and incorporate transporter distributions and kinetics with sophisti-

cated so-called random walk algorithms that faithfully mimic molecular motion on those geometries.
We present results from two models. In one, we explore the nature of Na+ transients localized to plasma membrane-sarcoplasmic reticulum (SR) nanospaces and occurring upstream of the Na^+/Ca^{2+} exchanger-mediated Ca^{2+} influx that causes contractile activation of VSM via asynchronous Ca^{2+} waves. In the second instance, we examine the possibility that focal Ca^{2+} release by a nicotinic acid adenosine dinucleotide phosphate (NAADP)-sensitive receptor channel in the lysosomes could trigger a Ca^{2+} wave when lysosomes and SR form a nanospace.

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SA07

Mitochondrial architecture and mobility in resistance arteries: changes and influences in smooth muscle proliferation

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The subcellular location of mitochondria controls cell function via changes in ATP production and Ca^{2+} handling. For example, in smooth muscle, mitochondria regulate the Ca^{2+} signals (Ca^{2+} puffs) which arise from the inositol-trisphosphate-sensitive channels (IP_3R), by operating within an IP_3R cluster. Since Ca^{2+} puffs are rapid transient events, a close apposition of IP_3R and mitochondria is required for this control to take place which is incompatible with rapid free movement of mitochondria observed in several cell types. We find mitochondria in fully differentiated smooth muscle cells to be individual, ovoid, immobile structures which lack directed motion. Brownian-like movement was observed but was so restricted in nature that a displacement of individual organelles did not occur. On the other hand, in proliferative smooth muscle mitochondria are much more diverse and exist as small spheres, rod-shapes, filamentous threads and networks. In addition the organelles undergo almost continuous changes in shape and position in the form of both long distance and complex local movements. In intact resistance arteries studied at physiological pressures *in vitro*, mitochondria are largely stationary, but in a small number of cells mitochondria display mobility like that observed in single proliferative smooth muscle cells. When smooth muscle in the intact resistance artery is encouraged to proliferate, in organ culture, mitochondrial mobility increases dramatically. Significantly, when mitochondrial mobility is reduced pharmacologically smooth muscle proliferation decreases. Mitochondrial dynamics may be required to facilitate the equal segregation of mitochondria into daughter cells during cell division. The changes in mitochondrial dynamics as smooth muscle enters a proliferative state may offer a target for altering proliferation in vascular disease.

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SA08

PEA-15: a calcium-activated protein with a regulatory role in smooth muscle gene expression

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Arterial hyperplasia occurs as a result of increased vascular smooth muscle (VSM) cell proliferation. It is now understood that this is initiated by modulation of VSM cells towards a more proliferative and diseased phenotype. An important event in this phenotypic change is the active repression of smooth muscle (SM) marker proteins associated with contractility, such as SM22α, calponin and smooth muscle α-actin. This decrease in SM marker proteins is controlled by growth factor-induced gene expression via activation of the mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK)1/2. When an appropriate growth signal is received by the VSM cell, this leads to phosphorylation of ERK1/2 which in turn regulates specific transcription factors including Elk-1. Elk-1, localised in the nucleus, has a critical role in gene expression and in VSM cells is responsible for repressing SM marker genes. We have recently revealed a novel mechanism regulating ERK1/2 and subsequently Elk-1 in VSM cells which is likely to be important in regulating VSM cell phenotype. An essential step in ERK1/2-induced changes in gene expression is the translocation of ERK1/2 from the cytoplasm to the nucleus (Hunter et al, 2011). As we have demonstrated, if this translocation is prevented in VSM cells, the ability of growth factors to repress SM marker genes is inhibited. Our experimental evidence now indicates that a protein previously uncharacterized in VSM cells, phosphoprotein enriched in astrocytes-15 (PEA-15), has an essential role in regulating ERK1/2 nuclear localization. PEA-15 has a cytoplasmic distribution and one of its primary roles is to function as an ERK1/2-binding protein (Ramos, 2008). Studies have now revealed that PEA-15 binds and sequesters ERK1/2 in the cytoplasm and we have confirmed this in VSM cells. In cultured human coronary artery smooth muscle cells, knockdown of PEA-15 results in ERK1/2 nuclear localization, which can be reversed by PEA-15 overexpression. Importantly, PEA-15 binding to ERK1/2 is regulated by phosphorylation of PEA-15 at sites phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). In an unstimulated cell PEA-15 is unphosphorylated and bound to ERK1/2. Our results demonstrate that an increase in intracellular Ca^{2+} and activation of CaMKII is essential for PEA-15 phosphorylation and subsequently ERK1/2 localization to the nucleus. This phosphorylation event is the “release” mechanism which uncouples ERK1/2 from PEA-15 thereby allowing ERK1/2 translocation. In a typical growth factor-induced response in VSM cells, phosphorylation of tyrosine kinase receptors activates ERK1/2 and simultaneously releases intracellular Ca^{2+} via phospholipase Cγ1 (PLCγ1). Knock-

down of PLCγ1 inhibits PEA-15 phosphorylation although does not affect growth factor-induced ERK1/2 activation. PLCγ1 knockdown however prevents ERK1/2 nuclear localization, Elk-1 activation and repression of smooth muscle marker genes such as smooth muscle α-actin. A rise in intracellular Ca^{2+} is therefore required in growth factor-induced proliferation as this directly leads to PEA-15 phosphorylation and permits normal ERK1/2 signalling to occur.
As PEA-15 has an important role in regulating VSM cell proliferation, changes in PEA-15 expression could have profound effects on blood vessel structure and function. Our results reveal that in mouse injured arteries PEA-15 expression is reduced in the neointima compared to the medial layer. We predict that a decreased PEA-15 expression allows increased ERK1/2 translocation to the nucleus thereby inducing VSM cell proliferation and contributing to hyperplasia in injured arteries. Physiological regulation of PEA-15 expression in the context of vascular disease will be further discussed.

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SA09

NFAT as a novel target for the treatment of atherosclerosis and vascular dysfunction in diabetes?

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Proteins of the NFAT family consist of four well characterized calcium (Ca^{2+})/calcineurin-sensitive transcription factors (NFATc1-c4), originally described as transcriptional activators of cytokine and immunoregulatory genes in T cells but now known to play a role in several cell types outside the immune system. NFAT signaling is required for normal heart valve formation and vascular patterning during embryogenesis, but the role of NFAT in the vasculature during adult life is less clear.
Over the past decade we have focused on dissecting the Ca^{2+} signaling requirements for NFAT activation in the vasculature, on the identification of kinases that regulate the export of NFAT from the nucleus and on finding NFAT-target genes in this tissue. It is now also known that NFAT activation promotes migration and proliferation of cultured vascular smooth muscle cells (VSMC), and that blockade of NFAT signaling reduces neointima formation in a rat carotid artery injury model. Also, that NFAT activation enhances VSMC excitability and vasoconstriction and the expression of inflammatory markers (i.e.IL-6, tissue factor, COX-2, VCAM-1).
NFAT and vascular complications of diabetes?
Diabetes is a lifelong, incapacitating disease affecting 246 million people worldwide. Presently, it can neither be prevented nor cured and it is associated with

devastating chronic macrovascular complications including coronary heart disease and stroke as well as microvascular disorders (nephropathy, retinopathy and neuropathy). The underlying pathogenesis is not clear, but hyperglycemia has been identified as an important risk factor.
We have recently shown that modest elevations of extracellular glucose effectively activate NFAT in VSMCs of large arteries (aorta and cerebral) and in small (retinal) microvessels in mice, both *ex vivo* and *in vivo*. This effect involves the local release of extracellular nucleotides, such as UTP and UDP, acting on purinergic-2Y receptors, leading to increased $[Ca^{2+}]_i$ and subsequent activation of calcineurin and NFATc3. In addition to promoting NFATc3 nuclear translocation, high glucose also reduces nuclear export of NFAT by inhibiting GSK-3β and JNK, providing additional mechanisms for glucose-induced NFAT activation. Once activated, NFAT promotes the expression of osteopontin (OPN). Diabetic mice showed increased expression of OPN in the ascending and thoracic aorta, vascular segments particularly prone to development of atherosclerosis and this was prevented by *in vivo* treatment with the NFAT inhibitor A-285222 or by lack of NFATc3 protein in arteries from NFATc3$^{-/-}$ mice. OPN is highly expressed in human atherosclerotic lesions and is not only a marker of inflammation but also an active player in the progression of atherosclerosis and restenosis. OPN expression is increased in the media of diabetic arteries, plasma levels of OPN have been shown to significantly correlate with progression of diabetic nephropathy and OPN levels in the vitreous are enhanced in patients with diabetic retinopathy.
Thus, NFAT may act as a glucose-sensor in the vascular wall, translating changes in Ca^{2+} signals and kinase activity into changes in gene expression. We hypothesize that this signaling pathway represents an unexplored mechanism underlying diabetic vascular disease.

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SA10

Mechanistic insights on the contribution of Kv1.5 to Kv1.3 switch to vascular smooth muscle proliferation

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Phenotypic modulation of VSMCs requires dramatic changes in gene expression profile. These changes involve a switch in ion transport mechanisms, as they are key elements required to redirect cell metabolism to new functional tasks. However, the large diversity of ion channels and the heterogeneity of their expression

pattern across different vascular beds has hindered the identification of candidate channels implicated in the phenotypic switch across different VSMCs preparations. The previous work of our group sought to circumvent this limitation by using high-throughput real-time PCR, to obtain a global portrait of ion channel expression in contractile versus proliferating VSMCs. We use several mouse vascular beds and several proliferative models. Changes in mRNA expression showed a good correlation between the two proliferative models, with only two genes, Kv1.3 and Kvbeta2, increasing their expression upon proliferation. In addition, these mRNA changes translate into similar changes in protein expression and function upon proliferation. While in contractile VSMCs Kv1.5-mediated currents are predominant, in proliferating VSMCs Kv1.3 channels are the main contributors to the Kv1-mediated component of the Kv current1. Besides, functional studies show that Kv1.3 current up-regulation in these cells is an essential component of their migratory and proliferative phenotype.

The objectives of the present study were twofold: (1) to explore whether this Kv1.3 up-regulation is a conserved landmark of VSMCs proliferation in different vascular beds from different species, and (2) to explore the molecular determinants and the signalling cascade linking the functional expression of Kv1.3 channels to cell proliferation.

We have analyzed VSMCs from different human vascular beds. In all cases we found a switch from a predominant expression of Kv1.5 in the contractile phenotype to a predominant expression of Kv1.3 channels in the proliferative phenotype. This Kv1.5 to Kv1.3 switch is functionally relevant, as the selective blockade of Kv1.3 currents produced a significant decreased of VSMCs proliferation rate in all the preparations. These results point to Kv1.3 channels as good therapeutical targets to avoid unwanted VSMC remodelling.

To explore the molecular mechanisms linking Kv1.3 expression to proliferation, we tried to reproduce the pro-proliferative effect of Kv1.3 heterologously expressed in HEK293 cells. Both cell counting and BrdU incorporation assays demonstrated that Kv1.3 over-expression promotes HEK cells proliferation, while Kv1.5 over-expression inhibits it. The use of Kv1.3 mutant channels in which either gating or permeation is affected indicate that the pro-proliferative effect of Kv1.3 is not dependent on its contribution to set resting membrane potential, but is abolished when voltage-dependent conformational changes are absent. These data suggest that Kv1.3 may be a moonlighting protein that regulates intracellular signalling pathways leading to cell proliferation by a mechanism that is independent of potassium flux.

1. Cidad et al., (2010) Arterioscler Thromb Vasc Biol. 30:1203-1211

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SA11

Calcium signalling and angiogenesis

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Several important genes that are involved in tissue remodeling and angiogenesis are switched on by virtue of CRE response elements in their promoters. Endothelin (ET) is an important vasoactive mediator that plays roles in vascular remodelling and angiogenesis, by activating 7 transmembrane G protein-coupled receptors (GPCR). Here I will describe studies carried out in our lab characterising the mechanisms ET-1 uses to regulate CRE-dependent remodeling genes in pulmonary vascular smooth muscle cells. We found that ET-1 activated several CRE response genes in vascular smooth muscle cells through two pathways. The first was an autocrine loop involving cPLA2, arachidonic acid release, COX-2-dependent PGI(2) synthesis, and IP receptor-linked elevation of cAMP leading to CRE transcription activation. The second involved a calcium-dependent, COX-2 independent, pathway involving calcium influx through T type voltage-dependent calcium channels. These studies give important insights into the upstream signaling mechanisms used by G protein-coupled receptor-linked mediators such as ET-1, to activate CRE response genes involved in angiogenesis and vascular remodelling.

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SA12

Endothelial cells and vascular disease

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Endothelial progenitor cells (EPCs) are a heterogeneous cluster of cells which have received a growing interest since the seminal work by Asahara et al., in 1997. These cells are born in the bone marrow and are released to the blood in response to ischemia; they subsequently attach to damaged vessels and repopulate the endothelium or support local angiogenesis.
Unfortunately, there is not a consensus definition of "EPC" based on surface markers or proliferate capabilities; however, most studies so far have assessed these cells by flow cytometry as CD34+/KDR+ events, although many cells included under this definition behave as angiogenic monocytes. Nevertheless, EPCs and angiogenic cells seem to work together in order to maintain a healthy endothelial function. EPCs and angiogenic monocytes are originated from the bone marrow and guided

by their chemokine receptor CXCR-4, which binds to ischemic-related SDF-1. In this journey, these cells subsequently acquire CD34, CD133, KDR, CD144 or VE-cadherin and von Willebrand factor. After reaching their destination, these cells secrete angiogenic factors to support local endothelial cells. This paracrine angiogenic role seems to be the key effect of these cells, according to the recent experimental data. This paracrine function is able to promote endothelial cell survival and subsequent maintenance of vascular tone, haemostasis and lack of inflammation. Thus, a healthy vascular function is repaired or maintained.

Of note, a healthy vascular function has been demonstrated to be deeply and early disrupted in atherosclerosis, the first global cause of death. Atherosclerotic vascular disease is able to decrease the number and function of EPCs. This decrease is directly linked to coronary obstruction beyond classical cardiovascular risk factors. In the last years it has been becoming increasingly evident that EPCs are initially increased after vascular damage, being further depleted in advanced cardiovascular disease. Many research efforts are being made in order to elucidate which factors determine an abnormal release of EPCs from their bone marrow origin and mediate this EPC exhaustion in atherosclerosis. It is expected that bone marrow will become the last frontier for our understanding of EPC dysfunction in atherosclerosis, and may set the basis for a putative unification of the vasculogenic and immune hypotheses of atherosclerosis.

EPCs and related cells are easily measured from peripheral blood by flow cytometry. Further clinical EPC assessments under a common protocol will set the basis for their usage as a sensitive and specific diagnostic and prognostic marker, since a single cell may be understood as the result of thousands of molecular interactions. EPCs may also be used as a therapeutic agent. Clinical trials show a promising value in terms of ejection fraction and survival, although the elucidation of the best EPC population and technical procedure will still take several years of effort. Eventually, classical cardiovascular drugs (eg. statins, pioglitazone) and target-designed ones are able to stimulate EPC function. This goal may also be achieved by target engineered scaffolds for cardiovascular surgery and endovascular stents.

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