SA1

In vivo Ca^{2+} imaging

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We have developed mice in which a purposed designed Ca^{2+} indicator is expressed in a lineage-specific fashion for the examination of Ca^{2+} signalling *in vivo*. The indicator, GCaMP2, is stable at physiological temperatures and displays excellent signal strength, dynamic range, and on/off kinetics *in vivo*. Lines of mice expressing GCaMP2 in the heart under control of a tetracylcline transactivator have been created. These mice report cellular Ca^{2+} transients over all regions of the heart with every beat. Experiments have characterized the kinetic limitations of GCaMP2 in simultaneous Rhod2 recordings in isolated, perfused hearts from humanely killed animals. Finally, we have used cardiac GCaMP2 mice to examine early embryonic development of the heart. These experiments provide new insight into the development of the SA and AV nodal regions of the heart and demonstrate the utility of this approach for the examination of complex physiological functions in mammals, *in vivo*.

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

SA2

Coupling between receptors, channels and intracellular calcium signalling in smooth muscle of small intestine

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Parasympathetic control of gastrointestinal smooth muscles is mediated through muscarinic receptors (M_2 and M_3 subtypes), activation of which by acetylcholine causes depolarization of the smooth muscle cell (SMC) membrane via cationic channel opening. Membrane depolarization increases the frequency of action potentials (APs) and thereby facilitates Ca^{2+} entry through voltage-gated Ca^{2+} channels (VGCCs). Activation of M_2 receptors primarily gates the cationic channels via the α- GTP subunit of G_o protein, whereas activation of M_3 receptors is coupled via $G_{q/11}$ protein to stimulation of phospholipase C-β, which hydrolyses phosphatidylinositol-4,5-bisphosphate to diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). While DAG has no effect on muscarinic cationic channels, Ca^{2+} mobilized from intracellular stores in response to IP_3 and Ca^{2+} entry through VGCCs potentiates muscarinic cationic current (mI_{cat}).

The experiments were conducted on single SMCs freshly isolated from the longitudinal layer of the guinea-pig ileum. The animals were humanely killed. Using fluorescence confocal imaging we analysed the spatio-temporal profile of $[Ca^{2+}]_i$ changes following stimulation of muscarinic receptors with carbachol (CCh) and related the spatial pattern of CCh-induced $[Ca^{2+}]_i$ transients to the spatial distribution of the intracellular Ca^{2+} stores, IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs). By combining confocal imaging with simultaneous patch-clamp recording we related $[Ca^{2+}]_i$ dynamics to the kinetics of mI_{cat} and evaluated the effect of RyR- and IP_3R-mediated Ca^{2+} release on mI_{cat}. In SMCs loaded with the high-affinity Ca^{2+} indicator fluo-3 and voltage-clamped at –50 mV, initiation of CCh (10 μM)-induced Ca^{2+} waves preceded the appearance of mI_{cat} (n = 7), which reached a peak about 1 s after the maximal increase in $[Ca^{2+}]_i$ was observed (n = 17). Neither spontaneous Ca^{2+} sparks (n = 8) nor caffeine (5 mM)-induced (n = 14) RyR-mediated Ca^{2+} release had any effect on mI_{cat}. The latter cannot be explained by an equal but opposing inhibitory effect of caffeine on muscarinic cationic channels since application of 5 mM caffeine did not inhibit mI_{cat} when $[Ca^{2+}]_i$ was strongly buffered with Ca^{2+}/BAPTA buffer (n = 5). Nor can it be attributed to an effect of caffeine on other mechanisms possibly involved in the regulation of Ca^{2+} sensitivity of muscarinic cationic channels since in the presence of 5 mM caffeine, flash-release of Ca^{2+} upon cell dialysis with 5 mM NP-EGTA/3.8 mM Ca^{2+} potentiated mI_{cat} in the same way as in control (n = 5). In contrast, flash-release of IP_3 (30 μM of 'caged' IP_3 in the patch pipette) augmented mI_{cat} (n = 15). The kinetics of the flash-induced current closely followed the dynamics of intracellular $[Ca^{2+}]_i$ changes and a threshold $[Ca^{2+}]_i$ for activation of mI_{cat} in this case was much lower than that observed upon flash-release of Ca^{2+} (n = 10) when responses of fluo-3 to the rise in $[Ca^{2+}]_i$ started to develop and saturated prior to that of mI_{cat}, thus reflecting that muscarinic cationic channels have a lower affinity to Ca^{2+} than fluo-3. Possible activation of muscarinic cationic channels by IP_3 directly or by depletion of IP_3-sensitive Ca^{2+} store was ruled out, as flash-release of IP_3 failed to augment mI_{cat} when $[Ca^{2+}]_i$ was strongly buffered with Ca^{2+}/BAPTA buffer (n = 4) or Ca^{2+} store was depleted with 0.1 μM thapsigargin (n = 5). Intracellular calcium store visualised with the low-affinity Ca^{2+} indicator fluo-3FF consisted of the sub-plasmalemmal sarcoplasmic reticulum (SR) and some perinuclear formation (n = 35). Immunostaining revealed that type1 IP_3Rs are predominant in sub-plasmalemmal SR (n = 40) while RyRs are confined to the central region of the cell (n = 18). In non-patched SMCs loaded with fluo-4 AM, CCh evoked staircase-like increase in $[Ca^{2+}]_i$ (consistent with summation of APs) with an initial sub-plasmalemmal $[Ca^{2+}]_i$ rise in regions where SR was found (n = 19). This pattern of $[Ca^{2+}]_i$ dynamics persisted after inhibition of RyRs with 100 μM ryanodine (n = 4), but not after block of VGCCs with 10 μM nicardipine (n = 6) or IP_3Rs with 30 μM 2-APB (n = 3). These results suggest that: (1) IP_3Rs are major contributor to the $[Ca^{2+}]_i$ rise upon muscarinic receptor activation, (2) IP_3R-mediated Ca^{2+} release is potentiated by Ca^{2+} entry through VGCCs and plays a central role in the modulation of mI_{cat} and (3) IP_3 may sensitise muscarinic cationic channels to Ca^{2+}.

Supported by the Wellcome Trust (060659, 062926, 075112).

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

SA3

Pyridine nucleotides and the functional integration of multiple calcium stores

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Mobilisation of intracellular Ca^{2+} stores plays a pivotal role in the regulation of arterial smooth muscle function, paradoxically

during both contraction and relaxation. However, the underlying spatiotemporal pattern of Ca^{2+} signals may also contribute to the regulation of differential gene expression. Thus, it is important that we characterise the mechanisms that determine different signalling patterns. A significant advance in this respect was the realisation that different Ca^{2+} storing organelles may be selected by the discrete or co-ordinated actions of multiple Ca^{2+} mobilising messengers. When considering such messengers it is accepted that sarcoplasmic reticulum (SR) stores may be mobilised by inositol 1,4,5 trisphosphate (IP3). However, relatively little attention has been paid to the role of Ca^{2+} mobilising pyridine nucleotides in arterial smooth muscle, namely cyclic adenosine diphosphate-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP).

We have found NAADP to be a powerful Ca^{2+} mobilising messenger in arterial smooth muscle (threshold ≤10 nM), and that it initiates global Ca^{2+} waves via a two-pool mechanism (Boittin et al. 2002). Initially, NAADP evokes highly localised intracellular Ca^{2+} signals, namely Ca^{2+} bursts, by mobilising a thapsigargin-insensitive intracellular Ca^{2+} store other than the SR. These Ca^{2+} bursts were shown to initiate subsequent, global Ca^{2+} waves and contraction of myocytes through the recruitment of ryanodine receptors (RyRs) on the SR via Ca^{2+}-induced Ca^{2+} release (CICR; Boittin et al. 2002). By contrast, NAADP-induced Ca^{2+} bursts did not recruit IP3 receptors (IP3Rs) by CICR. Subsequent studies established that NAADP evokes Ca^{2+} bursts by mobilising a bafilomycin A1-sensitive, lysosome-related Ca^{2+} store. And that lysosomal stores facilitate the generation of a global Ca^{2+} wave by co-localising with a subpopulation of RyRs on the SR to comprise a highly specialized trigger zone for NAADP-dependent Ca^{2+} signalling. Importantly, Ca^{2+} signalling by NAADP was also found to be induced in an agonist-specific manner by the vasoconstrictor endothelin-1 (Kinnear et al. 2004).

Much higher concentrations of cADPR (20-100 μM) are required to initiate Ca^{2+} signals in pulmonary arterial smooth muscle. In marked contrast to NAADP, however, threshold concentrations (20 μM) of cADPR were found to induce a sustained increase in intracellular Ca^{2+} concentration by mobilising a cyclopiazonic acid (CPA)-sensitive SR store proximal to the plasma membrane. Further investigation established that adenylyl cyclase coupled β-adrenoreceptors may mediate vasodilation by cADPR-dependent Ca^{2+} release via RyRs in the SR proximal to the plasma membrane, leading to subsequent BKCa-dependent hyperpolarization and vasodilation (Boittin et al. 2003). Paradoxically, we had previously shown that cADPR-dependent SR Ca^{2+} release via RyRs in a CPA-insensitive SR store underpinned pulmonary artery constriction by hypoxia (Dipp & Evans, 2001). When taken together, these findings suggest that cADPR-dependent Ca^{2+} release via RyRs could lead to stimulus-dependent relaxation or contraction in arterial smooth muscle. Given that RyR subtypes 1, 2 and 3 are present in vascular smooth muscle, this paradox may be explained if: (1) β-adrenoreceptor signalling targets PKA-dependent cADPR synthesis to a particular RyR subtype in the peripheral SR that is in close apposition to BKCa channels in the plasma membrane, (2) cADPR-dependent vasoconstriction results from the activation of a discrete RyR subtype localized in the central SR proximal to the contractile apparatus and (3) the peripheral and central SR represent functionally segregated compartments (Boittin et al. 2003). Considering the above, it seems likely that future studies may establish that NAADP and cADPR have a combinatorial role in mediating smooth muscle contraction. This is clear from the fact that cADPR may either activate RyRs or lower the threshold for CICR via RyRs (Galione et al. 1991). Thus, cADPR may also modulate the threshold for initiation of global Ca^{2+} signals by NAADP and / or the frequency of subsequent Ca^{2+} oscillations.

By contrast to the effects of cADPR and NAADP, Ca^{2+} signalling by IP3 occurred in a manner independent of lysosomal Ca^{2+} stores. Moreover, IP3 was able to initiate a global Ca^{2+} wave in the absence of CICR via RyRs. Cells may therefore co-ordinate and restrict the relationship between lysosomal Ca^{2+} stores and Ca^{2+} release channels on the SR/ER in a manner suited to their function.

These findings further advance our understanding of how the selection of different organnellar stores by the discrete or combinatorial effects of different Ca^{2+}-mobilizing messengers may underpin differential Ca^{2+} signalling patterns.

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This work was supported by the Wellcome Trust and the BBSRC.

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

SA4

Mitochondrial regulation of store-operated calcium channels: impact on cell function

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In a variety of cell types, calcium influx is essential for regulating a host of kinetically distinct processes including exocytosis, enzyme control, gene regulation, cell growth and proliferation and apoptosis. Although several distinct calcium entry pathways have been described, in non-excitable cells the major calcium entry pathway is the store-operated one, in which the emptying of intracellular inositol trisphosphate-sensitive calcium stores activates calcium influx (store-operated calcium entry, or capacitative calcium entry). Several biophysically distinct store-operated currents have been reported, but the best characterized is the calcium release-activated calcium current, ICRAC. Recent work has revealed a central role for mitochondria in the regulation of ICRAC, and this is particularly prominent under physiological conditions. Mitochondria take up some of the calcium that has been released from the stores by inositol trisphosphate, resulting in more extensive store depletion and hence activation of ICRAC. ICRAC is subject to calcium-dependent inactivation but this is reduced by mitochondrial calcium buffering. Hence mitochondria regulate both activation and inactivation of CRAC channels. Through effects on CRAC channel gating, mitochondria are effective regulators of both calcium-dependent exocytosis and secretion of pro-inflammatory signals like leukotrienes. ICRAC therefore represents a dynamic interplay between endoplasmic reticulum, mitochondria and plasma

membrane and this interaction sculpts subsequent calcium-dependent responses.

Work in the author's laboratory is funded by a Medical Research Council Programme Grant, the Lister Institute and the British Heart Foundation.

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

SA5

Muscarinic cation current of the ileum: signalling events and channel gating

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Cholinergic excitation of the gastrointestinal smooth muscles is mediated by cation channels via a synergistic action of the predominant M_2 (~80%) and minor M_3 (~20%) muscarinic receptors. Cation channel of 57±7 pS conductance (means±S.E.M., n=55) carries most of the muscarinic inward current, mI_{CAT} (Zholos et al. 2004a). The gating signals apart from $[Ca^{2+}]_i$ elevation and membrane depolarization include Pertussis toxin sensitive M_2-mediated activation of Gαo protein (Yan et al. 2003) and concurrent M_3-dependent activation of phospholipase C, PLC. mI_{CAT} appears to be directly activated by the M_2 receptor since adenylyl cyclase is not involved while the nature of the M_3 receptor action remains enigmatic. Apart from $[Ca^{2+}]_i$-dependent potentiation of mI_{CAT} due to $InsP_3$-induced Ca^{2+} release it also includes another important but yet unidentified process, in which PLC products, diacylglycerols and $InsP_3$, as well as Ca^{2+} store depletion are not involved (Zholos et al. 2004b).

Experiments were performed on single collagenase-dispersed myocytes isolated from the small intestine of humanely killed adult guinea-pigs using a combination of patch-clamp recordings, confocal $[Ca^{2+}]_i$ imaging (0.1 mM fluo-3 signal) and flash photolysis of 'caged' $InsP_3$ (30 μM in the pipette) or Ca^{2+} (5 mM NP-EGTA/3.8 mM Ca^{2+}). We aimed to relate the multiple signalling pathways to the channel mechanism.

The 60 pS channel in outside-out patches exposed to 50 μM carbachol externally or 200 μM GTPγS internally showed transitions between 8 states with strong correlations between the dwell times of adjacent shut and open states. Thus, four pairs of connected states have been identified which showed voltage-dependent vertical transitions in each pair (Fig. 1, note that mean dwell times and fractional contribution of each open state to the channel open probability, P_O, are indicated). Prominent regular cycles of P_O occurred because of a variable number of long openings between consecutive long shuttings, that is due to the horizontal transitions between the 'low-P_O mode' in C1-O1 and the 'high-P_O mode' in C4-O4 states. These probably occurred in a ligand-dependent manner since horizontal redistribution between the channel states was voltage independent.

In Cs^+-rich (125 mM) divalent cation-free solution which maximizes mI_{CAT} spontaneous cation current, sI_{CAT}, was revealed with voltage dependence similar to mI_{CAT} (e.g. U-shaped I-V curve at negative potentials). Its amplitude was 192±9 pA at −40 mV increasing to 1590±209 pA at 80 mV (n=8). sI_{CAT} was insensitive to atropine (50 nM) suggesting that mAChR constitutive activity was not involved. sI_{CAT} relaxations during steps from −40 to −120 mV were fast consistent with the O1 mean open time; in 3 out of 8 cells a slower component with τ=4.7±1.2 ms was also detected. Photorelease of Ca^{2+} significantly potentiated this current in a voltage-dependent manner (760±120 and 4860±137 pA at −40 and 80 mV, respectively), while slower relaxations with the time constant in the range 2.8-9.5 ms (7.5±0.4 ms; n=18) became apparent in the macroscopic kinetics — the values typical for the O2 microstate.

Carbachol application (10 μM) initiated Ca^{2+} waves and mI_{CAT}, the latter occurred with a latency of 229±55 ms (n=7) and peak lagging by 1.22±0.11 s (n=17) compared to the fluo-3 signal. However, at later times mI_{CAT} mirrored changes in $[Ca^{2+}]_i$ induced by an additional photorelease of $InsP_3$ (time-to-peak 279±25 and 298±23 ms, respectively; n=7). Moreover, 5 mM caffeine-induced Ca^{2+} release was totally inefficient in modulating mI_{CAT} while photorelease of Ca^{2+} was poorly efficient compared to the $InsP_3$-mediated modulation of mI_{CAT}. This suggested an important synergy between $InsP_3$ and Ca^{2+}-dependent activation of the channel although $InsP_3$ alone was inefficient when $[Ca^{2+}]_i$ was clamped at 100 nM using 10 mM BAPTA.

Our results suggest that in the absence of ligands intrinsic voltage-dependent channel gating occurs in the 'low-P_O' C1-O1 mode. The 'high P_O' C4-O4 mode is probably dominated by the activated Gαo interaction with the channel since Pertussis toxin nearly abolishes mI_{CAT}. Intracellular Ca^{2+} and the M_3/PLC/$InsP_3$ system may therefore shift the gating through the intermediate states (Fig. 1).

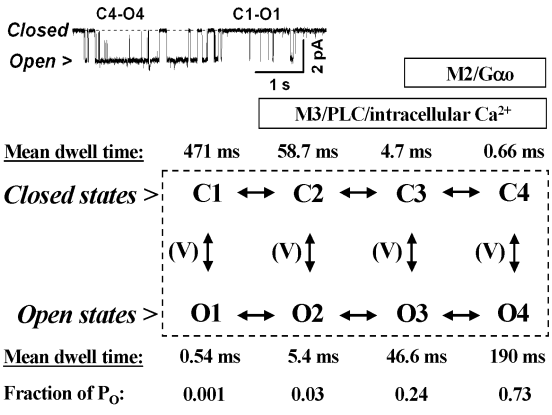

Fig. 1. Channel mechanism with mean parameters indicated. The inset shows gating typical for the C4-O4 and C1-O1 transitions.

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Supported by The Wellcome Trust (062926, 060659).

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

SA6

Regulation and gating of non-selective cation channels in vascular smooth muscle

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It is well established that Ca^{2+}-permeable non-selective cation channels have an important role in smooth muscle function. For example, many studies in diverse smooth muscle preparations have shown that agonist-induced excitatory responses involve non-selective cation channels which may also contribute to the resting membrane conductance. The purpose of this presentation is to describe the transduction pathways regulating these ion channels in freshly dispersed vascular smooth muscle cells. It will be shown that the non-selective cation current evoked by noradrenaline in rabbit portal vein involves G-protein stimulation of phospholipase C (PLC) and both diacylglycerol (DAG) and inositol 1,4,5-trisphosphate are required for optimal activation of the channel (a member of the tranisent receptor potential family, TRPC6).

In rabbit ear artery cells there is a constitutively active non-selective cation channel which contributes to the resting membrane conductance but is also enhanced by noradrenaline. Like the portal vein DAG is implicated in channel activation by a protein kinase C (PKC)-independent mechanism. However, a striking difference between the two preparations is that PLC is involved in the inhibitory regulation of this channel and that phospholipase D activity produces DAG via phosphatidic acid to produce channel opening. It is evident that this class of non-selective cation channel is linked to diverse transduction pathways.

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

SA7

Newly emerging Ca^{2+} channel molecules involved in vascular tone regulation

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The systemic blood pressure and local blood flow to various organs are critically determined by the momentarily changing tone of small arteries and arterioles, in which sustained Ca^{2+} influx activated by a variety of mechanisms plays a central regulatory role. Recent progress in molecular biological research has unraveled unexpectedly diverse and complex aspects of Ca^{2+} entry channel molecules involved in this Ca^{2+} influx. These include, in addition to voltage-gated Ca^{2+} channels, several members of a newly emerging non-voltage-gated Ca^{2+} entry channel superfamily, the transient receptor potential (TRP) proteins, such as TRPC1, TRPC4, TRPC6, TRPV1, TRPV2, TRPV4, TRPM4 and polycystins (TRPPs). Neither of these channels exhibits simple properties to fulfill a single particular role, because they are multimodally activated or modulated in amazingly diverse ways by receptor stimulation, temperature, mechanical stress or lipid second messengers generated from various sources, and may interact mutually. There is good evidence to believe that many of these channels play non-trivial roles in both acute vasomotor control and long-term vascular remodeling. It seems that, amongst them, of central physiological importance is the TRPC6 isoform, since it is the most widely distributed isoform in the vasculature and has been shown to serve as an integrative Ca^{2+} signaling molecule for sympathetic nerve activity, vasopressor hormones and increased intravascular pressure. Here, we will focus on the TRPC6 channel and highlight especially its novel regulatory mechanism by 20-hydroxyeicosatetraenoic acid (20-HETE), in an intriguing connection with a reflex constriction of small arteries induced by pressurization referred to as the 'myogenic response'.

Muscarinic stimulation with 100μM carbachol (CCh) in murine TRPC6-overexpressing HEK293 cells evoked a large cationic current (I_{TRPC6}). Exposure to hypotonic external solution (-90mOsm; HTS) of these cells produced a rapid and reversible increase in the amplitude of I_{TRPC6}, and concomitantly elevated the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or increased the rate of Ba^{2+} entry as evaluated by digital imaging microscopy. The potentiating effect of HTS on I_{TRPC6} was unaffected when intracellular Ca^{2+} was strongly buffered by inclusion of 10mM BAPTA/4Ca^{2+} in the patch pipette, or when I_{TRPC6} was more directly activated by internal perfusion with GTPγS (100μM) thus bypassing the receptor. However, 30min pretreatment with a 20-HETE production blocker HET0016 (3-10μM), which more selectively inhibits ω-hydroxylase than expoxygenase of non-hepatic vascular cytochrome P450, almost completely abolished the potentiating effect of HTS on I_{TRPC6} or Ba^{2+} entry evoked by CCh. Increasing the intraluminal pressure (≥20 mm Hg) of cannulated rat mesenteric arteries (RMA; 2nd or 3rd branches) itself elicited a weak pressure-dependent reflex vasoconstriction dependent on extracellular Ca^{2+}, but its extent was remarkably enhanced during α1-adrenergic receptor stimulation with low concentrations of phenylephrine (0.05-1.0μM). Pretreatment with HET0016 (10μM) greatly attenuated this reflex vasoconstriction, to an extent similar to that observed with Ca^{2+} elimination. These results collectively suggest that, at least in larger branches of RMA, 20-HETE production is a key intervening process for the development of myogenic tone at weak sympathetic excitation, in response to increased intravascular pressure. The mechanism underlying most likely involves 20-HETE-mediated potentiation of receptor-activated TRPC6 channels.

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

SA8

Pharmacology of TRP channels

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Our understanding of the biology of TRP channels has been hampered by a lack of specific and selective pharmacological tools with which to probe physiological function. Compounds,

such as SKF 96365, have been extensively used to identify possible TRP channel mediated functions, but they have had limited impact in pharmacologically sub-dividing functional responses. The molecular characterisation of this diverse family of cation channels has now provided a set of targets on which to profile potential pharmacological probes and screen for new agents with better selectivity.
Most success, to date, has been achieved with the TRPV1 channel where the early existence of agonist ligands and interest in this target as a route to new pain therapies has attracted substantial effort. New approaches initially shared structural features with the prototypic capsazepine, but the most recent patents disclose quite diverse templates. This demonstrates the success of functional high throughput screens in identifying a variety of novel chemical leads. These experiences with TRPV1, will encourage and aid more rapid development of novel ligands for other members of the TRP family.

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

SA9

Unforeseen trips (TRPs) to vascular smooth muscle: an endothelial connection

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Endothelial cells express a variety of TRP (transient receptor potential channel) channels, including TRPV4, TRPM4 and several canonical TRPCs. Here we focus on the functional role of TRPV4 in endothelial cells by using mouse aorta endothelial cells (MAEC cells) derived from humanely killed wild type (TRPV4+/+) mice and from TRPV4 knockout mice (TRPV4-/-). TRPV4 integrates a large variety of stimuli ranging from hypotonic cell swelling (HTS), temperature, and ligands e.g. 4α-phorbol 12,13-didecanoate (4α-PDD), to endogenous agonists such as arachidonic acid (AA), anadamide and epoxyeicosatrienoic acids. We show that TRPV4 is likely a target for activation of endothelium-dependent vasorelaxation, which can be modulated via the cytochrome P450 (CYP) pathway. The loss of TRPV4 expression in MAEC cells from TRPV4-/- mice attenuated or strongly diminished responses to all TRPV4 activating stimuli. Ca2+ imaging and patch clamp measurements show that TRPV4-dependent responses can be modulated via CYP enzymes, which metabolize AA to EETs. MAEC from TRPV4+/+ mice preincubated with nifedipine, which upregulates CYP2C expression, show a potentiated response to AA and cell swelling, whereas activation by 4α-PDD. Sulfaphenazole, an inhibitor of CYP2C9, decreased responses induced by AA and HTS. 1-Adamantyl-3-cyclo-hexylurea (ACU), an inhibitor of the soluble epoxide hydrolase which converts EETs to dihydroxyeicosatrienoic acids, increased the response induced by AA, HTS and EETs but not that induced by 4α-PDD and heat. All these data demonstrate that cytochrome P450-derived EETs modulate the activity of TRPV4 channels in endothelial cells.

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

SA10

Calcium signalling and excitability in the uterus

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Controlled uterine smooth muscle activity is essential for successful childbirth. Unfortunately, problems with uterine contractions, either elicited prematurely, or being of inappropriate strength, are quite common and remain a cause of maternal and neonatal morbidity, and an obstetrical challenge. There is a lack of knowledge of basic mechanism controlling uterine contractility, and to increase our knowledge and uncover mechanisms leading to uterine function and dysfunction is a physiological challenge.
Perhaps the most fundamental question to be addressed in uterine physiology and pathophysiology is, how is the electrical activity that triggers and modifies contraction initiated? This is a question that can be posed at several levels; is there a special type of cell within the myometrium that acts as a pacemaker? What are the properties of pacemaker cells, e.g. ion channels expressed? What are the roles of local and global Ca^{2+} signals in producing this excitation? How is the excitation spread to other cells? How do agonists, intracellular signalling pathways and local factors, such as pH, influence excitability? What changes occur in these mechanisms and properties within normal labour, and what happens in pre-term and dysfunctional labours? A daunting list.
I will present data that give us some insight into some of these questions. Recent studies of freshly dispersed myometrial cells from humanely killed pregnant rats have found that identical looking myocytes differ markedly in their ability to generate Ca^{2+} spikes, fire action potentials and produce outward current and thus under identical conditions healthy myocytes differ in their spontaneous activity. They also differ in the ion channels they express, e.g. in a study of inward current produced by Ca-activated Cl- (Cl_{Ca}) channels in rat myometrium, we found that only around 30% of cells (n>300) had a Cl_{Ca} current. Expression of such inward currents would be consistent with a pacemaking potential in these cells and their pharmacological inhibition produced a decrease in the frequency of uterine Ca2+ transients and contractions. In addition, we have recently examined cells from freshly dispersed human and rat uterus and in situ preparations, and discovered numerous interstitial-like cells (ILCs). These were multi-polar cells with spider-like projections and enlarged central regions. They were non-contractile, vimentin-positive and had numerous caveolae and mitochondria. Electron-microsopy of in situ preparations showed them making close contact with many myocytes and axons. Their role remains to be elucidated. The role of local sub-cellular Ca^{2+} signals e.g. Ca^{2+} sparks and puffs from the sarcoplasmic reticulum (SR) is virtually unknown in uterine smooth muscle. In other smooth muscles much progress has been made in investigating these events and relating them to excitability and contraction. All three isoforms of both the ryanodine receptor (RyR) and IP_3 receptor have been reported in human myometrium, but there is species variation e.g. mouse has only RyR3. There has been no investigation in myometrium of the nature and role of local Ca^{2+} signals, and their relation to global Ca^{2+} signals, e.g. Ca^{2+} waves and transients, is unclear. Similarly although Ca-activated K channels (BK) have been demonstrated in the myometrium, the role of

SR Ca^{2+} release vs. Ca^{2+} entry in activating the channels is unknown.

I will discuss two recent studies where we have data which link lab and clinical studies. Our in vitro studies had shown an inhibitory effect of acidic pH on uterine excitability and contractility. We therefore undertook a study of myometrial capillary pH in women suffering dysfunctional labour. Blood was taken at the first uterine incision in women having a Caesarean section (CS) either electively or as a consequence of fetal distress or dysfunctional labour. The results were startling and significant. The pH of myometrial capillary blood from women having a dysfunctional labour was significantly lower, at 7.35, than that from any other group. Furthermore lactate was higher and capillary oxygen saturation was lower. These data support the hypothesis that myometrial blood is more acidic in women labouring dysfunctionally, in a manner consistent with hypoxic episodes. We also determined that the pH drop determined in vivo, was sufficient in vitro, to change a normal pattern of uterine contractility into a dysfunctional one. In another set of experiments we have found that, in vitro, elevated cholesterol is deleterious to force and Ca^{2+} signalling in the myometrium. In a parallel study we have found that obese pregnant women, who have elevated cholesterol levels also are significantly more likely than lean women to suffer a dysfunctional labour, due to poor contractions. We are currently looking further into the mechanisms of both these exciting findings.

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

SA11

Two step mechanisms of Ca^{2+}-induced Ca^{2+} release in excitation-contraction coupling

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The significance of functional coupling between ryanodine receptor (RyR) and Ca^{2+}-activated ion channels in Ca^{2+} spark sites has been well established in various types of smooth muscle cells (SMCs)(Wellman & Nelson, 2003). In contrast, experimental results about the functional significance of Ca^{2+}-induced Ca^{2+} release (CICR) in excitation-contraction (E-C) coupling have been still controversial in SMCs (Collier et al. 2000; Herrera et al. 2000). We examined here i) the relative contribution of CICR versus direct Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) during single action potential (AP) to a contraction, ii) mechanisms underlying the spread of Ca^{2+} wave from local areas to whole cell, and iii) mechanisms of Ca^{2+} sequestration, during E-C coupling in single urinary bladder (UB) SMC of humanely killed mouse and guinea-pig. In addition to analyses of confocal Ca^{2+} images and membrane currents, following four approaches have been made in this series of studies.

1) CICR via RyR was completely blocked by 100 μM ryanodine. When UBSMCs were activated by 30 ms depolarization from –60 to 0 mV in the absence of ryanodine, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevated in several discrete small areas along cell membrane as hot spots in the early stage of depolarization (< 10 ms). Ca^{2+} hot spots, then, spread to whole cell area more slowly as Ca^{2+} waves even after repolarization (Ohi et al., 2001). A shorter depolarization (5 ms) elicited only a few Ca^{2+} sparks, which usually disappeared quickly. The number of Ca^{2+} hot spots elicited by depolarization closely related to the duration of depolarization up to ~20 ms. There was a sort of threshold duration around 10 ms to induce enough number of Ca^{2+} hot spots to spread globally as waves. Application of 100 μM ryanodine from a patch pipette changed neither the resting $[Ca^{2+}]_i$ nor the amplitude of VDCC current but abolished Ca^{2+} hot spots and markedly reduced peak $[Ca^{2+}]_i$ upon depolarization. Application of 3 μM xestospongin C, a blocker of inositol trisphosphate receptor (IP_3R) markedly reduced acetylcholine-induced Ca^{2+} release but did not affect Ca^{2+} hot spots and waves upon depolarization. Addition of 100 μM ryanodine to UB tissue segments reduced amplitude of contraction induced by direct electrical stimulation under moderate conditions by ~70 % in the presence of antagonists for transmitter receptors.

2) Ca^{2+} concentrations in cytosol ($[Ca^{2+}]_i$) and mitochondria matrix ($[Ca^{2+}]_m$) were monitored in a cell by fluo-4 and rhod-2, respectively. Upon depolarization, $[Ca^{2+}]_i$ was immediately elevated in hot spot sites and reached the peak at ~150 ms. In contrast, $[Ca^{2+}]_m$ was increased with a delay of ~50 ms after depolarization and peaked at ~500 ms. Upon repolarization, $[Ca^{2+}]_i$ returned to the resting level with a half decay time of ~500 ms, while $[Ca^{2+}]_m$ recovered more slowly (~1.5 s). The depolarization-induced increase in $[Ca^{2+}]_m$ in discrete mitochondria, where a Ca^{2+} hot spot was located closely, was significantly larger than that without the co-localization.

3) The contribution of VDCC to E-C coupling was examined in UBSMC of mutant mice, which lacked β3 subunit of VDCC ($\beta3^{-/-}$). In $\beta3^{-/-}$ UBSMC, Ca^{2+} current density was reduced by 45 %. The mRNA expression of RyRs and BK channel in UBSM of $\beta3^{-/-}$ was comparable to that in $\beta3^{+/+}$, respectively. The amplitude of contraction induced by moderate electrical stimulation in UB tissue segments of $\beta3^{-/-}$ was comparable to that in $\beta3^{+/+}$ but high K^+ induced contraction in $\beta3^{-/-}$ was significantly smaller than that in $\beta3^{+/+}$. The treatment with 100 μM ryanodine reduced contraction by the electrical stimulation in larger extent in $\beta3^{-/-}$ than in $\beta3^{+/+}$. Collectively, β3 knock-out resulted in reduction of VDCC density, which was functionally compensated by larger contribution of CICR to a twitch contraction.

4) The contribution of RyR type 2 (RyR2) to E–C coupling was examined in UBSMs from RyR2 heterozygous KO mice ($RyR2^{+/-}$), in which RyR2 mRNA expression decreased by over 50 % of wild type ($RyR2^{+/+}$). The number of Ca^{2+} hot spots and elevation of $[Ca^{2+}]_i$ upon depolarization were significantly smaller in $RyR2^{+/-}$ than those in $RyR2^{+/+}$. The density of VDCC and BK channel currents was not changed in $RyR2^{+/-}$. The contraction by moderate electrical stimulation and its decrease by ryanodine were significantly smaller in $RyR2^{+/-}$ than in $RyR2^{+/+}$.

In conclusion, CICR during E-C coupling elicited by single AP occurs in two steps in UBSMC. Ca^{2+} influx does not increase $[Ca^{2+}]_i$ markedly but elicits initial CICR in discrete hot spot sites via functional coupling between VDCC and RyR2. In the second step, Ca^{2+} release spreads as waves to other Ca^{2+} stores with amplification but more slowly in a way without mediating activation of IP_3Rs. Two steps of CICR are essential for a twitch contraction elicited by single AP in UBSMC.

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

SA12

Molecular determinants for calcium channel inhibition

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Calcium (Ca^{2+}) channel blockers such as 1,4-dihydropyridines (DHPs), phenylalkylamines (PAAs), diltiazem (Dil) and mibefradil (Mib) exert their antiarrhythmic and antihypertensive action by restricting Ca2+ entry through voltage gated Ca^{2+} channels (Cav) into myocardial and smooth muscle cells. Most Cav are heterooligomeric protein complexes consisting of an α1-subunit, auxiliary β-, α2-δ and in some channels of an additional γ-subunit. The α1 subunits sense the membrane voltage, form the pore and define the basic pharmacological properties of the channels. They are composed of four homologous domains (I-IV) formed by six transmembrane segments (S1 - S6). β-subunits (β1,β2,β3,β4) modulate the channel inactivation and other gating properties. Site directed mutagenesis revealed a number of amino acids in S5 and S6 segments of L-channel domains III and IV that determine the inhibition by DHPs, PAAs and Dil. Some of these point mutations either enhance or impair channel inactivation and simultaneously increase or reduce channel inhibition by Ca^{2+} channel blockers. Fast inactivating L-channel mutants tend to be more efficiently inhibited by PAAs, Dil and Mib than slow-inactivating ones. A similar conclusion can be drawn from an analysis of the drug-sensitivities of Cav composed of either 'accelerating' (e.g. β1 or β3) or 'decelerating' β2a-subunits. Again, subunit compositions promoting faster channel inactivation increase the apparent drug sensitivity of most constructs. Thus slowing channel inactivation by point mutations in different parts of the α1-subunit has almost the same effect as coexpression of the 'decelerating' β2a.

An open channel block mechanism can hardly explain the data. 'Decelerating' β2a-subunit increase the fraction of open channels which should enhance open channel inhibition but not decrease as observed. A modulated receptor concept would explain the correlation between inactivation and channel block by a higher affinity of PAAs, Dil and Mib to the inactivated state. The inactivation machinery of Cav is, however, sensitive to conformational changes in many different parts of the α1-subunit. Some inactivation determinants are located distant from the putative drug binding pocket in the pore. It is therefore more attractive to hypothesise that the numerous structural changes affect channel block in an indirect manner via a modulation of the inactivation mechanism and not by modulating drug-affinity.

A possible scenario is a drug-inactivation-synergism where Ca2+ channel blockers and "accelerating" β-subunits both stabilise an inactivated channel conformation (Hering, 2002). This hypothesis is not in contradiction with the notion that charged compounds access their receptor in the channel vestibule predominantly via the open channel conformation. Once these drugs interact with their binding site they are likely to promote inactivation.

The β-subunit composition of Cav is likely to affects drug sensitivity of native cells. Cav in smooth muscle of the uterus and trachea are formed by β2- and β3-isoforms (Reimer et al. 2000). The "accelerating" β3-subunit was found to associate with the α12.1 in the aorta which is expected to favour channel block in this tissue.

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Supported by FWF grant 15914-B05.

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

SA13

Role of voltage-independent calcium influx pathways in the response of pulmonary arteries to agonists and hypoxia

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Unlike systemic arteries, which dilate in response to hypoxia, the arteries of the pulmonary circulation constrict under hypoxic conditions. The mechanisms underlying this process, termed hypoxic pulmonary vasoconstriction, or HPV, remain controversial. Initial work by Post *et al.* (1992) suggested that hypoxia inhibited K^+ channels in pulmonary artery smooth muscle cells, causing depolarization and Ca^{2+} influx *via* L-type Ca^{2+}]channels. However, later work from our laboratory (Robertson *et al.* 2000) showed that HPV in isolated intrapulmonary arteries (IPA) was little affected by simultaneous depolarization and treatment with L-type channel blockers, indicating that voltage-independent Ca^{2+} influx was likely to play a pivotal role in raising $[Ca^{2+}]_i$ during HPV. Moreover, both rho kinase-mediated Ca^{2+} sensitization and the endothelium make important contributions to HPV (Aaronson *et al.* 2002).

In an effort to define the voltage-independent Ca^{2+} influx pathways present in rat IPA, we have recently investigated the mechanisms by which agonists raise $[Ca^{2+}]_i$ in these arteries, focusing mainly on $PGF_{2\alpha}$ which causes a powerful vasoconstriction. Our experiments show that low concentrations (0.1μM) of $PGF_{2\alpha}$ or the FP receptor agonist fluprostenol cause a transient rise in $[Ca^{2+}]_i$ associated with Ca^{2+} release, followed by a sustained rise in $[Ca^{2+}]_i$ which is abolished by pretreatment with 3μM U73122, 10μM La^{3+}, 75μM 2-APB and or 1μM thapsigargin. Conversely, a higher concentration (10μM $PGF_{2\alpha}$ and the TP receptor agonist U46619 (0.1μM) cause a sustained rise in $[Ca^{2+}]_i$ which is relatively insensitive to each of these drugs, and to the L-type Ca^{2+} channel blocker diltiazem. In addition, the Ca^{2+} influx pathway activated by U46619, but not the pathway activated by fluprostenol, is permeable to Sr^{2+}. These results suggest that FP receptor activation stimulates store-operated Ca^{2+}

entry, whereas TP receptor activation stimulates Ca^{2+}entry through a receptor operated channel.
In order to determine whether these pathways are also involved in the response to hypoxia, we have examined the effects on HPV of agents acting to modify or block either Ca^{2+} release or Ca^{2+} influx through non-voltage-gated Ca^{2+} entry pathways. HPV consists of an initial transient contraction (phase 1), followed by a sustained constriction (phase 2). Phase 1 HPV is abolished by 1μM La^{3+} or 1μM cyclopiazonic acid, but is less sensitive to dantrolene (50 μM) and 2-APB, whereas phase 2 is strongly inhibited by 2-APB and dantrolene and is partially inhibited by cyclopiazonic acid, but is insensitive to 1 μM La^{3+}. These results suggest that non-voltage-gated Ca^{2+} influx pathways are important in raising $[Ca^{2+}]_i$ in pulmonary artery smooth muscle during HPV, and that separate pathways probably contribute to each phase.

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This work was supported by the Wellcome Trust and the British Heart Foundation.

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

SA14

BKCa and ATP-dependent K channels in pregnant sheep myometrium: modulation by β-adrenoceptor activation

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Preterm labour, resulting in delivery before 37 weeks, occurs in 5-10% of all pregnancies and is the major cause of perinatal morbidity and mortality in the developed world. Various tocolytic agents that modulate uterine smooth muscle contractility, β-adrenoceptor agonists, nifedipine, magnesium sulphate and prostaglandin synthase inhibitors have been trialed and either delay labour by <72 h and/or have serious cardiovascular effects on both the mother and fetus. We have investigated the mechanisms underlying the reduced myometrial contractility evoked by β-adrenoceptor activation in pregnant sheep with a view to identifying potential new targets for prolonging pregnancy in the event of preterm labour. Tissues were collected from pregnant sheep under anaesthesia initially established with sodium thiopentone (20 mg kg^{-1} I.V.) and maintained with isoflurane (2% in oxygen) applied by intermittent positive pressure ventilation.
Intracellular microelectrode recordings from strips of sheep myometrium (late pregnancy, not in labour) revealed that spontaneous contractions were associated with bursts of action potentials. Salbutamol (0.01-1 μM) evoked concentration-dependent membrane hyperpolarization (to −80 mV at 0.5 μM) which prevented action potential discharge. Salbutamol-evoked hyperpolarizations were reduced to 7±3% (n=11) of control by glibenclamide (1 μM, blocker of K_{ATP} channels), and fully blocked by glibenclamide plus ouabain (10 μM, blocker of Na^+/K^+ ATPase). Activation of K_{ATP} channels using PCO400, evoked concentration-dependent hyperpolarization (to −85 mV). The amplitude of this hyperpolarization was not influenced by iberiotoxin (50-100 nM) which blocks large conductance, Ca^{2+}-activated K^+ (BKCa) channels. Salbutamol-induced hyperpolarization was reduced (to 64±5% of control, n=7) by inhibitors of protein kinase A, Rp-8-Cl cAMPS, Rp-8-CPT cAMPS and H89. Membrane hyperpolarization was also elicited by forskolin (0.1 μM, adenylyl cyclase activator), Sp-cBIMPS (50 μM, protein kinase A activator), and by IBMX (50 μM, phosphodiesterase inhibitor). Recordings from patches isolated from myometrial cells revealed the presence of K^+-selective ion channels with a conductance of 240 pS (in a symmetrical 130 mM K^+ gradient) which were readily activated by membrane potential, internal Ca^{2+} (23 nM to 1.4 μM) or NS1619 (30 μM) and blocked by 50-100 nM iberiotoxin, characterizing them as BKCa channels. Salbutamol (0.1 μM 10-20 min) applied to the bath or in the pipette increased BKCa channel activity in cell-attached patches due to a leftward shift in their voltage range of activation. In the presence of iberiotoxin, salbutamol and PCO400 activated an additional K^+ selective ion channel with a single channel conductance of 40 pS which was blocked by glibenclamide (1 μM).
These results demonstrate that stimulation of β-adrenoceptors in sheep myometrium leads to the activation, not only of BKCa channels, as previously reported by several laboratories, but also, to an hitherto undescribed activation of KATP channels. These K_{ATP} channels contribute to the resting membrane potential in myometrium and activation of K_{ATP} channels appears to provide the major membrane conductance increase underlying the hyperpolarization induced by β-adrenoceptor activation. In contrast BKCa channel activation appeared responsible for the reduced action potential amplitudes recorded in the presence of salbutamol. While the adenylyl cyclase/protein kinase A second messenger pathway may contribute to the hyperpolarization in response to β-adrenoceptor activation, it is unlikely to be the only mechanism involved.

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

SA15

Pulmonary vascular K^+ channels

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Potassium channels play an important role in pulmonary artery smooth muscle cells (PASMCs), maintaining a negative resting potential and preventing the cells from firing spontaneous action potentials. Consequently, PASMCs are electrically silent and this contributes to low intrinsic tone, which is important for maintaining the low arterial pressure that is characteristic of the healthy pulmonary circulation.
The molecular correlates of the K^+ channels that determine the resting potential are unclear. There is strong evidence that voltage-independent TASK-like channels are important (Gurney et al. 2003). A substantial body of evidence also implicates Kv chan-

nels, especially Kv1.5 and Kv2.1, although conflicting evidence argues against a major role for this class of channel in maintaining the resting potential (Gurney, 2003). Although it is widely agreed that the background K^+ conductance giving rise to the resting potential contains a voltage-dependent component, its biophysical and pharmacological properties are inconsistent with the involvement of Kv channels (Evans et al. 1996). Several of its properties, including low voltage threshold for activation, slow activation and absence of inactivation, are similar to the neuronal M-current, which is encoded by genes of the KCNQ family. This led us to investigate the possible contribution of KCNQ channel subunits to the background K^+ conductance, membrane potential and contractile function of PASMC.

At least three KCNQ channel subunits (KCNQ1, KCNQ4 and KCNQ5) were identified in pulmonary arteries, from humanely killed rat and mouse, by RT-PCR analysis of mRNA. A functional role for these channels is suggested by the finding that two blockers of KCNQ channels, linopirdine and XE991, caused rat and mouse pulmonary arteries to constrict at concentrations similar to those causing inhibition of recombinant KCNQ channels. The action was mediated by a direct effect on PASMC, because it was essentially unaffected by removal of the endothelium or by inhibiting the action of nerve-released noradrenaline with phentolamine. Moreover, the constrictor effect was abolished in the absence of extracellular calcium or in the presence of the calcium antagonist, nifedipine, or the K^+-channel opener, levcromakalim. This implies that pulmonary vasoconstriction caused by KCNQ blockers is mediated by membrane depolarisation and activation of voltage-dependent, L-type calcium channels. Thus KCNQ channel blockers appear to inhibit the background K^+ conductance, suggesting a role for these channels in maintaining the resting membrane potential.

We have identified several molecular candidates for the resting K^+ conductance in PASMC. Nevertheless, multiple K^+ channels are probably involved in regulating the resting membrane potential and their nature remains controversial.

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Grant support was provided by BBSRC and the British Heart Foundation.

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

SA16

Excitation-transcription coupling in vascular smooth muscle

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Alterations in the vasculature during hypertension include smooth muscle hyperplasia and hypertrophy, yet the changes in gene expression underlying these events are unknown. Previous data have shown that arteries from hypertensive animals exhibit elevated cytoplasmic Ca^{2+} and increased activation of both the Ca^{2+}/cAMP response element binding protein (CREB) and the MAP kinase, ERK. Multiple Ca^{2+} entry pathways induce CREB activation in vascular smooth muscle including entry through voltage-dependent Ca^{2+} channels (VDCC) and store-operated Ca^{2+} entry (SOCE). In the current study, microarray analysis was used to test the hypotheses that 1) Ca^{2+} entry induced by distinct stimuli differentially regulates gene expression in cultured human cerebrovascular smooth muscle cells and 2) cerebral arteries obtained from humanely killed Dahl S hypertensive rats exhibit increased expression of CREB- and ERK-regulated genes. Microarray results were validated and expanded using quantitative RT-PCR and parallel measurements of protein expression using western blot analysis and immunocytochemistry. Results indicate that the activation of different Ca^{2+} influx pathways stimulates transcription of both distinct and overlapping sets of genes and that the induction is prevented by the addition of corresponding Ca^{2+} channel blockers. In addition, arteries from hypertensive animals exhibit increased transcription of the Ca^{2+}- and proliferation-related genes c-fos, egr-2 and osteopontin, as well as eEF2K, a negative regulator of translation. We propose that during hypertension, overactivity of Ca^{2+} entry pathways and ERK in vascular smooth muscle leads to expression of specific sets of genes that regulate cell proliferation and hypertrophy. Together, these results suggest precise coupling of Ca^{2+} excitation pathways to transcriptional programs that may reveal future targets for therapies to prevent hypertension-related arterial pathologies.

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This research was supported by the National Heart, Lung and Blood Institute, the Totman Center for Cerebrovascular Research and the Vermont Genetics Network. The array experiments were performed and analysed by the University of Vermont DNA array and biostatistics core facilities, respectively.

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

SA17

MaxiK channels and smooth muscle partners

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Ca^2 -activated, voltage-dependent K channels also called MaxiK or BKCa because of their large conductance are abundant proteins in all smooth muscles. MaxiK channels are key regulators of vascular tone acting as a rheostat that fine tunes the membrane potential and intracellular Ca^2 concentration. In smooth muscle, they are formed by the coassembly of four α subunits that form the channel pore with four regulatory β1 subunits. β1 subunits profoundly modify how the channel responds to Ca^2, voltage, intracellular signalling and pharmacological agents. The α subunit is the product of a single gene but the potential to express plenty of splice variant isoforms. The β1 subunit belongs to a family of genes that are largely tissue specific, β1 being the smooth muscle archetype.

MaxiK channels are targets of vasoactive substances including vasorelaxants (e.g. nitric oxide-, arachidonic acid-pathways), and

vasoconstrictors (e.g. angiotensin II, 5-hydroxytryptamine, phenylephrine, thromboxane A2). A newly discovered pathway for vasoconstriction links constricting agonist stimulation to c-Src tyrosine kinase activation followed by phosphorylation and inhibition of MaxiK channel activity (Alioua et al. 2002), which would result in membrane depolarization, Ca^2 entry and contraction.

Besides the β1 subunit, MaxiK channels receive the aid of associated partner proteins that help in their function and to localize them in specific microdomains. Recent evidence indicates that at least a fraction of MaxiK channels are localized in caveolae structures and may interact with caveolin-1 in human myometrium (Brainard et al. 2005). Both MaxiK and caveolin-1 are important for vascular function as disruption of caveolae with β-methylcyclodextrine impairs agonist-induced vascular contraction and MaxiK α and β1 subunit gene ablation produces smooth muscle related disabilities (Brenner et al. 2000; Meredith et al. 2004). Thus, it is predicted that MaxiK channel forms functional units with receptors, tyrosine kinases and caveolin-1 in specialized microdomains.

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Supported by NIH (L.T. and E.S.) and AHA (M.E. and A.A.).

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SA18

Functions and regulated expression of vascular potassium channels

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As with studies of many cell types, investigations of vascular smooth muscle cells over the past twenty years have shown potassium channels to be the primary hyperpolarising drive on membrane potential. They have also revealed substantial diversity in potassium channel properties and heterogeneous expression between different vascular beds, and as the vasculature adapts to development and disease. Despite much work we only have a glimpse into the complexities of this hyperpolarising system. Nevertheless, there are some important facts known from the efforts of many different laboratories across the world- for example: K+ channels are necessary signal transduction elements in numerous vasodilatory responses, including those to nitric oxide. Subtypes of potassium channel have emerged as sensors of specific physiological signals - for example: BK-Ca senses intracellular calcium, K-NDP (the smooth muscle K-ATP variant) senses intracellular nucleotide diphosphates, inward rectifiers like Kir2.1 sense extracellular potassium, and delayed rectifiers like the Kv1 subunits sense myogenic and endothelin-evoked depolarisation. Although direct linkage of a potassium channel gene to vascular disease is lacking, there are intriguing associations of potassium channels with vascular abnormalities and indications that pharmacological agents targeted to potassium channels have, or might have, therapeutic benefit. For example: A blocker of the IK-Ca potassium channel suppresses smooth muscle proliferation, suggesting potential as an agent to suppress progression of neointimal hyperplasia or atherosclerosis. Experimental K-NDP potassium channel gene deletion evokes coronary vasoconstriction, conferring Prinzmetal-like angina on the mouse, which is consistent with the known anti-anginal properties of nicorandil - the K-NDP opener / nitric oxide donor. It has also emerged that Kv1 potassium channel expression is tailored to arterial calibre and down-regulates in the hypertensive rat, suggesting a role in regulating the myogenic set-point and thus in determination of the absolute value of blood pressure. These are important findings, but we do not know if potassium channels really have relevance to human vascular disease. Nor do we know the mechanisms by which vascular smooth muscle cells select specific members of the genomic potassium channel toolkit, or regulate the correct number of channels at the membrane. Over the past few years we have initiated studies to address these aspects of vascular biology. One focus has been on identifying transcription factors that bind and regulate potassium channel genes in vascular smooth muscle. In this regard we have, for example, discovered REST (repressor element-1 silencing factor) as a novel transcription factor of vascular smooth muscle that binds potassium channel genes and regulates their expression in blood vessels. We have also explored the role of potassium channels in failure of the human saphenous vein used as a coronary artery-bypass graft, a failure that occurs because of smooth muscle proliferation and migration into the lumenal space. The lecture will outline such recent findings in the context of general developments in the field.

We are grateful to support from the BHF, MRC and Wellcome Trust.

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

SA19

Origin of spontaneous rhythmicity in smooth muscle

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Rhythmic electrical activity is a feature of most smooth muscles but the contractile manifestation of this can vary from regular rapid phasic contractions to sustained contracture. The former contractile activity is well suited to fluid propulsion (as in the ureter or lymphatic vessels) while the latter is more suited to providing a variable resistance to flow (as in arteries and arterioles) or to the prevention of flow when this is undesirable (as in the urethra). For many years it was thought that spontaneous electrical activity originated in smooth muscle cells but recently it has become apparent that there are specialized pacemaker cells in many organs that are morphologically and functionally distinct from the surrounding smooth

muscle and that the former cells are the source of the spontaneous electrical activity which drives the, otherwise quiescent, smooth muscle cells.

We have recently shown that there are specialized pacemaking cells in the rabbit urethra which are similar in gross morphology and ultrastructure to the Interstitial Cells of Cajal found in the gastrointestinal tract (Rumessen & Thuneberg, 1996; Sanders, 1996). The urethral pacemaker cells are excitable, non-contractile, and contain abundant vimentin but no myosin filaments (Sergeant et al. 2000). They have an abundance of calcium-activated chloride current, exhibit regular spontaneous depolarizations which are increased in frequency by noradrenaline and blocked by perfusion with low calcium solution and by chloride channel blockers. The urethral smooth muscle cells, by contrast, are electrically quiescent and have very little calcium-activated chloride current. Electrical activity in the pacemaker cells consists in the regular firing of spontaneous transient depolarizations similar to those observed by Van Helden (1991) in veins. When interstitial cells were voltage clamped at -60 mV spontaneous transient inward currents (STICS) were observed. STICS reversed at ECl, were blocked by anthracene-9-carboxycylic acid as well as niflumic acid and were abolished by perfusion with calcium free Hanks solution suggesting that they were due to rhythmic activation of ICl(Ca). Under suitable recording conditions spontaneous transient outward currents (STOCS) could be recorded in the same preparation as STICS. Both currents were abolished by cyclopiazonic acid, caffeine, or ryanodine, suggesting that they were activated by intracellular Ca^{2+} release. When D-myo-inositol 1,4,5-trisphosphate (IP_3)-sensitive stores were blocked with 2-aminoethoxydiphenyl borate (2-APB), the STICs were abolished but, interestingly, STOCs were not. When measurements were made of intracellular calcium levels in fluo-4-loaded interstitial cells (Johnston et al. 2005) regular calcium oscillations corresponding to spontaneous transient inward currents were observed. Interference with IP_3-induced calcium release using 100 μM 2-APB, or the phospholipase C inhibitors NCDC or U73122 decreased the amplitude of spontaneous oscillations but did not abolish them. However, oscillations were abolished when RyR were blocked with tetracaine or ryanodine. Oscillations ceased in the absence of external calcium and frequency was directly proportional to external calcium concentration. Frequency of calcium oscillation was reduced by SKF96365 but not by nifedipine. Lanthanum and cadmium completely blocked oscillations. We conclude that spontaneous depolarizations in isolated rabbit urethral interstitial cells depend on global calcium waves caused initially by calcium release from ryanodine sensitive intracellular stores. These primary oscillations must be converted to a propagated calcium wave by IP_3-induced calcium release.

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

SA20

Muscarinic receptor-mediated cationic current in intestinal smooth muscle of M_2 or M_3 receptor knockout mouse

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Gastrointestinal smooth muscles exhibit the muscarinic cationic current (mI_{Cat}). Studies in guinea-pig ileum have suggested that both M_2 and M_3 subtypes of muscarinic receptor participate in the activation of mI_{Cat} through pertussis toxin (PTX)-sensitive G protein G_o and some isozyme of phopholipase C (PLC) other than regulated by G_q/G_{11} proteins (Zholos & Bolton, 1997; Komori et al. 1998; Yan et al. 2003; Zholos et al. 2004; Okamoto et al. 2004). To provide some insights into the activation mechanism of mI_{Cat}, we have studied the ileal smooth muscle mI_{Cat} in M_2 or M_3 subtype knockout (KO) or M_2/M_3-double KO mouse as well as wild type (WT). The mI_{Cat} was recorded from single ileal longitudinal myocytes using whole-cell patch clamp techniques, where the cells were bathed in a CsCl-based medium and dialysed intracellularly with another CsCl-based one containing a BAPTA/CaCl buffer (calculated Ca^{2+} = 100 nM), unless otherwise stated. All mice were humanely killed.

In WT-derived cells held under voltage-clamp at −50 mV, cumulative applications of nonselective muscarinic agonist carbachol (CCh; 1 to 300 μM) produced mI_{Cat} in a concentration-dependent manner. An M_2-preferring antagonist (methoctramine; 300 nM) caused a rightward parallel shift of the CCh concentration-response curve with an increased EC_{50} value which was consistent with M_2 mediation of mI_{Cat}, while an M_3-preferring antagonist (4-DAMP; 30 nM) severely depressed the E_{max} without a change in the EC_{50} value. Injection of PTX to WT animals (100 μg/kg, i.p.) 70-74 hrs before experiments caused marked reduction of mI_{Cat}. A bath application of a PLC inhibitor (U73122; 1 μM), but not an inactive analogue (U73343; 1 μM), almost abolished mI_{Cat}. Current-voltage (I-V) relation for mI_{Cat} exhibited a U-shaped curve in a voltage range of 0 and −120 mV. All the above features of mI_{Cat} resembled those described for mI_{Cat} in guinea-pig ileal myocytes.

In cells from the M_2-KO and M_3-KO types, only a small mI_{Cat} was evoked even by maximally effective CCh, of which amplitude at 100 μM was estimated to be 19.3 ± 3.7 pA (n=9) for the former type and 11.5 ± 1.5 pA (n=15) for the latter, much smaller than that in WT cells (195.0 ± 28.3 pA, n=13). No current was evoked in the M_2/M_3-double KO. Infusion of GTPγS (200 μM) via patch pipettes induced an mI_{Cat}-like inward current in all four types, and there was no noticeable difference in the current amplitude among them (150-260 pA).

CCh's effects in producing Ca^{2+}-activated K^+-current ($I_{K\text{-}Ca}$) via the M_3/G_q/PLC system and in reducing cAMP levels via the $M_2/G_{i/o}$/adenylate cyclase system were evaluated under appropriate experimental conditions. CCh (100 μM) evoked $I_{K\text{-}Ca}$ in

M_2-KO cells with a similar amplitude to that seen in the WT, but was without effect in the M_3-KO. CCh (1 μM) inhibited the isoprenaline-stimulated cAMP accumulation by 50 % in M_3-KO type and 30 % in WT, but rather enhanced by 30 % in the M_2-KO. Indicated from these results was that even with either M_2 or M_3 subtype lacking, either of the two muscarinic signaling systems is fully operative, in contrast to the mI_{Cat}-inducing system for whose full activation both M_2 and M_3 are indispensable. By non-stationary noise analysis of whole-cell current it was found that the unitary channel conductances underlying the mI_{Cat} (at 100 μM CCh) in M_2-KO and M_3-KO cells were 10 pS and 2 pS, respectively. These values clearly differed from the corresponding value (40 pS) estimated for WT mI_{Cat}. An inward current carried via 10 pS channels was evoked by a diacylglycerol (DAG) analogue (OAG). The I-V curve for mI_{Cat} in M_2-KO or M_3-KO cells was relatively linear compared with that for the WT mI_{Cat}. To see if mI_{Cat} is sensitive to a rise in cytosolic Ca^{2+}, some experiments were carried out where the BAPTA/CaCl buffer was absent inside the cell. There was seen a significant potentiation of mI_{Cat} upon voltage-gated Ca^{2+} entry in WT cells, but not in M_2-KO or M_3-KO cells.

Our data suggest that the ileal longitudinal myocytes exhibit three types of mI_{Cat}. The two of them arise because of the respective openings of 2-pS channels linked to M_2 subtype and of 10-pS channels linked to M_3 subtype via DAG-dependent pathways, while the other arises due to 40-pS channel opening for which both subtypes are absolutely needed and prominently contributes to CCh-evoked mI_{Cat} (in our experimental conditions). There may be a possibility that functional supermolecular units formed by M_2/M_3 hetero-complexes together with G_o protein, some PLC isozyme and 40-pS cationic channels may exist to control the channel opening. Instead, the functional units might involve 2-pS channels, which are transited to 40-pS channels and opened upon activation of the M_2/M_3 hetero-complexes.

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Supported by a Grant-in-Aid Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (No 16380199).

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

SA21

Regulation of spontaneous activity in gastric muscle: comparison of two pacemakers

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Properties of spontaneous electrical activity were investigated in smooth muscle tissues isolated from the stomach antrum of humanely killed guinea-pigs. Recording of electrical responses from smooth muscles using intracellular microelectrode reveals that there are three types of electrical response; pacemaker potentials, follower potentials and slow waves recorded from myenteric interstitial cells of Cajal (ICC-MY), longitudinal muscle and circular muscle, respectively (Dickens et al. 1999). In isolated circular muscles with no attached ICC-MY, slow potentials are also produced periodically (Suzuki & Hirst, 1999), as a result of summation of unitary potentials generated in intramuscular interstitial cells of Cajal (ICC-IM; Edwards et al. 1999). Pacemaker potentials are consisted of two components, a primary component with rapid and transient depolarization and following plateau component. The primary component may be formed by activation of Ca^{2+}-permeable channels, and the plateau component may be formed by activation of Ca^{2+}-activated chloride channels. Slow potential is sensitive to DIDS, suggesting that this potential is formed by activation of Ca^{2+}-sensitive chloride channels. Attempts were made to compare the properties between slow waves and slow potentials. Both potentials are recorded from circular muscles, and slow waves are composed of electrotonic component of pacemaker potentials and superimposing slow potentials, while slow potentials are formed by summation of unitary potentials generated in ICC-IM. Both potentials are formed primarily by summation of unitary potentials, and caffeine (1 mM) blocks only slow potentials but not slow waves, suggesting that the mechanism for generating spontaneous activity differs between ICC-MY and ICC-IM. Slow waves are more regular than slow potentials, and the frequency of slow waves is much higher than slow potentials. However, the frequency of the activity is increased by depolarization of the membrane with high-K^+ solutions or with current injection in both potentials, suggesting that the level of intracellular Ca^{2+} concentrations is one of the key factors for the regulation of frequency. Inhibition of either Ca^{2+}-ATPase at the internal stores by CPA, IP_3 receptors by 2-APB, or mitochondrial proton transport by CCCP results in the blockade of slow potentials but not slow waves. In slow waves, lowering temperature increases the duration and decreases the frequency of slow waves, with no alteration to the amplitude. In slow potentials, reduction in temperature again increases the duration, with no change in the amplitude and frequency. The frequency of these spontaneous activities is decreased by KCN, a metabolic inhibitor, suggesting that mitochondrial activity may be causally related to the generation of rhythmic activity. It is suggested that mitochondria take central role for the generation of rhythmic activity in stomach smooth muscle tissues.

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SA22

Excitability of lower urinary tract smooth muscles: functional implications

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The three functionally important smooth muscles of the lower urinary tract (ureters, detrusor and urethral smooth muscle)

normally show remarkably different patterns of activity. In ureters regular propagated action potentials originating from the renal pelvis trigger contractions propelling urine into the bladder. In the detrusor spontaneous action potentials arise in the muscle bundles but are not propagated far, allowing non-synchronized contractile activity that enables the bladder to sustain a compact shape during filling without significant rise in intravesical pressure, and the urethral smooth muscle maintains constant tone probably without developing action potentials, to prevent leakage of urine during filling. Although these activities were originally called
myogenic, since they persists in the presence of tetrodotoxin, it is becoming apparent that in all three tissues this spontaneous activity is either initiated or modulated by another class of cells with features similar to the interstitial cells of Cajal seen in gastrointestinal smooth muscles (ICC-like cells). These cells are thought to regularly inject depolarizing current through gap junctions into their adjacent smooth muscle cells, activating a variety of voltage sensitive channels leading to the characteristic electrical and mechanical activity seen.

The effects of activity in autonomic nerves to these three muscles also differ. Contractile activity cannot be initiated in the ureteric smooth muscles through autonomic motor nerves, although application of potential transmitters may modulate the action potential shape and resulting contraction. Detrusor smooth muscle is densely innervated by parasympathetic nerves, which can elicit action potentials and synchronized contraction of the detrusor to elevate bladder pressure. Urethral smooth muscles are innervated by multiple inhibitory and excitatory nerves that can suppress or enhance the contractile tone. Histological techniques suggest that both autonomic nerves and collaterals of sensory nerves may innervate both smooth muscle cells and ICC-like cells, and a variety of different receptors are present on both types of cell.

Many different molecular pathways have been implicated in neural modulation of contractile activity, including those by-passing the membrane, but many types of ion channel are present in smooth muscles and ICC-like cells, and may represent molecular targets for the transmitters.

This communication will discuss in particular the excitability of the detrusor muscle, since one of the most common disorders of the urinary system is bladder overactivity. In this condition increased spontaneous contractile activity may result in symptoms of urgency and elevation of bladder pressure, which may result in incontinence. Pharmacological control of this condition is not yet optimal, and considerable effort is being spent to develop drugs that can selectively reduce this activity. Increased understanding of the mechanisms involved in its generation is still necessary.

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