

SA1

TRP channels in vascular endothelium: TRPV4 in focus

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The TRP cation channel family consists of 6 mammalian subfamilies which comprise ~30 members. Endothelial cells express TRPV4, TRPM4, TRPP2 and several canonical TRPCs. We focus on the functional role of TRPV4 in mouse aorta endothelial cells, MAEC, from wild type TRPV4+/+ mice and from TRPV4 knockout mice TRPV4-/- . TRPV4 integrates a large variety of stimuli ranging from hypotonic cell swelling (HTS), shear stress, temperature, and α -phorbol ligands, to endogenous agonists such as arachidonic acid (AA) and epoxyeicosatrienoic acids. The binding site of the α -phorbol ligands have been identified as a complex binding pocket spanned by TM3 and TM4. TRPV4 is involved endothelium-dependent vasorelaxation, which can be modulated via the cytochrome P450 (CYP) pathway. The loss of TRPV4 in MAEC mice attenuated responses to all TRPV4 activating stimuli. TRPV4-dependent responses can be modulated via CYP enzymes, which metabolize AA to EETs. Upregulation of CYP2C expression by nifedipine in MAEC from TRPV4+/+ mice causes a potentiated response to AA and cell swelling. Sulphaphenazole, 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. All these data demonstrate that cytochrome P450-derived EETs modulate the activity of TRPV4 channels in endothelial cells. TRPV4-/- mice have a reduced flow-dependent vasorelaxation indicating that this channels may act as a mechano-sensor. As a model for mechano-sensing, we studied TRPV4 activation by cell swelling will be discussed in more detail. We show that N-terminal binding of Pacsin 3 to TRPV4 inhibits the mechano-activation by cell swelling. A possible mechanism and the underlying structural residues will be discussed.

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

SA2

TRP channels, membrane potential and vasomotor mechanisms in resistance arteriesJ. Brayden¹, S. Earley², M.T. Nelson¹ and S. Reading¹¹Pharmacology, University of Vermont, Burlington, VT, USA and²Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

Members of the Transient Receptor Potential (TRP) superfamily of cation channels are expressed by vascular smooth muscle cells, endothelial cells, and periarterial nerves. However, the functional significance of these channels in the vasculature is just beginning to be revealed. Members of the canonical TRPC subfamily contribute to calcium influx mechanisms triggered by depletion of vascular calcium stores or following receptor (adrenergic, purinergic) activation in vascular smooth muscle. In cerebral artery myocytes, receptor ligands activate TRPC3 channels.

In these cells, receptor-mediated Ca^{2+} entry occurs via direct permeation through the TRPC3 channels and also via voltage-dependent Ca^{2+} channels (VDCC); the smooth muscle VDCC are activated by TRPC3 channel-mediated cation influx which depolarizes the smooth muscle cell membrane (Reading et al. 2005). In systemic arteries, TRPC3 channels in smooth muscle are tonically active and contribute to resting membrane potential. TRPC6 channels appear to be receptor-coupled in the peripheral vasculature. The Ca^{2+} -activated TRP channel, TRPM4, is a member of the melastatin TRP subfamily and is present in vascular cells. TRPM4 plays an essential role in pressure-induced, smooth muscle cell depolarization, and development of myogenic tone *in vitro*. Increased intravascular pressure activates TRPM4 channels, which depolarizes the smooth muscle cells, increases VDCC Ca^{2+} influx and triggers myogenic tone (Earley et al. 2004). Cerebral blood flow measurements *in vivo* indicate that TRPM4 channels contribute to blood flow autoregulation via this myogenic mechanism. Protein Kinase C, which is activated by increased intravascular pressure, enhances TRPM4 activity in vascular smooth muscle cells by increasing the Ca^{2+} sensitivity of the channel. TRP channels of the vanilloid receptor subfamily (TRPV) also play important roles in vasomotor regulation. TRPV4 is present in endothelial and vascular smooth muscle cells and contributes to the endothelial response to vasodilator stimuli as well as the smooth muscle cell response to epoxyeicosatrienoic acids (EETs), which are one type of endothelium-derived hyperpolarizing factor. EETs activate TRPV4 channels. Direct Ca^{2+} entry through the activated TRPV4 channels induces Ca^{2+} release from the sarcoplasmic reticulum in the form of Ca^{2+} sparks; the increased Ca^{2+} spark frequency enhances the activity of BKCa channels, which hyperpolarizes and relaxes smooth muscle cells (Earley et al. 2005). The observations described above indicate that TRP channels play unique and important roles in control of tone in resistance arteries. Ongoing experiments are directed towards further understanding of the mechanisms by which TRP channels in the brain and the peripheral circulation are regulated.

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SA3

Store-operated channels, their molecular identity, mechanism of activation and the role in vascular smooth muscle function

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Store-operated channels (SOC) and Ca^{2+} entry (SOCE) have been described in a wide variety of vascular smooth muscle cells

(SMC), and were shown to play an important role in agonist-induced constriction and nitric oxide-induced relaxation in conduit vessels. Molecular identity and mechanism of SOC activation remain highly controversial, making it hard to assess the exact role of SOCE in microvascular function. Recent advancements in the SOCE field identified Orail as a new molecular candidate for SOC (Feske et al. 2006; Vig et al. 2006; Yeromin et al. 2006; Luik et al. 2006b), STIM1 as an activator of the SOCE pathway (Liou et al. 2005; Mercer et al. 2006; Wu et al. 2006; Baba et al. 2006; Luik et al. 2006a; Csutora et al. 2007), and Ca^{2+} -independent phospholipase A_2 beta (iPLA $_2$ β) as a crucial component for signal transduction from the stores to the plasma membrane channels (Smani et al. 2003; Smani et al. 2004; Vanden Abeele et al. 2004; Martinez & Moreno, 2005; Singaravelu et al. 2006; Boittin et al. 2006; Csutora et al. 2006; Csutora et al. 2007). However, alternative models suggest that SOC may be encoded by TRPC1 (Beech, 2005; Liu et al. 2003; Rosado et al. 2002; Ambudkar, 2007), and instead of iPLA $_2$ β and a diffusible messenger, conformational coupling of TRPC1 to the IP $_3$ receptor, STIM1, or other components of the endoplasmic reticulum may be responsible for SOCE activation (Boulay et al. 1999; Rosado & Sage, 2001; Rosado & Sage, 2000; Yuan et al. 2003; Huang et al. 2006; Lopez et al. 2006; Ong et al. 2007). In this review new experimental evidence will be presented on the crucial role of Orail, STIM1 and iPLA $_2$ β in SOCE mechanism and SMC function. A novel unifying model will be introduced that could account for most of the experimental evidence obtained so far by different groups of investigators that was originally used in favor of multiple mutually exclusive SOCE mechanisms. Using new knowledge and experimental tools we will address the questions on what is the role of SOC channels in Ca^{2+} entry and constriction of cerebral, mesenteric and carotid arteries, and how SOC, TRPC1 and voltage-gated L-type Ca^{2+} channels may peacefully play together, and could all be very important for SMC and microvascular function.

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SA4

TRP channels and lipid second messengers

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Transient receptor potential (TRP) superfamily of cation channels includes about 30 related proteins which are responsive to a large array of physical and chemical stimuli, typically in a poly-

modal manner. However, one common emerging feature of TRP regulation appears to be their promiscuous interaction with various phospholipids, which often alters the voltage dependence of the channel gating (Nilius et al. 2007). Several members of the TRPV and TRPM sub-families have been well characterised, especially in terms of their interaction with phosphatidylinositol 4,5-bisphosphate (PIP $_2$), the ubiquitous anionic lipid. Apart from PIP $_2$, lysophospholipids (LPLs) such as lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI) could both activate the cold- and menthol receptor TRPM8. We recently showed that TRPM8 activity reduced in inside-out patches due to PIP $_2$ depletion could be fully restored and even augmented above the cell-attached level following application of LPC or LPI to the intracellular side of the membrane. Ca^{2+} store depletion resulting in Ca^{2+} -independent iPLA $_2$ activation/LPLs production appears to be the main coupling mechanism in this alternative to cold "chemical" mode of TRPM8 activation (Abeele et al. 2006).

Regulation of members of the "canonical" TRPC subfamily by PIP $_2$ is much less characterised. Here we focused on one member, TRPC4, which is typically activated by phospholipase C (PLC)-coupled receptors (Plant & Schaefer, 2003). Its two most abundant transcripts, TRPC4 α and TRPC4 β (the latter lacks 84 amino acids in the cytosolic C terminus), are widely expressed in various smooth muscles (Walker et al. 2001), and in the gastrointestinal tract TRPC4 is an essential component of the cation channel activated by muscarinic receptor stimulation (Lee et al. 2005). Both for the heterologously expressed channels and the native current the signal transduction downstream of PLC activation is still debated. We thus investigated a possible role of PIP $_2$ in the regulation of TRPC4. Two mouse isoforms, TRPC4 α and TRPC4 β , were expressed in HEK293 cells and studied with the patch-clamp recording techniques using Cs⁺-rich (125 mM) external and internal solutions (internal [Ca^{2+}]_i was clamped at 100 nM using 10 mM BAPTA to avoid Ca^{2+} -dependent channel modulation). Infusion of 200 μM GTP γS via pipette slowly activated cation currents with peak densities at -50 mV of -93.1 ± 34.8 pA/pF and -28.9 ± 11.3 pA/pF for the TRPC4 α and TRPC4 β , respectively (n=7-11). The currents showed characteristic doubly-rectifying current-voltage relationships. The non-metabolisable PIP $_2$ analogue diC8-PIP $_2$ added to the pipette solution (20 μM) strongly suppressed TRPC4 α activation (peak current was reduced to -14.7 ± 3.5 pA/pF, n=7; $P < 0.05$ by non-paired *t*-test), but had no effect on TRPC4 β activation (peak current of -25.4 ± 7.6 pA/pF, n=11). Activation of both TRPC4 isoforms was strongly inhibited by the PLC blocker U73122 (2.5 μM , n=4; U73343 used as a negative control had no effect, n=4) as well as by pre-treatment with pertussis toxin (100 ng/ml for 16-18 hrs) indicating involvement of distinct G proteins. Consistent with these findings, in *in vitro* binding study we showed that PIP $_2$ binds directly to the 84 amino acid C-terminal domain that is only present in TRPC4 α . Since PIP $_2$ can act as an anchor for various signalling molecules and cytoskeleton we further investigated possible causal relation between PIP $_2$ effects and cytoskeleton disruption. Treatment of HEK293 cells with cytochalasin D (5 μM for 2 hrs) completely prevented inhibition of TRPC4 α by diC8-PIP $_2$. Finally, we confirmed by Western blotting that both channel variants were expressed in guinea-pig ileal myocytes in which carbachol-induced currents showed essentially similar properties (i.e. diC8-PIP $_2$ suppressed the current while cytochalasin D treatment accelerated its activation, produced a negative voltage shift of channel gating and abolished the inhibitory effect of diC8-PIP $_2$).

Our results suggest that TRPC4 channel is constitutively inhibited by PIP_2 , and its primary mechanism of activation may thus involve release from inhibition by PIP_2 hydrolysis evoked by PLC-coupled receptor stimulation. Cytoskeleton also plays a crucial role in maintaining TRPC4 inactivation.

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SA5

Endothelium-dependent signalling pathways underlying conducted vasodilatation

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An increase in tissue blood flow requires relaxation of smooth muscle cells along entire branches of vascular resistance networks, which are comprised of feed arteries and the arterioles they supply. Our goal is to define signalling pathways intrinsic to the endothelium which coordinate vasodilatation along feed arteries and arterioles and thereby increase tissue perfusion. In skeletal muscle, contractile activity produces vasodilatation that 'ascends' from arterioles into their feed arteries and is essential to attaining high levels of muscle blood flow, which is otherwise restricted by proximal resistance. Vessels of the heart and brain respond similarly to an increase in metabolic demand. To study the mechanism(s) underlying this vascular communication, feed arteries supplying the retractor muscle of hamsters were isolated and pressurized (75 mmHg) to develop spontaneous myogenic tone (resting diameter, 50 to 60 μm ; maximal diameter, 90 to 100 μm). Using acetylcholine (ACh) as a stimulus, we tested the hypothesis that the signal for conducted vasodilatation travels along the endothelium. A brief (≤ 1 s) pulse of ACh delivered from a micropipette onto the distal end of a feed artery (resting membrane potential, -30 to -35 mV) evoked hyperpolarization (10 to 15 mV) and vasodilatation (15 to 20 μm) that conducted rapidly (several mm/s) along the entire vessel (length, 3 to 4 mm). Following selective disruption of endothelial cells (light-dye treatment) within a segment (~ 250 μm long) midway along the vessel, hyperpolarization and vasodilatation spread up to but not through the damaged region. In contrast, selective smooth muscle cell damage had no effect on conducted responses. Thus, the endothelium provides a cellular pathway for conducting hyperpolarization and vasodilatation. To test the hypothesis that endothelial cells promote smooth muscle relaxation through

direct electrical coupling, 2 microelectrodes were used to simultaneously impale an endothelial cell and a smooth muscle cell separated by 500 μm along the vessel. Both cells displayed equivalent membrane potentials at rest and during responses to ACh. Microinjection of negative current into either cell caused hyperpolarization of the other cell along with conducted vasodilatation, confirming that respective cell layers are electrically coupled to each other. Moreover, focal electrical field stimulation evoked similar responses, indicating that endothelial cells can be excited electrically to produce hyperpolarization and vasodilatation. Thus, electrical signals initiated by and conducted along the endothelium can be transmitted directly to the surrounding smooth muscle to evoke electromechanical relaxation. Immunolabeling for gap junction proteins revealed robust expression of connexins 37, 40 and 43 along borders of endothelial cells. Electron microscopy confirmed the presence of myoendothelial gap junctions for heterocellular transmission of electrical signals. As complementary signals (e.g. nitric oxide) have been implicated in the conduction of vasodilatation, we tested the hypothesis that Ca^{2+} waves propagate from cell to cell along the endothelium. During Ca^{2+} imaging, ACh triggered an increase in endothelial cell Ca^{2+} at the site of stimulation which preceded two distinct events: (1) a rapid synchronous decrease in smooth muscle Ca^{2+} along the entire vessel, and (2) an ensuing Ca^{2+} wave that propagated bidirectionally for > 1 mm along the endothelium at ~ 111 $\mu\text{m/s}$. To investigate the functional role of Ca^{2+} waves, a vessel was perfused with charybdotoxin + apamin at the site of ACh stimulation. Remarkably, this local inhibition of K Ca channels (to prevent hyperpolarization) unmasked a 'slow' conducted vasodilatation that traveled at ~ 21 $\mu\text{m/s}$. Recorded 500 μm upstream from the ACh stimulus, a rise in endothelial cell Ca^{2+} preceded dilatation by ~ 10 s. When nitric oxide synthase and cyclooxygenase were inhibited, the slow vasomotor response was abolished while Ca^{2+} waves remained intact. Experiments performed in mice demonstrate similar behavior for arteriolar networks in vivo. Our findings collectively resolve 2 distinct yet complementary signalling pathways for the conduction of vasodilatation along microvascular endothelium in response to ACh: (1) a rapid electromechanical relaxation of smooth muscle cells along the vessel initiated by K Ca channels, and (2) an ensuing slow 'wave' of Ca^{2+} along the endothelium that releases autacoids to promote pharmacomechanical relaxation and sustain the coordinated vasomotor response.

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SA6

Endothelial cell ion channels and microvascular function

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Endothelium-dependent hyperpolarization assumes increasing functional significance as artery size decreases. As such, it makes a significant contribution to the physiological regulation of blood pressure and flow, independently of nitric oxide.

Hyperpolarization is initiated in the endothelium by an increase in cytoplasmic calcium concentration, which activates small and intermediate conductance calcium-activated K^+ channels (SKCa and IKCa channels). This key event is followed by a transfer of the hyperpolarization to the smooth muscle, consequently closing voltage-dependent calcium channels and leading to smooth muscle relaxation and vasodilatation. How the transfer of hyperpolarization occurs has been the subject of considerable debate, with a number of putative hyperpolarizing factors, or EDHFs, proposed to be responsible alongside the possibility that spread of hyperpolarization occurs passively. Passive spread of hyperpolarization could occur through myoendothelial gap junctions which link the endothelial and smooth muscle cells.

This lecture will provide an overview of the current state of knowledge in this area, discussing recent evidence suggesting that endothelial KCa channels are rationally localized within discrete regions of the endothelial cell, with consequences for the functional mechanisms responsible for EDHF dilatation. The possibility that EDHF-mediated dilatation reflects the combined action of a diffusible factor and spread of hyperpolarization through myoendothelial gap junctions will also be discussed in relation to radial spread into the artery wall, and longitudinal spread over distance along the artery wall, important for coordinating flow through vascular beds.

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SA7

Local potassium signalling couples astrocytic activity to brain microvascular functionM.T. Nelson¹, S.V. Straub¹, J.A. Filosa^{1,2}, K.M. Wilkerson¹, A.L. Meredith^{3,4}, R.W. Aldrich^{3,5} and A.D. Bonev¹

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Functional hyperemia - the linkage between neuronal activity and local blood flow that serves to satisfy neuronal metabolic demands - is a phenomenon that has been appreciated for over

100 years; however, the underlying mechanisms are poorly understood. Recent studies have illuminated a potentially central role for astrocytic calcium (Ca^{2+}) signals as mediators of this process. Astrocytes make hundreds to thousands of contacts with neurons, and their processes ('endfeet') encase the brain microcirculation. Neuronal stimulation causes a rapid elevation in intracellular Ca^{2+} which propagates to the endfeet, and is associated with subsequent reduction in Ca^{2+} in the smooth muscle cells (SMCs) of the penetrating arterioles (Filosa et al. 2004). Utilizing high spatiotemporal resolution confocal calcium imaging of cortical brain slices, it was found that inositol trisphosphate ($InsP_3$) receptors are present within astrocytic endfeet and are activated following induction of neuronal activity. The generation of an endfoot-delimited Ca^{2+} increase in an individual endfoot, through rapid spatially restricted photo-release of caged $InsP_3$, was sufficient to induce local vasodilatation of an adjacent arteriole (Straub et al. 2006). Since the $InsP_3$ -induced vasodilatation was restricted to a short stretch of the vessel centred on the endfoot, it suggests that endfeet function as individual 'vasoregulatory units' in the brain. One potential target for a Ca^{2+} signal in the astrocytic endfoot is the large-conductance, Ca^{2+} -sensitive K^+ (BK) channel, which when activated would release K^+ ions from the endfoot onto the adjacent smooth muscle of the arteriole. Modest elevation of extracellular potassium (K^+) activated inward rectifier K^+ (Kir) channels, and caused membrane potential hyperpolarization and vasodilatation of intracerebral arterioles, in isolation, and in cortical brain slices. Blocking Kir channels or BK channels reduced neuronally-evoked vasodilatation by about 70%, and caused complete abrogation in the presence of a COX inhibitor (Filosa et al. 2006). These results support the concept that neuronal activity is translated into an $InsP_3$ -mediated calcium signal in astrocytes, which is decoded by BK channels in the endfoot to locally release K^+ into the perivascular space to activate SM Kir channels, and cause vasodilatation.

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SA8

Potassium channels and the regulation of arteriolar function

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Vascular smooth muscle (VSM) cells and endothelial cells (EC) that form the walls of arterioles express a diverse array of ion channels that play important roles in the function of these cells

and the microcirculation in both health and disease. Potassium channels, in particular, importantly regulate the function of these cells primarily through their impact on membrane potential. In cells that possess voltage-gated calcium channels (VGCC) such as VSM and perhaps some EC, membrane potential importantly controls the open state probability of the VGCC, which determines intracellular calcium and hence the function of these cells. Membrane potential also importantly impacts the electrochemical gradient for diffusion of calcium into cells through non-voltage gated channels such as those in the transient receptor potential family of ion channels. Finally, membrane potential, through its ability to be transmitted between cells through gap junctions, also acts as an important signal for cell-cell communication in the arteriolar wall. Microvascular VSM cells express at least four different classes of potassium channels including inward-rectifier potassium channels (KIR), ATP-sensitive potassium channels (KATP), voltage-gated potassium channels (KV) and large conductance calcium-activated potassium channels (BKCa). VSM KIR participate in dilation induced by elevated extracellular potassium and may also be activated by C-type natriuretic peptide, a putative endothelium-derived hyperpolarizing factor (EDHF). Vasodilators acting through cAMP or cGMP signalling pathways in VSM may open KIR, KATP, KV and BKCa causing membrane hyperpolarization and vasodilatation. VSM BKCa also may be activated by epoxides of arachidonic acid (EETs) identified as EDHF in some systems. Conversely, vasoconstrictors may close KATP, KV and BKCa through protein kinase C, Rho-kinase or c-Src pathways and contribute to VSM depolarization and vasoconstriction. Despite the inhibitory effects of these signaling pathways on KV and BKCa, the net depolarization, and in the case of BKCa, the increase in intracellular calcium caused by vasoconstrictors activate both KV and BKCa channels which act in a negative feedback manner to limit the vasoconstrictor-induced depolarization and increase in VSM intracellular calcium and hence prevent vasospasm. While calcium transients through ryanodine receptors (calcium sparks) control BKCa channel function in some smooth muscles, calcium entry through VGCC appears to play a more important physiological role in controlling BKCa function in some microvascular VSM. Microvascular EC express at least five classes of potassium channels including small (sKCa) and intermediate (IKCa) conductance calcium-activated potassium channels, KIR, KATP and KV. Both sK and IK are opened by endothelium-dependent vasodilators that increase EC intracellular calcium to cause membrane hyperpolarization that may be conducted through myoendothelial gap junctions to hyperpolarize and relax arteriolar VSM. Endothelial KIR serve to amplify sKCa- and IKCa-induced hyperpolarization and allow active transmission of hyperpolarization along EC through gap junctions. Endothelial KIR channels also may be opened by elevated extracellular potassium and participate in potassium-induced vasodilatation. Endothelial KATP channels may be activated by vasodilators as in VSM and EC KV channels may provide a negative feedback mechanism to limit depolarization in some endothelial cells. Thus, potassium channels play a central role in the regulation of many aspects of microvascular cell function.

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SA9

Control of potassium channel function by alternative splicing

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The pore-forming subunits of potassium channels are encoded by more than 70 distinct genes. Expression of distinct genes allows potassium channels with diverse properties to be encoded several of which are implicated in the physiological control of vascular function. However, a variety of posttranscriptional mechanisms can further extend the phenotypic diversity of potassium channels including heteromeric assembly of subunits, association with accessory subunits, assembly with distinct signalling complexes and alternative pre mRNA splicing. Alternative pre mRNA splicing is particularly prevalent in single gene families as exemplified by large conductance voltage- and calcium-activated potassium (BK) channels encoded by the KCNMA1 gene. Here we discuss how dynamic control of alternative pre mRNA splicing of BK channels can dramatically modify their functional properties and regulation by divergent signalling pathways. The implications of alternative splicing for analysis of the control of the microcirculation will be discussed.

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

SA10

Vascular ion channel switching and regulation

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The vascular smooth muscle cell is an example of a cell that retains its capability to switch phenotype through adult life – from a contractile cell to a non-contractile, proliferating and migratory cell. This capability is important in physiology but also contributes substantially to vascular diseases including neointimal hyperplasia. An emerging feature of the phenotypic switch is that it is accompanied by change in the selection of ion channels made by the cell. Some of the change is known to be functionally important because blocking key ion channels suppresses proliferation, migration and neointimal hyperplasia (Kumar et al. 2006; Xu et al. 2006). Other cell types undergo similar changes and so there may be common underlying mechanisms. Regulatory networks at the transcriptional level are clearly implicated and emerging features will be reviewed. The lecture will focus on REST (repressor element-1 transcription factor), recently shown to be a repressor of the KCNN4 gene, which encodes a critical potassium channel (KCa3.1) in cell proliferation (Cheong et al. 2005). REST is down-regulated in cell proliferation and regulates expression of other relevant ion channels. We are working on the hypothesis that REST has a role in orchestrating

changes in ion transport associated with cell phenotype switching.

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SA11

Retinovascular physiology and pathobiology: New approaches, new ideas

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Evidence is accumulating that pericyte-containing microvessels, which constitute the largest component of the circulatory system, actively regulate local perfusion. Because the retinal vasculature is specialized for the local control of blood flow, study of its microvessels is proving useful in the quest to elucidate the mechanisms by which local perfusion is regulated. The microcirculation of the retina is also a focus of attention due to its vulnerability to diabetes, which is a leading cause of vision loss. This talk will give an overview of our recent experimental findings with an emphasis on unexpected observations and new ideas concerning the regulation of retinal microvascular function (1). We use perforated-patch pipettes to monitor ionic currents, fura-2 to measure calcium levels and time-lapse photography to visualize changes in mural cell contractility and lumen diameter in microvessels freshly isolated from the adult rat retina. For the study of diabetes, rats are injected with streptozotocin. To isolate microvascular complexes, retinas were rapidly removed post mortem and then incubated in a papain-containing solution. Subsequently, a retina is gently sandwiched between two glass coverslips; adhering to the coverslip contacting the vitreal side of the retina are complexes that include smooth-muscle encircled arterioles and pericyte-containing capillaries (Fig. 1).

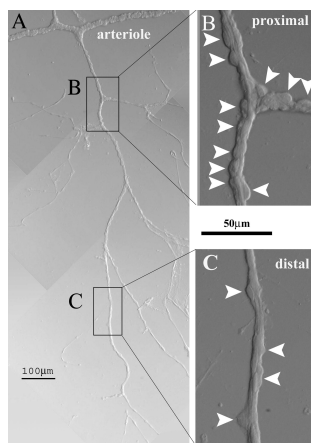


Figure 1. A vascular complex freshly isolated from the retina of an adult rat.

Recently, we began to assess how putative vasoactive signals regulate ion channels, intracellular calcium and mural cell contractility (Fig. 2). Unexpectedly, we found that in addition to evoking the release of stored calcium, activating non-specific cation channels and causing pericyte contraction, angiotensin II, ATP and endothelin-1 potently inhibit cell-to-cell communication within pericyte-containing microvessels. This regulation of gap junction pathways is a newly appreciated mechanism by which extracellular molecules regulate microvascular function.

Indicative of the importance of gap junction-mediated transmission, our recent study of the electrotonic architecture of the pericyte-containing microvasculature revealed that locally induced voltage changes are efficiently transmitted throughout a vascular network. As a result, a localized exposure to angiotensin, ATP or endothelin initially evokes depolarization throughout a microvascular complex. However, when gap junctions close, intercellular transmission ceases. Thus, it appears that the spatiotemporal dynamics of the microvasculature's response to extracellular signal may be more complex than previously thought.

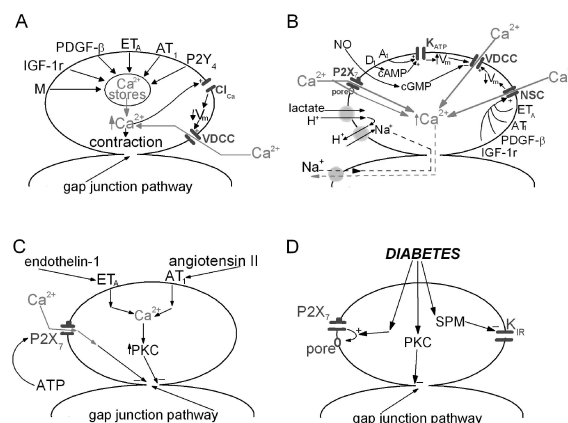


Figure 2. Schematic summaries of recently characterized mechanisms mediating the response of retinal microvessels to extracellular signals.

Another unexpected observation was that pericyte-containing retinal microvessels express P2X₇ purinoceptors, whose activation causes vasoconstriction (Fig. 2), but also results in the formation of large transmembrane and the triggering of microvascular cell death. Interestingly, soon after the onset of diabetes, the concentration of P2₇ needed to open pores and to trigger apoptosis decreases by approximately 80-fold. This increased formation of pores appears to be due to a diabetes-induced enhancement of the transition from activated P2X₇ receptor/channels to opened pores. In this way, normally non-lethal concentrations of P2X₇ ligands may trigger cell death in microvessels of the diabetic retina.

In other studies, we detected a topographical heterogeneity of K_{IR} currents. At distal sites within pericyte-containing retinal microvessels, the K_{IR} current shows strong inward rectification, but proximally the K_{IR} current is only weakly rectifying. Because of the efficient electrotonic transmission within retinal microvessels, the outward current of the proximal K_{IR} channels plays a significant role in setting the membrane potential of cells located throughout the microvasculature. Of potential pathobiological significance, K_{IR}

heterogeneity is lost soon after the onset of diabetes, as rectification of the proximal K_{IR} current increases. This diabetes-induced effect is reversed by an inhibitor of polyamine synthesis and is mimicked by spermine, whose concentration is elevated in the diabetic eye. These new findings raise the possibility that functional changes in K_{IR} channels contribute to the dysregulation of blood flow detected early in the course of diabetic retinopathy.

Recent studies reveal previously unrecognized mechanisms by which the function of the retinal microvasculature may be regulated in health and disease.

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

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Ion channels in retinal arterioles: physiology and pathophysiology

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Variations in the tone of retinal arterioles plays an important role in auto-regulation of retinal blood flow. Our research aims to understand the physiological control of retinal arteriolar tone and how this control is altered in diabetes mellitus. Animal and clinical studies have shown that retinal vasoconstriction and reduced retinal blood flow precede the onset of diabetic retinopathy (Bursell et al. 1996). The studies summarised here provide evidence that this may result from down-regulation of the β_1 -subunit of the large conductance Ca^{2+} activated K^+ (BK) channels. This reduces BK-channel activity, which normally hyperpolarizes and relaxes vascular smooth muscle cells. Understanding these changes may allow earlier diagnosis of retinopathy and suggest novel therapeutic strategies. Experiments were carried out using freshly isolated arterioles from male Sprague-Dawley rats (Scholfield & Curtis, 2000). Diabetes was induced by intra-peritoneal injection of streptozotocin and confirmed by measurement of blood glucose and glycosylated haemoglobin. Animals were terminally anaesthetized with CO_2 and retinal vessels isolated 3 months after injection. Tissues were compared with those from age-matched controls. Ca^{2+} -measurements and imaging were carried out using isolated segments of retinal arteriole pre-loaded with fura-2 or fluo-4, respectively. Whole cell electrophysiology using the perforated patch technique (amphotericin B in the pipette) was applied to myocytes embedded within arteriole segments but electrically isolated by partial enzymatic digestion. Single channel recordings were made using inside-out patches. Data have been summarised as the mean \pm SEM. Myocytes in control arterioles generate spontaneous Ca^{2+} -sparks, waves and oscillations (Tumelty et al. 2006). This opens up the possibility for feedback via Ca^{2+} -sensitive ion channels, since both Ca^{2+} -activated K^+ and Ca^{2+} -activated Cl^- conductances are

present in these cells (Scholfield et al, in press). Application of the BK blocker penitrem A (100 nM) decreased the diameter of pressurised retinal arterioles by more than 25% ($P<0.05$, paired t-test), indicating that BK activity normally limits constriction in these vessels. The BK current evoked by caffeine in voltage clamped myocytes from diabetic vessels was reduced by approximately 90% at 0 mV ($P<0.05$). This was selective for the BK current as there were no parallel decreases in the caffeine activated Cl^- current or the caffeine-evoked $[Ca^{2+}]_i$ transients. BK channel mediated spontaneous transient outward currents were also reduced in diabetic cells ($P<0.001$ at 0mV), even though the frequency of the Ca^{2+} sparks believed to activate these currents was unchanged and their amplitude (maximal change in normalised fluorescence) was actually increased from a control average of 0.42 ± 0.03 to 0.92 ± 0.06 in diabetic vessels ($P<0.001$). These data all suggest that Ca^{2+} -activated BK activity was reduced by a mechanism downstream of the Ca^{2+} release process. Two observations suggest that this was not simply the result of decreased expression of the BK channels themselves. Quantitative PCR showed no change in mRNA levels for the pore forming α -subunit in diabetic arterioles and the amplitudes of Ca^{2+} -independent BK currents elicited by depolarizing voltage steps were similar in control and diabetic arterioles (115 ± 30 pA/pF and 114 ± 26 pA/pF, respectively; NS). Single channel conductance was also unaltered but the Ca^{2+} sensitivity of single BK channels was markedly reduced. There was a rightward shift in the in the open probability vs. $[Ca^{2+}]$ relationship at +80mV for channels in inside-out patches from diabetic retinal arteriolar myocytes, with a parallel reduction in the Hill slope. When the voltage-dependence of channel activation was determined at a fixed $[Ca^{2+}]$ of $10\mu M$, this was shifted to the right by >100 mV in diabetic tissues. These observations are consistent with down-regulation of BK β_1 activity, since this subunit confers Ca^{2+} -sensitivity to BK channel complexes (Brenner et al. 2000). Transcript levels for BK β_1 were appreciably lower in diabetic retinal arterioles and protein expression, as estimated using immunohistochemistry and confocal microscopy, was also reduced. The mean open times and the sensitivity of BK channels to tamoxifen were also decreased in diabetic cells, observations which are also consistent with a down-regulation of BK β_1 -subunits. Overall, these results support the hypothesis that diabetes mellitus down-regulates expression of the BK β_1 -subunit within retinal arteriolar myocytes and that this may explain the retinal vasoconstriction seen in the early phases of diabetic retinopathy.

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