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WHAT'S WHERE AND WHY AT A VASCULAR MYOENDOTHELIAL MICRODOMAIN SIGNALLING COMPLEX

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SUMMARY

1. Modulation of vascular cell calcium is critical for the control of vascular tone, blood flow and pressure.

2. Specialized microdomain signalling sites associated with calcium modulation are present in vascular smooth muscle cells, where spatially localized channels and calcium store receptors interact functionally. Anatomical studies suggest that such sites are also present in endothelial cells.

3. The characteristics of these sites near heterocellular myoendothelial gap junctions (MEGJs) are described, focusing on rat mesenteric artery. The MEGJs enable current and small molecule transfer to coordinate arterial function and are thus critical for endothelium-derived hyperpolarization, regulation of smooth muscle cell diameter in response to contractile stimuli and vasomotor conduction over distance.

4. Although MEGJs occur on endothelial cell projections within internal elastic lamina (IEL) holes, not all IEL holes have MEGJ-related projections (approximately 0–50% of such holes have MEGJ-related projections, with variations occurring within and between vessels, species, strains and disease).

5. In rat mesenteric, saphenous and caudal cerebellar artery and hamster cheek pouch arteriole, but not rat middle cerebral artery or cremaster arteriole, intermediate conductance calcium-activated potassium channels (IK_{Ca}) localize to endothelial cell projections.

6. Rat mesenteric artery MEGJ connexins and IK_{Ca} are in close spatial association with endothelial cell inositol 1,4,5-trisphosphate receptors and endoplasmic reticulum.

7. Data suggest a relationship between spatially associated endothelial cell ion channels and calcium stores in modulation of calcium release and action. Differences in spatial relationships between ion channels and calcium stores in different vessels reflect heterogeneity in vasomotor function, representing a selective target for the control of endothelial and vascular function.

Key words: cell signalling, endothelium, ion channel, smooth muscle, vasodilation.

INTRODUCTION

The control of vascular tone and, thus, blood flow and pressure is dependent on the balance between vasoconstrictor and vasodilator actions, which, in turn, are dependent on neural, humoral and physical stimuli.[1] Endothelium-derived vasoconstrictors include peptides such as endothelin-1 and angiotensin II, metabolites of arachidonic acid (e.g. thromboxane A_2) and superoxide anions,[2] whereas endothelium-derived vasodilators include nitric oxide (NO), prostacyclin (PGI_2) and the non-NO/PGI_2 endothelium-derived hyperpolarization (EDH) mechanism.[3–5] The relative contribution of these different vasoconstrictor and vasodilator mechanisms varies within and between vascular beds, species, strains, sex, development, ageing and disease, as well as with experimental conditions.

In addition to the above, functional variability in arterial smooth muscle cells (SMCs) is associated with the separation of the signalling pathways involved in the control of tone,[6] with electrical current and second messenger molecule movement between vascular cells via gap junctions also being critical for the maintenance of tone. Changes in calcium in both SMCs and endothelial cells are essential for the maintenance of vascular tone.[1,3,4,6–9] Aspects of the specific mechanisms involved in arterial SMC function, and particularly those related to modulation of intracellular calcium, appear to depend on the spatial compartmentalization of ion channels and receptors and associated calcium stores at 'microdomain' sites.[6,10,11] Such sites enable microdomain-specific channel and receptor activation,[6,10]

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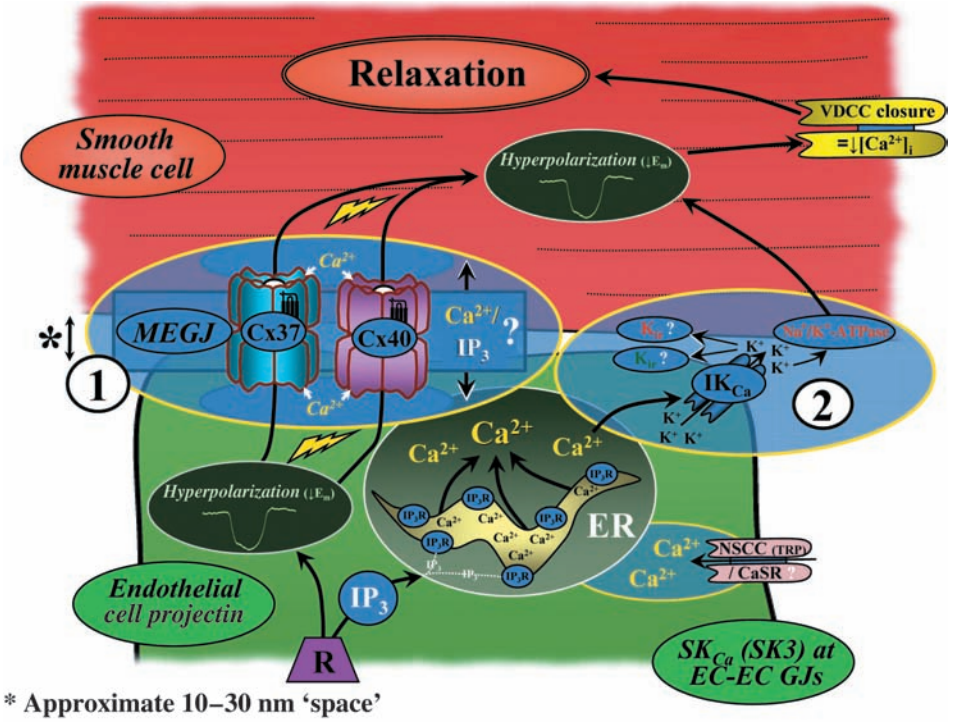

Fig. 1 Mechanism of endothelium-derived hyperpolarization (EDH): endothelial to smooth muscle signalling in rat mesenteric artery. In response to agonist (R), intracellular endothelial cell (EC) calcium release occurs from inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R)-mediated[29,30] endoplasmic reticulum (ER) stores, which are in close proximity to myoendothelial gap junction (MEGJ) connexin (Cx) 37 and Cx40 (mechanism 1)[32] and intermediate conductance calcium-activated potassium channels (IK_{Ca}; mechanism 2).[32] The net result of such activity is hyperpolarization of the adjacent smooth muscle, closure of voltage-dependent calcium channels (VDCC), subsequent reduced smooth muscle cell (SMC) calcium and vessel relaxation. Mechanism 1 involves transfer of EC-derived hyperpolarization via MEGJ Cx37 and Cx40, whereas mechanism 2 involves localized potassium release from IK_{Ca}, activation of smooth muscle Na^+/K^+-ATPase, with endothelial (and perhaps smooth muscle) inward rectifying potassium channels (K_{ir})[23,24] regulating such localized potassium activity. Replenishment of EC calcium putatively occurs via the non-selective cation channel (NSCC; as a transient receptor potential (TRP);[82,104,105] see also Fig. 4, inset) or calcium-sensing receptor channel[106] (CaSR) activity. Endothelial to smooth muscle (or vice versa; Fig. 4) movement of calcium and/or IP_3 may also be involved in the modulation of mesenteric artery tone,[29,30,42] with such a suggestion being by culture studies showing Cx modulation of IP_3 transfer.[107] In addition, calcium modulation of gap junction Cx function[108,109] may regulate current and/or IP_3 transfer, thus being critical for the control of vessel tone. Mechanisms 1 and 2 may operate independently or in a concomitant manner. The use of characterized antibodies to small conductance calcium-activated potassium channels (SK_{Ca})[12,110] demonstrates the presence of SK3 at adjacent EC-EC gap junctions (GJs), but not at MEGJs, in rat mesenteric artery. The 'space' between EC and smooth muscle cell membranes at the MEGJ-related site is approximately 10–30 nm (asterisk), although such an apparent distance will likely be dependent, in part, on the methodology used for the preparation of samples.

although the spatial arrangement and physiological role of such sites in endothelial cells is a new area of study. Of interest, the connexins (Cxs) comprising the myoendothelial gap junctions (MEGJs) are in close spatial association with intermediate conductance calcium-activated potassium (IK_{Ca}) channels in rat mesenteric artery,[12,13] potentially mediating specific functional aspects of EDH activity.[14]

The focus of the present brief review is to describe the limited current knowledge on the microdomain signalling mechanisms associated with contact-mediated gap junction communication between endothelial cells and SMCs at MEGJs.

HETEROCELLULAR MEGJ COUPLING

Endothelium to smooth muscle signalling: EDH

Consensus on the mechanism of EDH involves agonist-induced release of endothelial cell calcium, subsequent activation of endothelial cell small (S) K_{Ca} and IK_{Ca} channels, release of epoxyeicosatrienoic acids (EETs), K^+ and/or current, which is transferred to the adjacent smooth muscle with subsequent hyperpolarization, closure of voltage-dependent calcium channels and vessel relaxation (Fig. 1). A proposed role for C-type natriuretic peptide (CNP) and hydrogen peroxide in EDH is not supported by current evidence, with the original studies being based on questionable experimental design and data.[3,15–18]

In a limited number of vascular beds, namely coronary, renal and cerebral beds of some species, and in response to selected stimuli, EETs are synthesised by cytochrome P450 2C or 2J epoxygenases.[4,5] The EETs may then act to regulate gap junctions[19] and/or be released from endothelial cells to activate SMC large (B) K_{Ca}, although the precise mechanism of EET activation of BK_{Ca} is not fully characterized.[4] EET-independent BK_{Ca}-dependent EDH activity has also been reported in mouse cremaster arterioles and spontaneously hypertensive rat mesenteric artery.[20,21]

With regard to K^+, it was originally proposed that K^+ released from endothelial cells during channel opening accumulated in the space occupied by the internal elastic lamina (IEL) and surrounding SMCs in a concentration sufficient to activate inward rectifying potassium channels (K_{ir}) and the Na^+/K^+-ATPase (Na^+–K^+ pump) on SMCs.[4,22] However, this scenario is implausible,[23,24] because such a space is physically too large to achieve the dynamic K^+ flux required for the characterized rapid EDH response. Alternatively, as outlined in the present review, diffusible K^+ may act at specialized microdomain signalling sites associated with MEGJs (Fig. 1).

Spatial and temporal modulation of calcium dynamics is critical for vascular function, and anatomical and functional studies suggest an association between sites of calcium release, targets of calcium action and gap junction Cxs.[12,13] In rat mesenteric artery, adjacent endothelial cells are coupled by Cx37, Cx40 and Cx43,[27] which are spatially close to densities of SK_{Ca},[12] whereas MEGJ Cx37 and

Cx40 are spatially associated with IK_{Ca},[12,13] potentially corresponding to different facets of the functional EDH response.[28] Other data demonstrate a close spatial relationship between Cxs, inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R) and endoplasmic reticulum (ER) within endothelial cell projections through holes in the IEL, with MEGJs being located between endothelial cells and SMCs on such endothelial cell projections. Intense IP_3R labelling is present near the endothelial side of MEGJs in rat mesenteric artery (Figs 1,3a–e), consistent with the integral role for IP_3 and IP_3R in EDH.[29,30] Such sites likely reflect IP_3-mediated 'calcium pulsars' within IEL holes, at putative MEGJ sites, as are present in pressurized mouse mesenteric artery.[31,32] The close spatial localization of sites of calcium release and vascular Cxs suggest the potential for a causal functional relationship in that these sites of current transfer and calcium modulation likely interact. Thus, the relationship between IK_{Ca} and MEGJs differs between vascular beds and species (Fig. 2a–l), with IK_{Ca} density corresponding to the density of MEGJ-related endothelial cell projections in some, but not all, vascular beds.

In rat mesenteric, saphenous and caudal cerebellar artery, IK_{Ca} are localized on endothelial cell projections (Fig. 2a–l), corresponding to MEGJ incidence.[26,33,34] Furthermore, such a relationship is present in hamster cheek pouch arteriole, but not rat middle cerebral artery or cremaster arteriole (SL Sandow, unpubl. obs., 2006). The hamster cheek pouch arteriole exhibits a high density of localized IK_{Ca}, consistent with the high MEGJ density in this vessel.[35] Of note, although MEGJs are relatively common in rat cremaster arteriole and middle cerebral artery[36,37] (approximately 25% of IEL holes have MEGJs in rat middle cerebral artery), IK_{Ca} densities at IEL holes are absent and, thus, not localized to MEGJs in these two vessels. Indeed, although MEGJs generally occur in a similar location on endothelial cell projections that pass through holes in the IEL, not all such holes have endothelial cell projections with MEGJs passing through them. In fact, only 0–50% of such holes have projections with MEGJs, with the variation occurring within and between vessels, species, strains and disease.[38] In general, MEGJ density is decreased with increased vessel size,[3] concomitant with the contribution of EDH to vasodilation.[3] In this regard, MEGJs are rare to absent in adult male saphenous and femoral artery, respectively, where EDH is absent.[33,34] However, even in these vessels, IEL holes exist and are prevalent.[38] In the absence of heterocellular coupling, these holes may act as low-resistance pathways for the diffusion of vasoactive substances between endothelial cells and SMCs (or vice versa), or they may signify previous developmental history or subsequent ageing and disease involving changes to MEGJs and associated signalling mechanisms.

An ongoing problem within the EDH field is the restriction of studies to the use of pharmacological blockers of relevance only to certain pathways of specific interest, with failure to consider the use of inhibitors that may implicate a role for alternative EDH mechanisms. Such behaviour results in bias in favour of one EDH mechanism over others that could otherwise be implicated. This situation is particularly prevalent in studies considering the role of CNP and hydrogen peroxide in EDH.[3,16,17] A further ongoing issue with the EDH field (as with many fields) is the lack of specific characterized blockers. Indeed, 1-[(2-chlorophenyl)diphenyl-methyl]-1H pyrazole (TRAM-34), a key IK_{Ca} antagonist used in many EDH studies, has recently been suggested to have effects at non-selective cation channels (NSCCs), such as transient receptor potential (TRP) channels.[25] Further, Cx-mimetic 'Gap' peptides, key blockers of electrically mediated EDH, have additional effects on intracellular calcium stores.[26] This latter effect may be related to the close spatial association of vascular Cxs and sites of calcium modulation, discussed below (Figs 1–4).[12] Unfortunately, such observations complicate the interpretation of data in EDH studies.

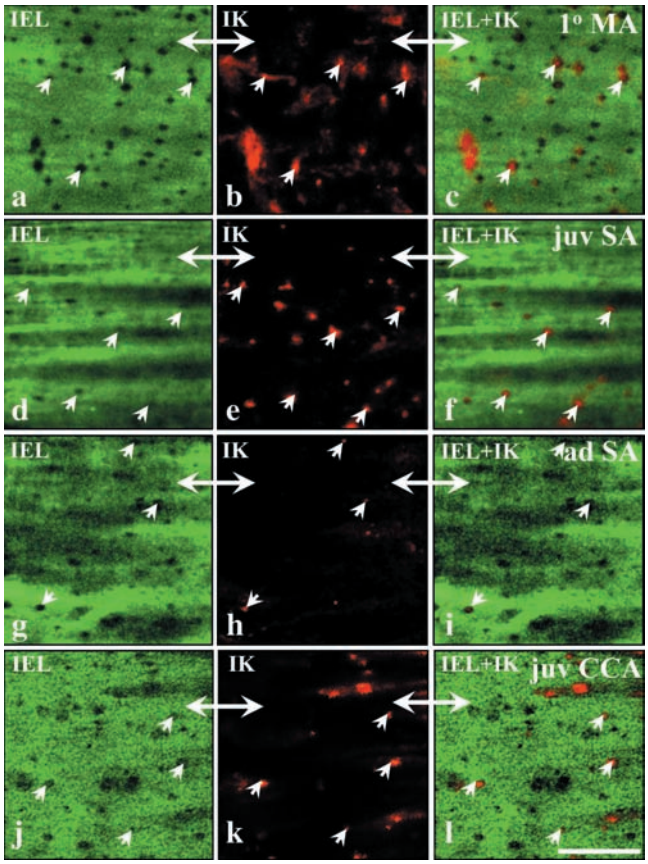

Fig. 2 Distribution of intermediate conductance calcium-activated potassium channels (IK_{Ca}; red) at the internal elastic lamina (IEL; green) hole (dark spots) to smooth muscle cell interface, as potential myoendothelial gap junction (MEGJ) sites (examples arrowed), in selected resistance arteries. Confocal microscopy using antibodies to IK1 (SK4), developed and characterized by Neylon *et al.*[111] and Chen *et al.*,[110] demonstrate IK_{Ca} distribution corresponding to MEGJ density in rat primary mesenteric (a–c; 1° MA), juvenile and adult saphenous (d–f (juv SA), g–i (ad SA), respectively) and caudal cerebellar arteries (j–l; CCA). Approximately 40 and 46% of IEL holes are associated with localized IK_{Ca} densities and MEGJ incidence in rat primary mesenteric and caudal cerebellar arteries, respectively, and 46 and 4% of juvenile and adult saphenous arteries, respectively (MEGJ density from published studies;[26,33,112] see also table 6 in Sandow *et al.*[38]). Primary antibodies were conjugated to Alexa633 secondary, with immunohistochemistry and controls conducted as per standard.[12,13] Endothelial cells are arranged left to right. Bar, 20 μm.

Smooth muscle to endothelium signalling

As outlined above, myoendothelial endothelial cell to SMC communication is essential for vasodilator control of arterial diameter and, by association, blood flow and pressure. Studies have focused on how endothelial cells regulate the diameter of underlying SMCs via the release diffusible factors, such as NO and by direct electrical coupling via MEGJs. However, it is becoming evident that the

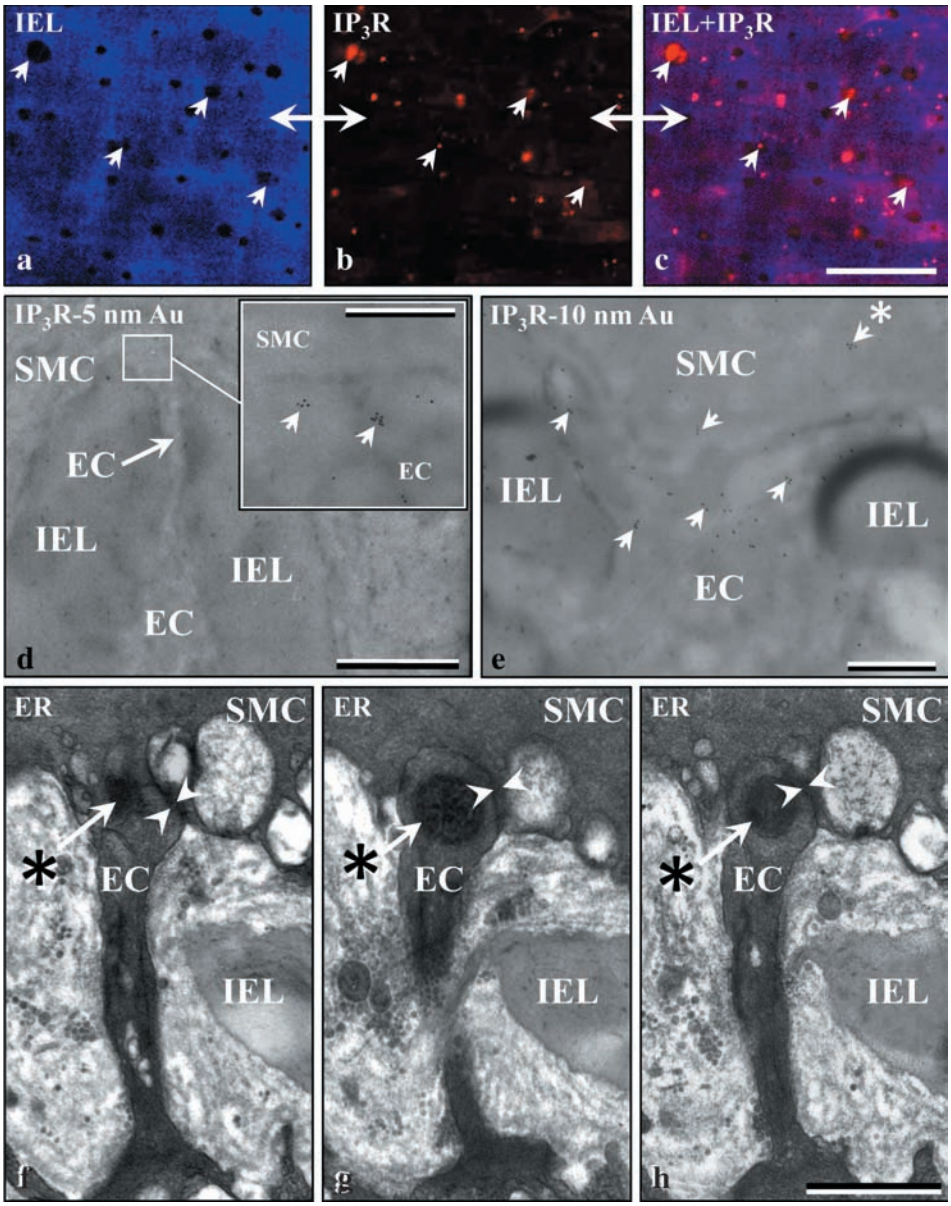

Fig. 3 Distribution of inositol 1,4,5-trisphosphate receptor (IP_3R; red and Au particles, examples arrowed) and endoplasmic reticulum (ER) at potential myoendothelial gap junction (MEGJ) sites in rat mesenteric artery. Confocal microscopy using antibodies to IP_3R (pan, Chemicon (Billerica, MA, USA), AB1622, a–d; IP_3R1, Alomone (Jerusalem, Israel), ACC019, e), demonstrate IP_3R distribution corresponding to MEGJ density (a–c) at the internal elastic lamina (IEL; blue) hole (dark spots, examples arrowed; a–c) to smooth muscle cell (SMC) interface, as potential myoendothelial gap junction sites. Ultrastructural data confirm the localization of IP_3R to discrete regions of the endothelial cell (EC) projections (d,e; antibody conjugated to 5 and 10 nm Au, respectively; high-pressure frozen, freeze-substituted and low-temperature embedded tissue[12,13,26,113]), shown previously to be associated with MEGJ connexins.[12,13,26] Localized, but apparently sparse, IP_3R were also present in the adjacent smooth muscle (e; arrow with asterisk), suggesting sarcoplasmic reticulum (SR) localization. Conventional ultrastructural preparation[33] with post-fixation in $KFeCN/OsO_4$ (in a similar manner to SR staining[114]) show labelling of apparent ER (f–h; arrowed), consistent with IP_3R localization (d,e) at such sites. Regions between arrowheads (f–h; being every second section of a series of sections) are small areas of pentalaminar membrane, consistent with the presence of gap junctions at such sites. No discernible space is present between the endoplasmic reticulum and the point of myoendothelial contact in one section (h; area adjacent to arrowheads). Of note, such ER are only present in approximately 14% of MEGJ-like projections (72 examined from three vessels, each from a different rat). The likely reason for this is that such structures do not consistently take up the label. For confocal and immunoelectron microscopy, primary antibodies were conjugated to Alexa633 and 5 and 10 nm Au secondaries, respectively, and procedures conducted as per standard.[12,13,26,113] Bar, 20 μm (a–c), 0.5 μm (d,f–h,), 100 nm (e, inset).

converse pathway, namely the transfer of chemical and/or electrical signals from SMCs to endothelial cells via MEGJs, may be important for the regulation of arterial diameter in response to stimuli that cause SMC contraction.

Constriction of resistance arteries in response to elevations in intraluminal pressure, stimulation of sympathetic nerves and application of contractile agonists is limited by endothelium-derived NO and EDH (Fig. 4).[39–41] In addition, changes in vessel diameter that occur when calcium oscillations become synchronized among adjacent SMCs, such as in vasomotion,[26,42–45] are suggested to be modulated by NO and/or EDH in many vessels, such as rat mesenteric and basilar artery and hamster aorta.[26,42–47] Conversely, synchronization of calcium oscillations and vasomotion is suggested to be dependent on EDH, but not NO, in rat mesenteric artery,[48,49] whereas yet other studies emphasize an essential role for a cGMP-dependent chloride current.[43] A question yet to be addressed is how does SMC contraction stimulate the production of NO and/or EDH in endothelial cells? An MEGJ-mediated feedback mechanism is the most likely explanation.[12]

Generation of both NO and EDH depends on an increase in endothelial cell calcium levels.[50,51] Agonist-induced SMC contraction, such as that caused by the α-adrenoceptor agonist phenylephrine, is associated with increased global endothelial cell calcium levels in intact arteries, leading to the suggestion that calcium may move from activated SMCs to adjacent endothelial cells.[42,52] However, movement of calcium from one cell type to another has yet to be demonstrated directly.

The movement of calcium within cells is slow and spatially restricted,[53] which means that it is unlikely that bulk diffusion of this

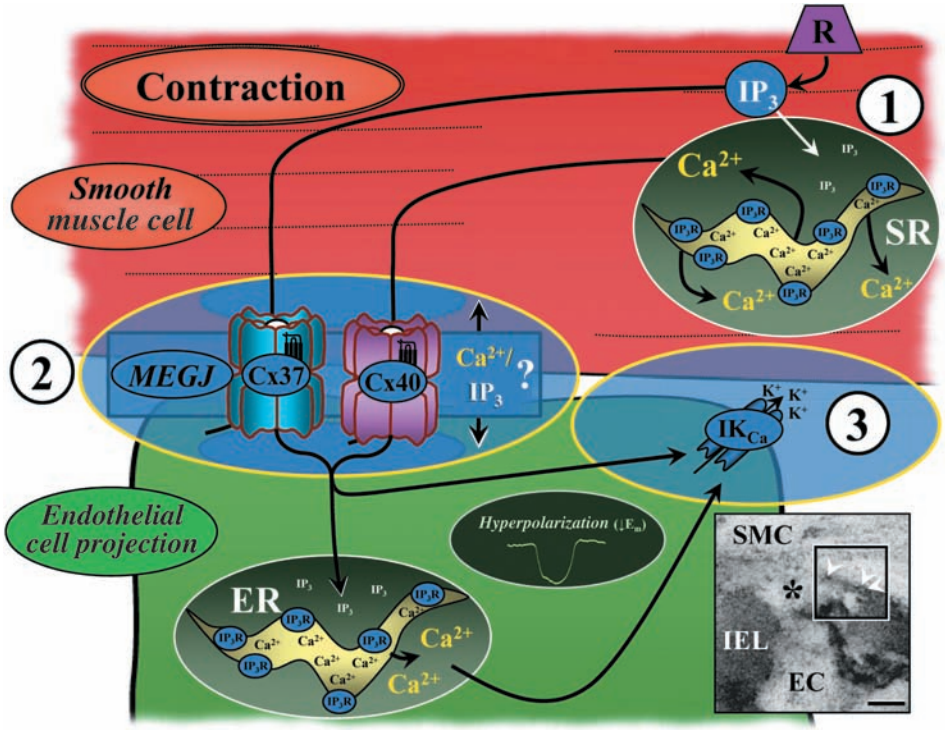

Fig. 4 Smooth muscle cell (SMC) to endothelial cell (EC) signalling mechanism in rat mesenteric artery. Contractile agonist (R) activation results in increased SMC calcium and inositol 1,4,5-trisphosphate (IP_3) levels (1) and movement through myoendothelial gap junctions (MEGJs), (2) leading to increased endothelial cell intracellular calcium ($[Ca^{2+}]_i$) within spatially restricted endothelial cell projections. Subsequent IK_{Ca} activation (3) hyperpolarizes endothelial cells, facilitating calcium entry through non-selective cation channels (NSCC; as a transient receptor potential (TRP) channel) to refill stores and potentially activate endothelial nitric oxide synthase. Spread of endothelial cell hyperpolarization back to SMCs limits further contractile activation. TRPC3 shows apparent localization to the MEGJ-related endothelial cell projection (inset; Alomone (Jerusalem, Israel), ACC-016; 1 : 200, with matching 10 nm Au secondary; arrowheads indicate the position of the Au label; high-pressure frozen, freeze-substituted and low-temperature embedded tissue,[12,13,26,113] although note that characterization of this antibody has not been conducted). The asterisk indicates MEGJ-related endothelial cell projection. Bar, 100 nm. Cx, connexin; IEL, internal elastic lamina; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum.

second messenger from SMCs can account for the increase in global endothelial cell calcium levels observed following contractile stimulation of intact vessels. Furthermore, discrete calcium changes can regulate signalling pathways independent of global calcium changes, which may occur at the same time.[54–56] Thus, the role of the increase in bulk calcium levels in endothelial cell in stimulating the production of NO and/or EDH in response to SMC contraction needs to be defined.

Of note, the use of agonists that activate a global receptor population to elevate SMC calcium, such as those acting at extrajunctional receptors and junctional α-adrenoceptors,[57] should be treated with caution because their use may not necessarily reflect a physiological state. Thus, it is more pertinent, although technically more difficult, to examine such responses under conditions of basal myogenic tone or in response to local application of vasoconstrictors that mimic discrete neurotransmitter release and not in the presence of globally acting preconstricting agents, such as phenylephrine.

Smooth muscle cell contraction is associated with membrane depolarization, which can spread to endothelial cells via MEGJs in rat basilar artery and juvenile (1–2-month-old) aorta.[58,59] This mechanism may play a role in endothelium-dependent coordination of vasomotion,[26] but is unlikely to contribute to increased endothelial cell calcium because depolarization would be predicted (depending on its size) to decrease calcium through NSCC action, a major calcium entry route in arterial endothelial cells.[60] In support of this prediction, SMC depolarization following inhibition of voltage-dependent K^+ channels causes endothelial cell depolarization and inhibition of acetylcholine (ACh)-evoked, endothelium-derived NO-mediated relaxation in rat basilar artery.[58] Because SMC contraction to depolarizing stimuli such as neurotransmitters is limited by the generation of endothelium-derived NO and/or EDH, another mechanism must be activated to oppose the transfer of depolarization to endothelial cells during SMC contraction.

In both SMCs and neurons, cellular compartmentalization provides for microdomain-specific activation of ion channels,[6,61] although the spatial arrangement and physiological role of such signalling domains in endothelial cells has received little attention. Selective activation of spatially distinct populations of K_{Ca} by vasodilators such as ACh in rat mesenteric artery,[28,62] ATP and ACh in rat aorta[63] and bradykinin and substance P in porcine coronary artery[64] have been described. However, only recently have immunohistochemical studies provided a structural basis for these observations by revealing the specific localization of K_{Ca} subtypes in endothelial cells of intact arteries (Figs 1,2a–l).[12,26]

As above, immunohistochemical studies show discrete and intermittent expression of IK_{Ca} and nearby IP_3R within IEL holes, as potential MEGJ sites, in intact rat mesenteric artery (Figs 1–4). Ultrastructural studies confirm the presence of IP_3R within endothelial cell projections in this vessel and demonstrate the presence of ER within approximately 14% of endothelial cell projections in the same vessels (Fig. 3f–h). In a similar manner, Isakson *et al*. suggest that ER (as endoplasmic and smooth sarcoplasmic reticulum, respectively) is juxtaposed to the plasma membranes of both endothelial cells and SMCs at MEGJs in mouse cremaster arteriole.[65]

As well as being involved in EDH-mediated signalling, we propose that the distribution of IK_{Ca}, IP_3R and ER in close spatial association with MEGJ Cxs[12,13] provides a mechanism for the SMC to endothelial cell communication that underlies endothelium-dependent modulation of SMC contraction. The localization of IP_3R within endothelial cell projections places them in an ideal position to be activated by small

amounts of IP_3 diffusing through MEGJs from contracting SMCs. The subsequent calcium release from the ER would give rise to a local increase in calcium within the spatially restricted endothelial cell projections, resulting in IK_{Ca} activation. Subsequent IK_{Ca}-mediated hyperpolarization of endothelial cell membrane potential would spread rapidly across and between endothelial cells (via extensive gap junctions at such sites[12,27,66]) to facilitate calcium entry through NSCCs, as well as spreading through MEGJs back to electrically coupled SMCs to limit further contraction (Fig. 4). Further studies are required to determine the precise spatial arrangement of SMC sarcoplasmic reticulum as a source of IP_3 and MEGJs in this model.

Diffusion of IP_3 is less spatially restricted than that of calcium[53] and a role for this second messenger in SMC to endothelial cell communication is supported by observations that pharmacological inhibition of IP_3 generation within SMCs or blockade of endothelial cell IP_3R prevents the vasoconstrictor-evoked increase in bulk calcium in endothelial cells in both rat intact mesenteric artery and mouse aortic endothelial cell–SMC coculture, indicating that diffusion of IP_3 through MEGJs may contribute to SMC to endothelial cell communication.[65,67,68]

Central to the smooth muscle to endothelial MEGJ communication model is the hypothesis that a localized increase in calcium at the MEGJ, within restricted endothelial cell sites, rather than a global increase in endothelial cell calcium is crucial to SMC to endothelial cell communication and to endothelium-dependent modulation of SMC contraction. To date, studies of changes in endothelial cell calcium levels within intact arteries have focused almost exclusively on the measurement of bulk calcium,[42,52,69] a situation resulting mainly from technical limitations that may hinder elucidation of mechanisms underlying endothelial cell function and, in particular, those relating to myoendothelial communication. For example, in contrast with cultured endothelial cells,[70–73] measurements of bulk calcium in endothelial cells isolated from rat middle cerebral artery and cremaster arteriole[74,75] suggest that agonist-evoked changes in endothelial cell calcium are not modulated by endothelial cell membrane potential. However, elevations in intracellular calcium, particularly in response to calcium influx, are rarely homogeneous throughout the cell. For instance, invoking store-operated calcium in SMC-derived A7r5 cells produces a modest increase in bulk calcium but increases subplasmalemmal calcium levels up to 300-fold.[76] Furthermore, cultured endothelial cells expressing the calcium indicator cameleon, targeted to the plasma membrane, provide evidence for the spatial restriction of changes in calcium at the plasma membrane, rather than an increase in bulk calcium, which may be required for NO production.[77] Thus, the use of more sophisticated imaging techniques is required to resolve the importance of localized changes in calcium for endothelial cell function.

In support of the proposal that specifically localized changes in calcium are key mediators of endothelial cell function, Ledoux *et al.* recently described spontaneous, IP_3-mediated calcium release events ('calcium pulsars') within IEL holes as putative MEGJ projection sites in pressurized mouse mesenteric artery.[31,32] Expression of IP_3R and IK_{Ca} in MEGJ-related endothelial cell projections confirms previous reports of selective localization of these proteins to an area of the cell crucial to SMC to endothelial cell communication.[12] Furthermore, inhibition of calcium pulsars provides evidence of a functional link between the release of calcium from ER stores, the generation of pulsars and IK_{Ca} activation. The frequency of calcium pulsars is increased by the endothelium-dependent vasodilator ACh, indicating a potential role for these localized calcium events in endothelial cell to SMC signalling, although the effect of SMC contraction on endothelial cell calcium pulsars was not investigated. The frequency of spontaneous local endothelial cell calcium events following contractile stimulation of SMCs in rat mesenteric artery is increased,[67] although the location of these calcium events was not defined. Thus, the proposal that signalling microdomains at MEGJs provide a novel mechanism by which increased local calcium events link SMC contraction to endothelial cell function awaits further investigation. If local calcium events are key to SMC to endothelial cell communication, then the relationship between these events, activation of IK_{Ca} and the generation of NO and/or EDH needs further examination.

Functional evidence to support a role for IK_{Ca} in SMC to endothelial cell communication comes from preliminary studies that show that the IK_{Ca} inhibitor TRAM-34 (1 μmol/L) blocks endothelium-dependent modulation of agonist-evoked rat mesenteric artery contraction (Fig. 5),[78] a vessel in which IK_{Ca} are localized to MEGJs[12] (Fig. 2a–c) and where IK_{Ca} are found only on endothelial cells and not on SMCs.[12] In contrast, apamin, an inhibitor of SK_{Ca}, was without effect on agonist-evoked contraction in endothelium-intact segments of the same vessel.[78] Using characterized SK3 antibodies, SK_{Ca} were found to be localized to endothelial cell–endothelial cell junctions and not to MEGJs in this vessel, suggesting that they may play a different physiological role to IK_{Ca}.[12] However, as mentioned above, TRAM-34 has been shown to block NSCCs in inflammatory cells[25] and, thus, further confirmation of IK_{Ca} involvement is required.

In addition to IP_3R and IK_{Ca}, NSCCs are potential contributors in SMC to endothelial cell signalling domains at MEGJs. The NSCC inhibitors SKF 96365 and $NiCl_2$ and the sodium-calcium exchanger (NCX) inhibitor KBR 7943 reduce spontaneous, localized calcium events within endothelial cells in pressurized rat mesenteric artery, suggesting that Na^+ influx through NSCCs leads to activation of the reverse mode of the NCX to cause calcium influx; the process thus being necessary to maintain these events.[67] However, interpretation of this finding is complicated by the observation that KBR 7943 can also block store-operated calcium channels[79] and TRP channels.[80]

The molecular identity of endothelial cell NSCCs remains to be defined, but these channels are most likely composed of homo- and/or heteromultimers of TRP channels.[81] Interestingly, as in cocultured mouse aortic endothelial cells and SMCs,[82] preliminary studies suggest that TRPC3 channels are localized to MEGJs in rat mesenteric artery (Fig. 4), placing them an ideal position to participate in SMC to endothelial cell communication. However, further investigation of their functional role may be limited by the poor selectivity of putative TRP channel blockers and by the expression of these channels on SMCs; thus, molecular approaches, such as the use of dominant negative strategies or short interference (si) RNA, may be required. In addition, the apparent poor specificity and characterization of a number of the commercially available TRP antibodies currently precludes detailed examination of their distribution (TH Grayson *et al.*, unpubl. obs., 2006).

In response to vasoconstrictor stimuli, SMC to endothelial cell communication likely plays an important role modulating changes in arterial diameter and, thus, blood flow and pressure. We propose a model in which specific localization of IP_3R and IK_{Ca} at MEGJs provides a signalling microdomain linking SMC contraction to endothelial cell activation,[61,62] with the spatial complexity of endothelial

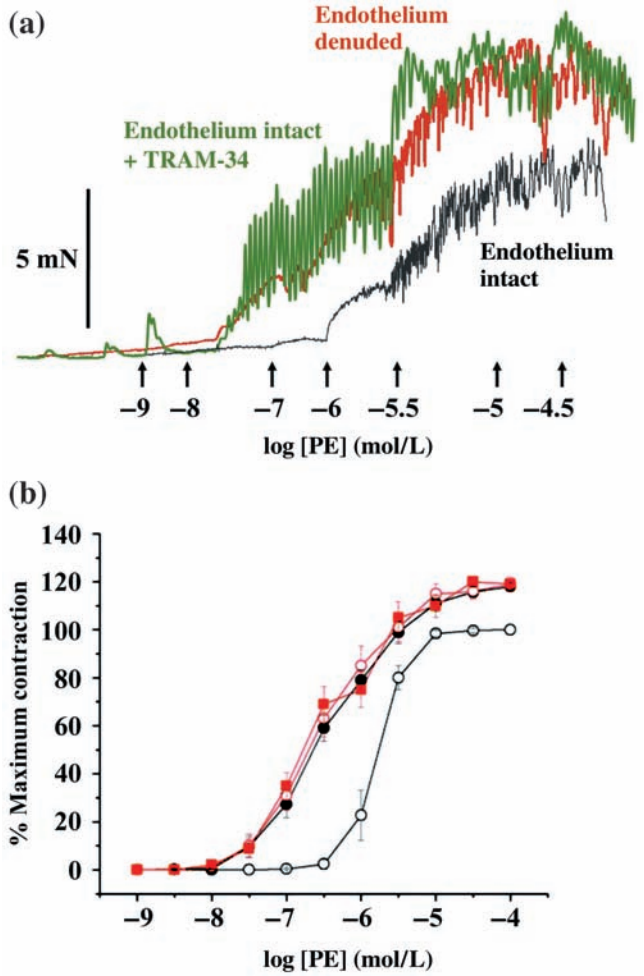

Fig. 5 Endothelium-dependent modulation of vasoconstriction in rat mesenteric artery is inhibited by 1-[(2-chlorophenyl)diphenyl-methyl]-1H pyrazole (TRAM-34). (a) Representative traces of cumulative concentration–response curves to phenylephrine (PE) in segments of third-order rat mesenteric artery mounted in a wire myograph. Contractile responses are shown for an endothelium-intact artery, an endothelium-denuded artery and an endothelium-intact artery pre-incubated with TRAM-34 (1 μmol/L; 10 min), an inhibitor of IK_{Ca} channels. (b) Mean concentration–response curves for PE in endothelium-intact (○, ●) and -denuded (○, ■) segments third-order rat mesenteric artery in the presence (filled symbols) and absence (open symbols) of TRAM-34 (1 μmol/L). As shown, TRAM-34 causes endothelium-dependent enhancement of contractile responses to PE ($n = 4$).

cell signalling only now becoming apparent (Fig. 4). Selective expression of ion channels and receptors at the luminal or abluminal endothelial cell surface may confer functional polarity, analogous to that in epithelial cells,[83] permitting differential activation of signalling mechanisms by localized changes in calcium from spatially distinct sources, thus providing an additional level of control of endothelial cell function.

Conduction over distance

The focal application of ACh to small resistance vessels initiates a vasodilatory response that conducts robustly along the vessel wall.[92,93] This so-called 'conducted' response is present in a range of vascular beds and begins with the initiation of hyperpolarization and its subsequent spread along endothelial cells.[84,94,95] At sites remote to the point of agent application, endothelial hyperpolarization is thought to effect SMC relaxation through one of two mechanisms. First, in similar studies on larger vessels, conducted hyperpolarization augments endothelial cell calcium, elevating the production and release of NO or EETs.[36,93,96] Such factors would presumably induce smooth muscle relaxation by activating a K^+ conductance.[36,93,96] However, further studies suggest that paracrine agents are of limited importance in this process and that remote smooth muscle relaxation reflects direct charge transfer between the two cell layers.[94,97,98] Although it is difficult to fully summarize the diversity of data and opinion, there is increasing consensus that charge transfer via MEGJs is the principal means of effecting smooth muscle relaxation at remote conducted sites. This consensus reflects findings from a range of studies that, cumulatively, have shown: (i) blockers of both NO synthase and EETs have only a modest effect on conduction;[36,93,96] (ii) MEGJ sites are present in many vascular beds;[3,8,98,99] and (iii) current can pass directly between the two cell layers.[98]

As a consequence of the preceding work, current investigations are increasingly examining how other elements of myoendothelial communication could shape the conducted response. One area receiving substantive attention is whether the spread of second messengers, such as calcium or IP_3, through MEGJs is sufficient to influence how electrical signals spread along an arterial wall.[85] Duling first raised this idea as a means to explain why depolarizing responses initiated in smooth muscle fail to conduct like their endothelial counterparts.[85,100] Although an appealing concept, evidence of second messenger flux influencing conduction is limited and incomplete. Indeed, existing studies are noted for their limited presentation of controls and a host of conceptual and theoretical inconsistencies. Particularly problematic is whether limited second messenger flux from a small number of activated SMCs generates sufficient endothelial current to alter membrane potential. Another area of emergence centres on whether the Cx composition or phosphorylation state alters the conducted response.[101,102] Testing this concept has proven difficult, with the consequences of altered myoendothelial function not being entirely obvious. This is exemplified by work that has tried to link augmented conduction decay in Cx40-knockout mice with either decreased cell coupling in the endothelial cell layer or at MEGJs.[101,102] Although elevated resistance of MEGJs would diminish the ability of the endothelium to drive smooth muscle membrane potential, it should not promote conduction decay.[97] Indeed, by limiting charge loss to the smooth muscle, increased myoendothelial resistance would, in theory, induce the opposing effect. Thus, further work is required in this emerging field.

ENDOTHELIUM-DERIVED HYPERPOLARIZATION *IN VIVO* VERSUS *IN VITRO*: AN ANAESTHETIC ISSUE?

Studies of intact vessels in chronically anaesthetized animals *in vivo* and of the same isolated pressurized vessels *in vitro* yield apparently contradictory results, namely the absence of myoendothelial coupling *in vivo* and the presence of such coupling *in vitro* (hamster cheek pouch[84,85] and mouse cremaster[86,87] arteriole *in vivo* cf. *in vitro*, respectively), despite the demonstration of MEGJs in these vessels.[35,87] This apparently contradictory observation is likely related to the use of anaesthetic in the *in vivo* studies. Indeed, the potential for

anaesthetics to produce effects that inhibit specific aspects of dilator and constrictor function has considerable implications for the interpretation of data in many previous studies.

The effects of anaesthetics on dilator or constrictor function generally result in an increase or decrease in blood pressure, with the currently poorly characterized aetiology of these effects[88] likely related to differences in anaesthetic action within and between vascular beds. Studies of vessel function *in vivo* require experimental animals to be chronically anaesthetized and the apparent lack of MEGJ coupling in this state is consistent with anaesthetics having effects on endothelium-dependent vasodilator activity (cf. data for hamster cheek pouch[84,85] and data for mouse cremaster[86,87]). Indeed, the *in vivo* use of isoflurane, halothane, ketamine, pentobarbitol and etomidate results in antagonism of NO and EDH.[89,90] Under specific conditions, similar antagonism of NO and EDH occurs *in vitro* via a mechanism that includes blocking intracellular calcium release in endothelial cells,[91] which underlies the predominant mechanisms of endothelium-dependent vasodilation. Thus, a primary underlying factor having an effect on the apparent myoendothelial coupling derived from *in vivo* versus *in vitro* studies is the type of anaesthetic used in chronic *in vivo* experiments. Interestingly, *in vivo* urethane is without apparent effect on endothelium-dependent EDH and NO;[90] further studies being required to clarify the mechanisms that underlie the effects of anaesthetics on endothelial function.

CONCLUSION

Heterocellular MEGJ coupling in arteries plays a key role in the maintenance of vascular tone, blood flow and pressure and thus has implications for the aetiology of vascular disease.[4,14] Potential selective bidirectional MEGJ signalling at local sites and MEGJ signalling associated with conduction of responses over distance[9,103] are the key processes associated with such coupling, with heterogeneity in MEGJs and associated structures conferring additional functional specialization at these sites. Anatomical and functional studies support the proposition of a close spatial relationship between channels and the distribution of receptor-mediated calcium stores, and the related dynamic functional modulation of calcium release and action, as a key mechanism that underlies heterogeneity in arterial function, thus representing a selective target for the control of endothelial and vasomotor function. The possibility that differential channel, receptor and store activity may underlie different aspects of MEGJ function, and in particular EDH, is being investigated in other resistance vessels and in disease, such as that associated with diet-induced obesity.[14]

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