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REVIEW

Coordination of Vasomotor Responses by the Endothelium

Kim A. Dora, PhD

Increases in the diameter of small resistance arteries and arterioles occur secondary to processes that can be dependent or independent of changes in membrane potential. Hyperpolarization reduces the opening of voltage-gated calcium channels and thereby the stimulus for contraction of these resistance vessels. The stimulus for smooth muscle cell (SMC) hyperpolarization can occur directly via opening K^+-channels expressed within those cells, but can also occur in response to stimulation of endothelial cells (ECs). This endothelium-dependent hyperpolarization (EDH) of smooth muscle often occurs in response to agonists that stimulate a rise in the Ca^{2+} concentration of ECs, which in turn can open Ca^{2+}-activated K-channels to hyperpolarize the ECs, and if present, patent gap junctions connecting ECs to SMCs (myoendothelial gap junctions) can potentially enable direct electrical coupling. There is also evidence to suggest a diffusible factor or factors hyperpolarizes SMCs (EDHF pathways). Furthermore, whether evoked in ECs or SMCs, hyperpolarization can spread a considerable distance to neighboring cells via gap junctions, causing remote dilatation termed 'spreading' or 'conducted' dilatation. This process is endothelium-dependent and likely relies on both homo- and heterocellular gap junctions. This review will focus on the cross-talk between ECs and SMCs that coordinates the spread of hyperpolarization and thus modulates smooth muscle tone. (*Circ J* 2010; **74:** 226–232)

Key Words: Arteries; Conduction; Endothelium-derived factor; Ion channels; Vasodilation

Coordination of vasomotor responses is a fundamental property of resistance vessels and enables uniform changes in diameter within segments of arteries. An integral aspect of these responses is the ability of both current and small molecules to pass through the gap junctions that connect the cells within the vessel wall. In arteries, where the endothelium and smooth muscle cells (SMCs) are well coupled, a signal for hyperpolarization can originate in either the endothelial cells (ECs) or the SMCs, and increase the membrane potential in both cell types. Hyperpolarization of SMCs can be evoked via a variety of direct and indirect pathways, to include direct activation of SMC ATP-sensitive K-channels (K_{ATP}-channels) with levcromakalim, and secondary to activation of EC calcium-activated K-channels (K_{Ca}-channels) by acetylcholine (ACh). The hyperpolarization then leads to vasodilatation, mainly by reducing Ca^{2+} influx into SMCs via voltage-gated calcium channels. A key fundamental difference between agonists that can evoke hyperpolarization and those that do not is the ability to evoke 'spreading' or 'conducted' responses in addition to 'local' dilatation at the site of agonist delivery. This phenomenon has been well studied within the microcirculation, but increasing evidence also supports an important role for spreading dilatation in the small resistance arteries of the body.

Local Hyperpolarization

Endothelium-dependent hyperpolarization is perhaps best known as involving endothelium-dependent hyperpolarization factor or factors (EDHF), which describes a ubiquitous pathway for dilatation predominant in the smaller resistance arteries of the body. Although originally assumed to be a single, unidentified diffusible factor analogous to endothelium-derived relaxing factor (EDRF ie, nitric oxide (NO)), EDHF describes a complex pathway involving diffusible factors and/or the spread of hyperpolarization radially through myoendothelial gap junctions (MEGJs). As such, it is perhaps more accurately described simply as endothelium-dependent hyperpolarization or EDH.[1–4] Despite considerable past confusion in the field, 3 NO/PGI_2-independent EDH pathways linking ECs to myocyte hyperpolarization and relaxation have now emerged. (1) First, K^+ efflux through EC K_{Ca}-channels into the myoendothelial extracellular space causes smooth muscle hyperpolarization via Na^+/K^+ATPase and possibly K_{IR}-channels (2) K_{Ca}-channel activity in the endothelium evokes hyperpolarization passing to the muscle through MEGJs. (3) $[Ca^{2+}]_i$ increase in the endothelium leads indirectly to the formation and release of arachidonate metabolites of cytochrome P_{450} (epoxyeicosatrienoic acids), and subsequent activation of BK_{Ca}-channels in smooth muscle. With the latter, evidence suggests a fairly restricted role, as a component of EDH in (mainly large) coronary arteries, and

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Department of Pharmacology, University of Oxford, Oxford, UK
Grants: British Heart Foundation Senior Basic Science Research Fellowship; Wellcome Trust (UK) Project and Programme Grants.
Mailing address: Kim A. Dora, PhD, Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, OX1 3QT, UK.
E-mail: kim.dora@pharm.ox.ac.uk
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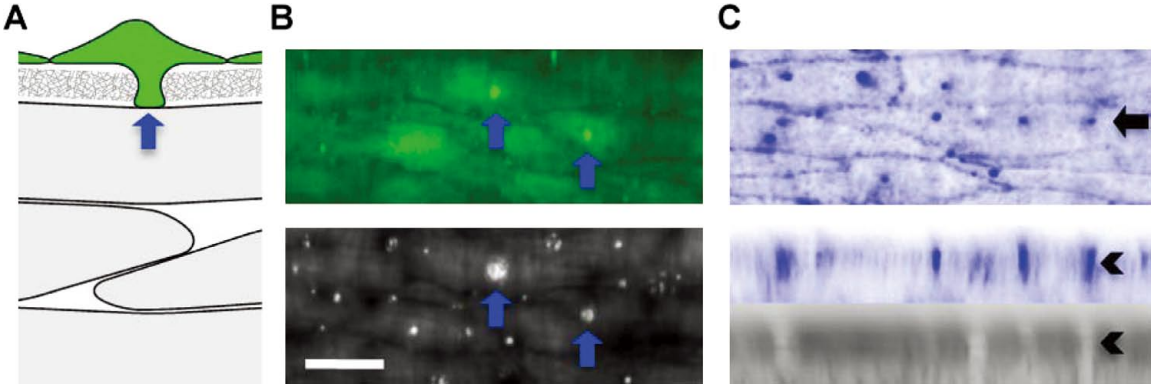

Figure 1. Resistance artery structure and K_{Ca}-channel expression. The endothelium and internal elastic lamina (IEL) are shown in radial cross section (**A**) and en face in a pressurized rat mesenteric artery (**B**, modified from Dora et al, 2008[11]), showing the position of endothelial cell (EC) projections through holes in the IEL (blue arrows). ECs green, smooth muscle cells grey, IEL hatch (white holes visible in **B**). (**C**) Another en face micrograph from a different artery showing the localization of $K_{Ca}2.3$-channels (blue) to EC borders and holes through the IEL. (Bottom panels) Fluorescence intensity reconstructed in the z-(radial) axis corresponding to a longitudinal line through the black arrow in the Upper panel. Blue indicates $K_{Ca}2.3$-channels, and the greyscale image, which was simultaneously acquired, shows the IEL with holes apparent at the points where channels can project through (arrowheads). Note that not every hole shows immunostaining. Data are modified from Dora et al, 2008,[11] which also showed strong immunostaining for $K_{Ca}3.1$-channels, $K_{ir}2.1$-channels and 2 isoforms of the Na^+/K^+-ATPase within the projections. (**B**,**C**) Bar=30 μm.

certainly a mechanism that is not as widespread in the vasculature as either (1) or (2). Apart from a restricted role as an EDHF, cytochrome P_{450} products may actually serve as intracellular second messengers to facilitate and transmit EDH.

Arguably the most important early advance in understanding EDH was the discovery that both $K_{Ca}2.3$- and $K_{Ca}3.1$-channels are normally responsible for hyperpolarization, and that these channels reside on the endothelium.[5–8] Then by simultaneous measurement of membrane potential and tension in mesenteric arteries, it was established that $K_{Ca}2.3$-channels alone explained hyperpolarization around and beyond the smooth muscle resting potential (approximately –55 mV), with $K_{Ca}3.1$-channels recruited as the smooth muscle depolarized and contracted.[9] One obvious explanation for differential activation was hypothesized to be that the Ca^{2+} events leading to activation of the K_{Ca}-channels, and/or the K_{Ca}-channels themselves, were confined to different microdomains within the ECs, most likely $K_{Ca}3.1$-channels near MEGJs.[9]

Signalling Circuit Within EC Projections (ECPs)

ECPs through the internal elastic lamina of resistance arteries can make contact with SMCs, and at these points can form MEGJs. Using confocal fluorescence microscopy, these ECPs can be visualized in living tissue by loading dyes into ECs (**Figure 1**).[10,11] The presence of MEGJs can be demonstrated using transmission electron microscopy of radially sectioned arteries and arterioles.[12–14] Using this technique, careful study of serial sections can demonstrate the approximate frequency and size of the MEGJs within an artery, and has been shown in distal rat mesenteric arteries to occur at intervals of at least 1 per EC.[15] It is worth noting that many ECPs were identified that did not form MEGJs,[15] suggesting that the incidence of projections is far higher than 1 per EC. Whether ECPs were not seen to form MEGJs because of physical disturbance during tissue preparation, dynamic changes in MEGJ formation or accurate morphology is not crucial when it is considered that the close apposition of the 2 membranes reduces the diffusion distance for any released signaling molecule, including a true EDHF.

In small mesenteric resistance arteries of the rat, the ECP microdomain contains intermediate-conductance $K_{Ca}3.1$-channels (as predicted[9]) and concentrated immunostaining for the Na^+/K^+ ATPase, a target for K^+ serving as a diffusible hyperpolarizing factor or EDHF,[11,16] and likely the $InsP_3$ receptor.[17] In this same region, connexin protein (Cx37, Cx40) is linked ultrastructurally to the presence of heterocellular MEGJs with the smooth muscle.[16,18] The spread of EDH to the muscle appears highly dependent on Cx40 in the MEGJ,[16] and in parallel to the spread of hyperpolarization through the MEGJs, efflux of K^+ through $K_{Ca}3.1$-channels contributes to muscle hyperpolarization and relaxation by activating Na^+/K^+ ATPase.[11] Pharmacologically, the ECP microdomain underlies selective recruitment of $K_{Ca}3.1$-channels to EDH during contraction of the artery, at which time it provides significant drive for smooth muscle relaxation.[9,11]

Thus the ECP is effectively a microdomain (**Figure 2**) that is critically positioned to influence vasodilatation, including the generation and spread of EDH into the media. This process alone can evoke 100% dilatation in rat mesenteric resistance arteries.[11,16,18]

EC Calcium Signaling and EDH

Although smooth muscle Ca^{2+} handling and its link to contraction/relaxation have been extensively studied, similar studies with ECs, particularly in situ and under physiological conditions are far less extensive. The endothelial monolayer is of course crucially important for normal function in the cardiovascular system, and dysfunction is an early and general feature of cardiovascular disease.[19–21] The endothelial influence usually relates to changes in cytoplasmic Ca^{2+}, for example in controlling vascular tone by the release of NO,

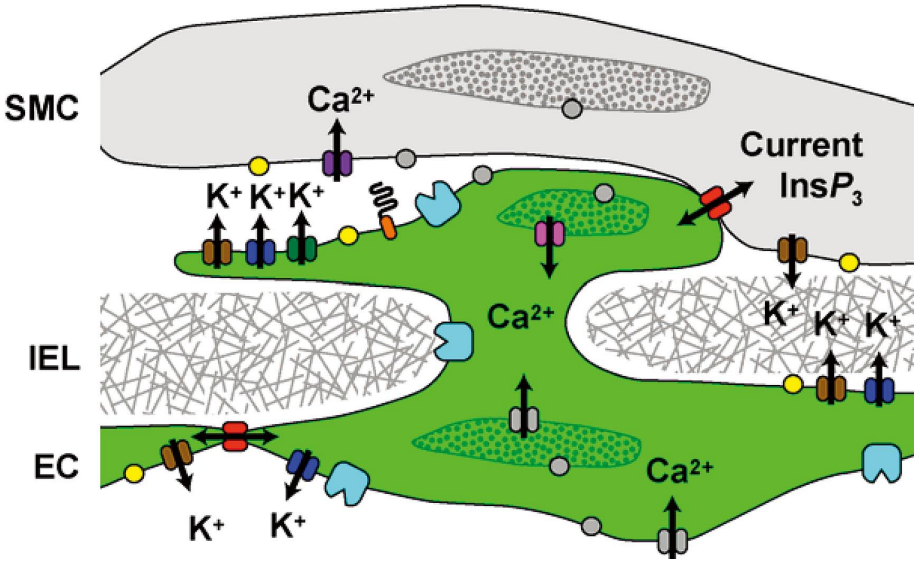

Figure 2. A simplified schematic of endothelium-dependent hyperpolarization (EDH)-dilatation signaling circuits. Increasing evidence supports various signaling circuits leading to endothelium-dependent hyperpolarization responses in small arteries such as rat and mouse mesenteric arteries.[11,17,42,79] In both tissues, endothelial cells (ECs) can project through holes in the internal elastic lamina (IEL) to make contact with smooth muscle cells (SMCs). The endothelial cell projections (ECPs) form a unique signaling circuit, with certain proteins involved in endothelium-dependent dilatation concentrated in this space.[11,18] Receptors for some agonists (cyan)[43] and inositol trisphosphate ($InsP_3$) receptors (pink)[17,42] appear concentrated within this signaling domain. Agonist-evoked rises in Ca^{2+} can activate $K_{Ca}3.1$-channels (green), which are inhibited by rises in extracellular Ca^{2+}, likely via the CaSR (orange) and PKA,[11] and $K_{Ca}2.3$-channels (blue). Small rises in extracellular K^+ in this space[5] can amplify hyperpolarization by activating K_{IR}-channels (brown) and Na^+/K^+-ATPase (yellow),[5] but does not preclude an effect within regions not at the ECP. Homo- and heterocellular coupling between ECs and SMCs can occur via gap junctions (red),[16,17] allowing the intercellular passage of both current and small molecules.[80] There are also signaling circuits between ECs, as both $K_{Ca}2.3$- and K_{IR}-channels are strongly expressed at EC borders,[11] at sites very similar to the connexins.[18,51,72] Together with Na^+/K^+-ATPase, this may serve to amplify the spreading dilatation response.[62,63,70] Various Ca^{2+} influx (eg, voltage-gated Ca^{2+}-channels in SMCs, purple) and release channels, transporters and Ca^{2+} pumps (all represented generically as grey) are found in both ECs and SMCs.

prostacyclin, and activation of EDH.[1,20,22] Cytoplasmic [Ca^{2+}] is modulated not only by the direct action of agonists and hemodynamic forces on the ECs, but also indirectly by communication with surrounding cells. Spontaneous and agonist-mediated rises in EC Ca^{2+} in intact arteries are usually observed as asynchronous waves of Ca^{2+} passing along individual ECs. These waves are not sensitive to blockade of ryanodine receptors, and together with sensitivity to inhibitors of phospholipase C, support an important role for $InsP_3$ receptors,[10,23] likely expressed along the entire surface of the endoplasmic reticulum. The rise in EC Ca^{2+} and associated hyperpolarization are maintained by Ca^{2+} influx,[23,24] although the exact pathways responsible are not yet fully elucidated.

As already noted, a key pathway for intercellular communication within resistance arteries and arterioles is direct cell-cell coupling via homo- and heterocellular gap junctions.[16,25–30] In addition to current passing between cells through the gap junctions, there is also evidence for Ca^{2+} signaling following elevations in SMC Ca^{2+} by agonists such as phenylephrine and KCl, first reported in arterioles.[31] The consequent secondary rise in global endothelial Ca^{2+} can enhance the production of both NO and EDH(F) and as a result influence vessel diameter.[31–34] In smooth muscle, although global changes in Ca^{2+} are fundamental to changes in tone, discrete, spontaneous Ca^{2+} events (Ca^{2+} sparks) appear to be extremely important functionally in certain arteries (eg, cerebral). They reflect activation of ryanodine receptors located next to BK_{Ca}-channels, and under normal conditions tonically activate these channels to suppress arterial tone.[35–37] In situ, ECs also generate spontaneous Ca^{2+} events, as reported in rat lung capillaries and ureter arterioles, and mouse cremaster arterioles.[38–41]

In mesenteric resistance arteries we recently demonstrated spontaneous Ca^{2+} events in ECs under physiological pressure.[10] Localized increases in cytoplasmic Ca^{2+} concentration originated from the endoplasmic reticulum, depended on $InsP_3$ (Ca^{2+}puffs), not RyR, and could be modulated not only by endothelial ion channels, but also by the SMCs via MEGJs, both basally and during agonist-evoked contraction. In mesenteric resistance arteries, spontaneous Ca^{2+} events in the endothelium have now been termed 'pulsars' and appear to align with ECPs.[42] $K_{Ca}3.1$-channels are restricted to these projections,[11,42] and are activated basally by the pulsars, causing suppression of the smooth muscle membrane potential by approximately 8 mV. As well as $K_{Ca}3.1$-channels, $InsP_3$-sensitive Ca^{2+} stores are also concentrated in the ECPs,[17,42] and ACh stimulates an approximate 2.5-fold increase in pulsar frequency.

Despite being able to resolve the Ca^{2+} changes within the ECPs, it has not been possible to resolve any subtle difference between the Ca^{2+} events activated by ACh under resting conditions compared with those in the presence of

phenylephrine tone. Indeed, under both conditions a clear rise in Ca^{2+} is observed in the head of the ECP,[11] which is consistent with expression of M_3 muscarinic ACh receptors within this microdomain.[43] Therefore this does not appear to be the explanation for the differential activation of $K_{Ca}2.3$- and $K_{Ca}3.1$-channels. However, changes in extracellular Ca^2 do affect changes in the activity of $K_{Ca}3.1$-channels, indicating quite clearly that the ECP microdomain and the input to EDH and thus vasodilatation is subject to dynamic modulation by the immediate intra- and extracellular environment in a complex fashion.

Interaction Between $K_{Ca}3.1$-Channels and Calcium: A Role for Ca-Sensing Receptors?

Input to EDH(F) dilatation from EC $K_{Ca}3.1$-channels is influenced dramatically by small, physiological changes in extracellular $[Ca^{2+}]$.[11,44] Similar concentration changes act on the calcium-sensing receptors (CaSRs), recently found in the endothelium and linked both to activation of $K_{Ca}3.1$-channels[45] and vascular relaxation.[46] As $[Ca^{2+}]_o$ increases above 1 mmol/L, $K_{Ca}3.1$-channel mediated hyperpolarization is suppressed, but reappears during Ca^{2+} influx into the adjacent smooth muscle during contraction.[11] Therefore, dynamic fluctuations in external $[Ca^{2+}]$ around ECPs represent a fundamental physiological control mechanism. Relative to the intracellular space, diffusion is restricted and buffering of low capacity, so large fluctuations in $[Ca^{2+}]_o$ will occur.

CaSRs have been reported in a number of blood vessels, and from a range of species, including humans.[47] On the endothelium, the link between activation of the CaSR and $K_{Ca}3.1$-channels is disrupted in mesenteric arteries from a model of type II diabetes, possibly reflecting decreased expression of CaSRs, although $K_{Ca}3.1$-channel protein, but not hyperpolarization, was also reduced.[48] The CaSR and $K_{Ca}3.1$-channel are colocalized in caveolin-poor regions of the EC membrane in those arteries.[45] $K_{Ca}2.3$-channels localize within the caveolae so abundant in ECs and, inter alia, act as signaling platforms. Knockout of the key structural protein caveolin-1 appears to ablate EDHF-mediated relaxation by disrupting Ca^{2+}-signaling involving TRPV4 and thus reducing connexin expression,[49] whereas TRPV4-deficient mice also lose EDHF relaxation, have reduced NO-relaxation and decreased blood pressure reduction to systemic ACh.[50]

Conducted Hyperpolarization

Whether initiated in the endothelium or smooth muscle, in many small arteries and arterioles local hyperpolarization can also spread axially to affect spreading vasodilatation, translating a focal dilatation to a more widespread drop in vascular resistance necessary to increase blood flow. A fundamental defining feature of this spreading dilatation is that it has not been observed following focal dilatation to NO,[26,51,52] which was thought not to evoke hyperpolarization, and the EDH(F) pathway leading to spreading dilatation is independent of NO release.[53] Indeed, there appears to be a correlation between agonists that open K-channels and an ability to reliably evoke spreading dilatation.[54]

Such spread of dilatation is extremely important physiologically. For agonists to achieve a significant improvement in tissue blood flow, any increase in diameter must significantly reduce vascular resistance. If dilatation only occurred focally, it would be unlikely to reduce resistance, as it is partly determined by the length of the artery. However, if dilatation can also spread upstream, blood flow is much more likely to significantly increase. Such an effect has been measured in response to ACh, but not NO, within the microcirculation in situ,[55,56] and demonstrates the physiological importance of spreading dilatation. This phenomenon can also be observed in isolated, pressurized arteries.[51]

The signal for spreading dilatation is thought to simply reflect the passage of current to adjacent cells not directly stimulated by the agonist.[13,57] This can occur bidirectionally along the length of the vessel, and is intrinsic to the wall of the artery, relies on the endothelium, and is not dependent on either nerves or blood flow change.[13,54,58–61] The phenomenon is well-characterized within the microcirculation, where studies in exteriorized vascular beds in situ,[55] and arterioles isolated from those vascular beds, have used the endothelium-dependent agonist ACh to evoke robust responses when applied to the outside of arterioles with micropipettes. Spreading dilatation can also be sustained in small resistance arteries, such as the rat mesenteric artery, which have 3–5 layers of muscle, illustrating its relevance across the vasculature.[51,62,63]

Importance of the Endothelium in Spreading Dilatation

By activating ATP-sensitive K^+ (K_{ATP}) channels, which are restricted to the smooth muscle in rat mesenteric arteries,[62,64] it has been demonstrated that the SMCs are not coupled sufficiently to allow spreading dilatation over long distances,[62] emphasizing the importance of the endothelium as the conduit for electrical coupling.[28,51,65,66] This research suggests that the mechanism leading to hyperpolarization is not crucial in determining spread, only in initiating cell–cell coupling. However, some agonists may stimulate additional signaling pathways that can either augment (eg, by cAMP)[67] or reduce (eg, by α_1-adrenoceptor stimulation; PKC)[68,69] the ability of hyperpolarization to spread between cells.

Spreading Dilatation Following Luminal Perfusion of Agonists

Circulating vasoactive agonists are crucially involved in the physiological control of regional blood flow, yet remarkably, few studies apply these agonists to the artery lumen when studying the mechanisms by which this integrative effect is achieved. Such an approach is of special importance when delineating the mechanism of action of agonists that have opposing actions via the endothelium and on the SMCs, as with purinergic (eg, ATP) and adrenergic (eg, adrenaline) agonists. Using a novel approach of cannulating arteries with a sidebranch to luminally infuse agonists it has been shown that dilatation evoked by luminal perfusion of ATP or UTP (uridine triphosphate) stimulated both local and spreading dilatation.[51] All the evidence to date supports an important and essential role for the endothelium in enabling the integration of vascular responses to the coordinated control of local blood flow. Therefore, any dysfunction of this monolayer in conditions such as diabetes, obesity, and hypertension will markedly impair its ability intricately to control local tissue perfusion.

What Mechanisms Sustain Spreading Dilatation: Another Signaling Circuit?

Given the reliance of spreading dilatation on membrane

hyperpolarization, any agonist able to stimulate hyperpolarization will potentially evoke spreading dilatation, and will rely on homo- and heterocellular gap junctions to enable the necessary current spread. Of major interest is whether current passing between cells of the arterial wall decays passively, or some sort of amplification mechanism exists, and of course whether this mechanism is the same in all vessels. The available evidence in isolated, pressurized arteries supports a role for an as yet not fully defined amplification process, enabling current to spread further than predicted by passive decay. This has been elegantly demonstrated in cannulated hamster retractor small arteries (1–2 layers of smooth muscle), where the decay of injected current was faster than the decay of hyperpolarization to ACh, despite similar local hyperpolarization (length constants 1.2 and 1.9 mm, respectively).[60] Therefore, the intercellular spread of current via gap junctions appears to be sustained by an undefined mechanism. Candidate ion channels involved in this process currently favor an important role for inwardly rectifying K^+ (K_{IR}) channels.[63,70,71] However, this does not appear to be universal, as Ba^{2+} alone (to inhibit K_{IR} channels) has no effect on spreading dilatation to either ACh or levcromakalim in rat mesenteric arteries,[62] but the Na^+/K^+-ATPase, which we know can also be activated by modest increases in extracellular $[K^+]$,[5] may play an as yet to be determined role. This raises the possibility that the release of K^+ from vascular cells during hyperpolarization may act as an amplification mechanism, with a distinct parallel to its action as an EDHF.[5] This is supported by the expression of both K_{IR}-channels and $K_{Ca}2.3$-channels at the borders of ECs,[11] at sites resembling those of interendothelial cell gap junctions.[51,72] It is also highly likely that an isoform of the ATPase is also expressed within this space. Thus, release of K^+ through any of these channels could act on adjacent channels, pumps and/or cells to amplify hyperpolarization.

In addition, there are other candidates that may act together with or in parallel to K^+. Given its central importance in vascular function, NO could play a role,[73,74] although inhibition of NO synthase does not appear to reduce spreading dilatation.[51,53,75] It has been previously shown that EC Ca^{2+} does not detectably increase in response to local[24,76] or spreading[62] hyperpolarization in either rat mesenteric or cremaster arteries. However, recent evidence shows that the presence of tone can unmask a detectable, slow, conducted intercellular Ca^{2+} wave, both in vitro[74] and in vivo,[77] although it is important to note that this wave is not a requirement for the rapid spread of hyperpolarization and associated dilatation.[62,77]

Conclusions

EDH is a fundamental physiological control mechanism. It is now clear that a continual, basal EDH provides a significant and physiologically relevant suppression of blood pressure that is independent of both NO and prostacyclin (PGI_2). This, together with the fact that endothelial dysfunction, including changes in basal and evoked EDH, is a fundamental feature of cardiovascular disease,[20,21,78] highlights the importance of this discovery and the need to define how and to what extent the EC microdomains of ion channels and pumps can modulate the elaboration of hyperpolarization and its ability to evoke vasodilatation.

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