EDH: Endothelium-dependent hyperpolarization and microvascular signaling

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Running title: EDH and artery dilatation

Summary

Endothelium-dependent hyperpolarizing factor (EDHF) is a powerful vasodilator influence in small resistance arteries and thus an important modulator of blood pressure and flow. As the name suggests, EDHF was thought to describe a diffusible factor stimulating smooth muscle hyperpolarization (and thus vasodilatation). However, this idea has evolved with the recognition that a factor(s) can operate the spread of hyperpolarizing current from the endothelium to the vascular smooth muscle (VSM). As such, the pathway is now termed endothelium-dependent hyperpolarization (EDH). EDH is activated by an increase in endothelial $[Ca^{2+}]_i$, which stimulates two Ca^{2+}-sensitive K-channels, SK_{Ca} and IK_{Ca}. This was discovered because apamin and charybdotoxin applied in combination blocked EDHF responses, but iberiotoxin a blocker of BK_{Ca} was not able to substitute for charybdotoxin. SK_{Ca} and IK_{Ca} channels are arranged in endothelial microdomains, particularly within projections toward the adjacent smooth muscle, which are rich in IK_{Ca} channels, and close to inter-endothelial gap junctions where SK_{Ca} is prevalent. K_{Ca} activation hyperpolarizes endothelial cells, and K^+ efflux through them can act as a diffusible 'EDHF' by stimulating VSM Na^+/K^+-ATPase and inwardly-rectifying K-channels (K_{IR}). In parallel, hyperpolarizing current spreads from the endothelium to the smooth muscle through myoendothelial gap junctions located on endothelial projections. The resulting radial EDH is complemented by spread of 'conducted' hyperpolarization along the endothelium of arteries and arterioles to affect conducted vasodilatation (CVD). Retrograde CVD effectively integrates blood flow within the microcirculation, but how the underlying hyperpolarization is sustained is unclear.

Key words: EDHF, endothelium, NO, prostacyclin, vasodilatation, potassium channels, gap-junctions, SK_{Ca}, $K_{Ca}2.3$; IK_{Ca}, $K_{Ca}3.1$; connexins, hyperpolarization

Abbreviations: ACh, acetylcholine; BK_{Ca}, large conductance Ca^{2+}-sensitive K^+-channels; CaSR, calcium-sensing receptors; Cx, connexin; CVD, conducted vasodilation; EDH, endothelium-dependent hyperpolarization; EDRF, endothelium-dependent relaxing factor; EDHF, endothelium-dependent hyperpolarizing factor; IK_{Ca}, intermediate conductance Ca^{2+}-sensitive K^+-channels; InsP3, inositol trisphosphate; K_{Ca}, Ca^{2+}-sensitive K^+ channels; NO, nitric oxide; K_{ATP}, ATP-sensitive K^+-channels; K_{IR}, inwardly-rectifying K^+-channels; K_v, voltage-gated K^+-channels; MEGJ, myoendothelial gap junctions; Na^+/K^+-ATPase, sodium and potassium dependent adenosine triphosphatase; SK_{Ca}, small conductance Ca^{2+}-sensitive K^+-channels; TRP, transient receptor potential.

Endothelium-dependent hyperpolarization and EDHF: a historical perspective

That vascular hyperpolarization could arise within the endothelium became clear when smooth muscle hyperpolarization to carbachol was shown to depend on the integrity of endothelial cells. Based on this observation, it was suggested that muscarinic stimulation stimulates hyperpolarization by releasing a factor from the endothelial cells, causing hyperpolarization in the smooth muscle and relaxation/dilation by reducing the open-probability of voltage operated Ca^{2+} channels in the latter (Bolton et al., 1984). However, it was not clear if this factor was simply the EDRF discovered and reported by Furchgott in 1980 (Furchgott and Zawadzki, 1980).

That hyperpolarization was an entity separate from EDRF (known by 1988 to be NO) was shown when either methylene blue (used to block guanylyl cyclase and scavenge NO) or haemoglobin (which binds NO) failed to alter endothelium-dependent hyperpolarization in the rat aorta and pulmonary artery, or to depress ^{86}Rb efflux, a marker of K^+ movement. In contrast, the vascular smooth muscle relaxation in these arteries (tone induced with noradrenaline) was inhibited by well over 50% and cyclic GMP accumulation effectively abolished. None of these responses were altered by indomethacin ruling out input from eicosanoids (Chen et al., 1988). Although this work did not provide any evidence for a discrete factor *per se,* the authors suggested *'the properties of this [hyperpolarizing] agent suggest it should be designated endothelium-derived hyperpolarizing factor (EDHF).'* Evidence that a factor was involved derived from a separate study, recording smooth muscle hyperpolarization in endothelium-denuded small coronary arteries. Hyperpolarization

was only observed in the coronary smooth muscle if the arteries were co-incubated with femoral artery segments with intact endothelium. However, at the time the authors did not suggest the hyperpolarization might be due to anything other than NO/EDRF. Importantly, they did find that hyperpolarization was blocked by ouabain (Félétou and Vanhoutte, 1988), an effect we come back to later.

The initial studies of endothelium-dependent hyperpolarization in the rat aorta and pulmonary artery did not suggest it was at all important for smooth muscle relaxation. It turns out that endothelium-dependent hyperpolarization becomes more important as the size of arteries decreases. This was shown by simultaneous measurement of membrane potential and tension in small resistance arteries. In these minute arteries acetylcholine (ACh) stimulated pronounced hyperpolarization and relaxation, which was not altered by oxyhaemoglobin, indomethacin or nitro-L-arginine. Thus, hyperpolarization could completely relax preconstricted resistance arteries (Garland and McPherson, 1992). The EDHF response was thus recognized as smooth muscle relaxation in blood vessels, evoked (usually by the endothelium-specific agonist ACh) in the presence of inhibitors of cyclooxygenases and NO synthase. Technically, microelectrode recording from vascular tissue is very difficult, so it is perhaps not surprising that the vast majority of 'EDHF' studies simply assess this response indirectly, by measuring vascular relaxation (of vasoconstriction to, for example, an α_1-adrenoceptor or thromboxane receptor agonist) with inhibitors for cyclooxygenases and NO synthase present. So although many papers refer to EDHF, they largely avoid recording the membrane potential changes that defined the pathway. Furthermore, in effect most studies have not investigated the consequences of hyperpolarization (defined as an increase in resting membrane

potential) but rather repolarization (a reversal of the depolarization evoked by vasoconstrictor agonists). This distinction has proved to be crucial when trying to understand the complexity of the mechanisms that are responsible for initiating and spreading endothelium-dependent hyperpolarization, as EC IK_{Ca} and SKC_a channels may be differentially active (Crane et al., 2003a). Although the acronym EDHF has been used most widely to describe both endothelium-dependent hyperpolarization and repolarization, in smaller vessels there is very good evidence now to suggest a factor is not always involved. As such, the term EDH (again to include repolarization) is really a more accurate description of what is a key pathway for vasodilatation in resistance arteries.

Endothelial cell SK_{Ca} (K_{Ca}2.3) and IK_{Ca} (K_{Ca}3.1) are the basis of EDH

EDH is activated by an increase in endothelial cell Ca^{2+}, which can potentially stimulate three types of K-channel, the small, intermediate and large conductance Ca^{2+}-sensitive K^+ channels (K_{Ca}); SK_{Ca} (K_{Ca}2.3); IK_{Ca} (K_{Ca}3.1) and BK_{Ca} (K_{Ca}1.1). SK_{Ca} and IK_{Ca} channels are generally located on the endothelium and account for most EDH responses; while in some vascular beds BK_{Ca} channels serve as a smooth muscle target for a diffusible EDHF (Garland et al., 2011).

Although K^+ efflux was clearly a fundamental aspect of the EDH response (Chen et al., 1988), it took quite a long time to identify the underlying K-channels. With hindsight, it is clear this was because two types of K-channel are involved. EDH-mediated responses were quickly shown to be insensitive to glibenclamide, excluding a role for ATP-sensitive K-channels (K_{ATP}) (Plane and Garland, 1993, Garland and McPherson, 1992). A key role for voltage-gated K-channels (K_V) also seemed

extremely unlikely, as the EDH response relied on pronounced and sustained hyperpolarization and this would inactivate channels whose open probability is raised by depolarization This was confirmed when EDH-like relaxation was shown to be insensitive to K_V block (Adeagbo and Triggle, 1993). K_{Ca} did have a role, as relaxation was attenuated either by the non-selective blocker tetrabutylammonium or apamin, the latter a specific SK_{Ca} ($K_{Ca}2.3$) blocker (Adeagbo and Triggle, 1993, Holzmann et al., 1994). Then, recording both hyperpolarization and relaxation simultaneously, we found not only apamin but also charybdotoxin could each reduce but not block these responses. We suspected that parallel signalling pathways may be operating, so applied both toxins in combination. For the first time, were able to block EDH hyperpolarization and relaxation (Waldron and Garland, 1994). This seminal observation was confirmed and crucially extended when iberiotoxin was found unable to substitute for charybdotoxin (Zygmunt and Hogestatt, 1996). As charybdotoxin blocks both IK_{Ca} and BK_{Ca} channels, the absence of any of block with iberiotoxin indicated it was IK_{Ca} channels, along with SK_{Ca}, that underpinned the EDH response.

At that time, it was wrongly assumed that both types of K-channel were located on the smooth muscle, and thus the subject of activation by a diffusible EDHF released by the endothelium. It was known that vascular smooth muscle contained BK_{Ca}, and we demonstrated this when we found high concentrations of NO could activate BK_{Ca} in mesenteric artery smooth muscle cells. However, we could find no evidence for SK_{Ca} activity (Mistry and Garland, 1998a, Mistry and Garland, 1998b). Then, in collaboration with Arthur Weston's group in Manchester, we used intracellular recording directly from *endothelial* cells *in situ* to reveal that ACh evoked

hyperpolarization was blocked by apamin and charybdotoxin in combination. So the most obvious explanation was that the K_{Ca} channels were focused on the endothelial cells. This of course made sense, as M_3 receptors signal via inositol trisphosphate ($InsP_3$), which by releasing Ca^{2+} will provide a signal to activate Ca^{2+}-sensitive, but voltage-insensitive, K_{Ca} channels. The discovery that SK_{Ca} and IK_{Ca} channels are present on the endothelium, not the smooth muscle cells, has now been confirmed in many laboratories (Busse et al., 2002). The second major outcome of our collaboration, was providing direct evidence to suggest that after efflux through endothelial cell (EC) channels, K^+ can act as a diffusible factor or EDHF (Edwards et al., 1998).

In hepatic arteries from the rat, where a factor plays a major role, as opposed to spread of hyperpolarization, both smooth muscle hyperpolarization and relaxation evoked with exogenous K^+ or EDHF (activated by EC stimulation with ACh) displayed a similar magnitude and sensitivity to inhibition with a combination of Ba^{2+} and ouabain (to block K_{IR} and Na^+/K^+-ATPase). However, in contrast to the smooth muscle cells, the endothelial cell hyperpolarization to ACh was not sensitive to Ba^{2+} and ouabain. Together these observations suggested the release of K^+ through endothelial SK_{Ca} and IK_{Ca} could activate smooth muscle K_{IR} and Na^+/K^+-ATPase causing hyperpolarization. This idea was supported by measuring directly an increase in $[K^+]_o$ at the endothelial-smooth muscle interface of around 10 mM during endothelial cell stimulation with ACh. The increase was sufficient to explain the measured EDHF hyperpolarization and relaxation (Edwards et al., 1998). The small arteries used were rat hepatic and mesenteric arteries, and while the EDHF response was blocked in the former with Ba^{2+} and ouabain, in the mesenteric artery a

significant component persisted, and we suggested myoendothelial gap junctions (MEGJs) between endothelial and smooth muscle cells may be responsible (Edwards et al., 1998).

The suggestion that endothelium-derived K^+ serves as a hyperpolarizing factor is now supported by studies in arteries from a range of species and locations, including human arteries. Failure by some others to evoke vasorelaxation to $[K^+]_o$, even in the rat mesenteric resistance artery (Lacy et al., 2000, Doughty et al., 2000), can be explained simply by the experimental design, which failed to take into account functional or physiological antagonism. This was demonstrated when relaxation and repolarization to elevated $[K^+]_o$ were measured in rat small mesenteric arteries during either submaximal or maximal stimulation with phenylephrine. As stimulation intensity increased towards maximal, repolarization and relaxation to $[K^+]_o$ was blocked (Dora and Garland, 2001), because stimulation of the muscle α_1-adrenoceptors, by increasing intracellular Ca^{2+} ion, activates iberiotoxin-sensitive BK_{Ca} channels resulting in efflux of K^+ so the ion accumulates in the extracellular space saturating Na^+/K^+-ATPase activity and blocking responses to any additional (exogenous) K^+ ion (Dora et al., 2002). When efflux from the smooth muscle was reduced, by the presence of iberiotoxin, this functional antagonism was removed and exogenous K^+ ion was again able to evoke repolarization/hyperpolarization and relaxation, or increase diameter in pressurized arteries (Dora et al., 2002). The extracellular accumulation of K^+ that underlies this physiological antagonism was termed the 'K^+-cloud' (Weston et al., 2002).

Myoendothelial gap junctions (MEGJs) – a bridge between muscle and endothelium

The presence of myoendothelial junctions between cells in the vascular wall had been recognized for many years (Rhodin, 1967), and a key role for gap junctions in conducted vasodilatation to ACh clearly demonstrated in arterioles of the microcirculation (Segal and Duling, 1989). That at least in part the EDH response spreads via gap junctions was shown by whole-cell patch clamp experiments in arterioles, with hyperpolarization to ACh in the endothelium of intact arterioles demonstrated to be identical in the adjacent smooth muscle cells (Yamamoto et al., 1999). But if the gap junctions were blocked, ACh only induced an outward current in the ECs. Furthermore, extracellular Ca^{2+} removal or charybdotoxin reduced EC hyperpolarization. However, the functional significance of gap junctions, just like K^+, seemed to vary between arteries and was not always apparent (Beny and Schaad, 2000). This indicated that the functional input through MEGJs might increase in importance as vessels decreased in size, and as such help to explain the observation that EDH-evoked relaxation increases in importance as artery diameter decreases (Garland et al., 1995, Shimokawa et al., 1996). Support here came from a morphological study that attempted to quantify muscle-endothelial and EC-EC gap junctions in rat mesenteric arteries where MEGJs were found and shown to be smaller than the homocellular gap junctions between the ECs. They also appeared larger in number in distal compared with to proximal vessels, consistent with an increasing role as artery size decreased (Sandow and Hill, 2000). The larger endothelial cell gap junctions displayed a pentalaminar structure and were distributed extensively throughout the mesenteric arterial bed. MEGJs occurred on projections of the endothelial cells (MEPs), which in the rat pass through perforations in the internal elastic lamina, although not all MEPs possess a junction (Sandow and Hill, 2000). As with all gap junctions, MEGJs are formed by docked connexins in the

membranes of connecting cells, proteins with four trans-membrane domains which come together as hexamers to make up a hemichannel (a connexon) in each of the coupled cells (Johnstone et al., 2009). In the vasculature, connexons can be formed from Cx37, Cx40, Cx43 and/or Cx45 (de Wit and Griffith, 2010, Goto et al., 2004, Mather et al., 2005), and the development of Cx40 deficient mice showed this protein to be essential for CVD, a response discussed later that relies on the spread of hyperpolarization between ECs and VSMs, so raising the possibility Cx40 may be important in MEGJs (de Wit et al., 2000).

A role for MEGJs removes the need to rely on generation of a diffusible factor to explain the EDHF response. So as hyperpolarization is initiated within the endothelium a more accurate descriptor for this pathway, and one now generally accepted, is EDH. Unraveling the functional contribution of gap junctions in EDH dilatation is not easy, mainly because the pharmacological gap junction uncouplers available have multiple effects. To probe a functional role for Cx40, which is present in the endothelium, we developed a technique selectively to load the endothelium of pressurized rat mesenteric resistance arteries with antibodies (by pinocytosis). Using this approach, we were able to block EDHF-dilatation with anti-Cx40 antibody, but only if the parallel 'EDHF' action of K^+ was blocked (Mather et al., 2005). In contrast, antibodies against intracellular regions of Cx37 or Cx43, or mimetic peptide for the intracellular loop region of Cx37, were inactive. Furthermore, Cx40 was visualized (by immunohistochemistry) within the terminal region of MEPs, supporting our functional experiments (Mather et al., 2005).

So EDH-mediated dilatation can be explained by the generation of hyperpolarization

in endothelial cells, which then passes to relax the adjacent smooth muscle by K^+ efflux and by conduction through MEGJs and involving Cx40. During submaximal vasoconstriction, both pathways contribute to dilatation, but as constriction intensifies so does the 'density' of the extracellular K^+ cloud, ensuring the smooth muscle target is saturated, and at this time MEGJs alone sustain EDH vasodilatation.

The concept of endothelial membrane microdomains

The possibility that MEPs might represent a signalling microdomain arose from the observation that endothelial cell SK_{Ca} and IK_{Ca} can operate independently. Although both K-channels are central to EDH, in isolated mesenteric arteries simultaneous measurements of tension and membrane potential showed that SK_{Ca} alone were responsible for hyperpolarization (from resting potential *circa* -55 mV). However, input from IK_{Ca} also became apparent once the arteries were stimulated with phenylephrine to evoke smooth muscle depolarization and contraction (Crane et al., 2003b). Such differential K_{Ca} channel activation might be explained by the intracellular Ca^{2+} sensitivity of the two channel types and/or their exposure to cytoplasmic Ca^{2+}, perhaps due to a separate, distinct subcellular localization of the channels. The latter seems to be the explanation, as IK_{Ca} channels are restricted within the MEPs passing through perforations in the internal elastic lamina towards the smooth muscle (Dora et al., 2008, Sandow et al., 2006). In contrast, SK_{Ca} channels were distributed throughout the endothelial cell membrane, and appeared to be in particularly high concentration close to the large EC-EC gap junctions (Dora et al., 2008, Sandow et al., 2006). It has subsequently become apparent that the IK_{Ca} channel forms part of a complex signalling microdomain within MEPs. This domain includes the Na^+/K^+-ATPase, Cx37, Cx40 (integral to the MEGJs), and

calcium-sensing receptors (CaSR). CaSRs co-localize with IK_{Ca} and transiently activate, then inhibit channel activity as $[Ca^{2+}]_o$ increases to levels > circa 1 mM (Mather et al., 2005, Dora et al., 2008, Yarova et al., 2013). MEPs also contain $InsP_3$-sensitive Ca^{2+} stores, which spontaneously release Ca^{2+}, events that have been termed 'pulsars' (Ledoux et al., 2008). They activate the closely aligned IK_{Ca} channels, generating hyperpolarization and suppressing smooth muscle membrane potential by around 8 mV (Ledoux et al., 2008).

More recently, MEPs have been shown to contain clusters of transient receptor potential vanilloid 4 (TRPV4) channels associated with IK_{Ca} (Sonkusare et al., 2012, Bagher et al., 2012) Figure 1. Single TRPV4 channels mediate spontaneous elementary Ca^{2+} influx events in mouse mesenteric artery ECs, and these events have been named "TRPV4 sparklets" by analogy with Ca^{2+} sparklets in the heart (Sonkusare et al., 2012). Although TRPV4 sparklets are not only found in MEPs, in this region they appear to operate in clusters, a property that appears to depend on the presence of the A-kinase anchoring protein (AKAP150), which is concentrated exclusively within the MEPs (Sonkusare et al., 2014, Bagher and Garland, 2014). AKAP150 somehow enhances gating co-operativity between units of up to four TRPV4 channels within these thin EC projections. AKAP150 is a scaffolding protein that brings together and anchors key signaling molecules at the plasma membrane. In ECs, the signalling complex assembled by AKAP150 effectively and precisely targets Ca^{2+} influx through co-operatively gated TRPV4 channels to activate IK_{Ca} channels and evoke EDH vasodilation. An absence of AKAP150 in this domain limits this interaction, highlighted in angiotensin II–induced hypertension and in $AKAP150^{-/-}$ mice. Consistent with these data, angiotensin II–induced hypertension does not

occur in AKAP150$^{-/-}$ mice (Sonkusare et al., 2014).

Further evidence that signalling microdomains within MEPs are key to the physiological regulation of small arteries/arterioles comes from studies with pressurized and myogenically active arterioles (Bagher et al., 2012). A drop in intraluminal pressure was found to increase the frequency of spontaneous EC Ca^{2+} events, reflecting TRPV4 channel activation. The events were discrete, and due to both TRPV4-sparklet- and non-sparklet evoked increases in MEP Ca^{2+}, which then activated IK_{Ca} channels to evoke EDH-vasodilation that actively reduced myogenic tone at pressures <60 mmHg (Bagher et al., 2012).

Finally, EC signalling can also be activated during vasoconstrictor agonist stimulation of VSM. This was first reported in arterioles, when vasoconstrictor agonist evoked increases in VSM Ca^{2+} was shown also to signal an increase in EC $[Ca^{2+}]_i$, which was sufficient to release NO and suppress VSM contraction (Dora et al., 1997). Subsequently, the EDH pathway was also shown to be activated and to provide similar negative feedback (Dora et al., 2000b). Whether it is Ca^{2+} or IP_3 that provides the signal passing from the VSM to activate EC vasodilator signalling has been the subject of some debate, as this is a very difficult issue to resolve unequivocally with the tools currently available (Tran et al., 2012).

EDH and arterial conducted vasodilatation (CVD)

So far, we have focused on the ability of EDH to move radially into the artery wall to affect vasodilation. However, the fact that extensive homo- and heterocellular gap junction coupling is present in the artery wall means that vascular hyperpolarization

can also spread axially to cause vasodilatation Figure 2. As such, the axial spread of hyperpolarization is able to translate focal dilatation into the more widespread drop in vascular resistance that is necessary to increase blood flow. For agents to stimulate a significant increase in tissue blood flow, an increase in artery diameter must significantly reduce vascular resistance. As resistance is a function of vessel length a limited, focal dilatation will not do this. But if focal dilatation in, for example, an arteriole can spread upstream a significant increase in blood flow will follow (Dora et al., 2000a, Kurjiaka and Segal, 1995). The signal for spreading dilatation is thought to be the passage of hyperpolarizing current to adjacent cells; that is to cells not directly stimulated by a hyperpolarizing agonist (Emerson and Segal, 2000, Dora et al., 2000a). Spread occurs mainly via the endothelium, and can occur bi-directionally along the length of the vessel from a defined point of stimulation. It is not dependent on either nerves or a change in blood flow (Emerson and Segal, 2000, Winter and Dora, 2007, Segal, 2005, Dora et al., 2000a, Delashaw and Duling, 1991, Rivers, 1997), but is influenced by the open-state probability of EC and VSM K^+ channels. Activation of vascular K channels limits the spread of hyperpolarizing current and the associated vasodilation (Beleznai et al., 2011, Behringer and Segal, 2012).

The endothelium is crucial for conducted vasodilatation

The ability of the endothelium to act as the primary route for hyperpolarizing current reflects both the orientation of the cells and their extensive coupling to each other, through homocellular gap-junctions (Takano et al., 2004, White and Hiley, 2000, Dora et al., 2008, Yamamoto et al., 1999, Winter and Dora, 2007, Haas and Duling, 1997, Emerson and Segal, 2000). The K^+ channel underlying a given magnitude of hyperpolarization is not of any real relevance as far as the subsequent spread is

concerned. It's only role is to increase membrane potential, which because of cell-cell coupling means current can spread. Thus, activation of either endothelial or smooth muscle K^+ channels will evoke CVD. Albeit that some agonists may stimulate additional signalling pathways that may either augment (e.g. by cyclic AMP; (Popp et al., 2002) or reduce (e.g. by α_1-adrenoceptor stimulation, protein kinase C; (Bao et al., 2004, Haug et al., 2003) the ability of hyperpolarization to spread between the vascular cells.

A key question is whether hyperpolarizing current passing between arterial cells decays passively, or whether an amplification mechanism is present to sustain conduction and the associated vasodilatation. The available evidence in isolated, pressurized arteries supports a role for a mechanism that limits current decay, as current appears to spread further than predicted by simple passive decay alone. This was shown directly in cannulated small arteries (1-2 layers of smooth muscle), where the decay of injected current was faster than the decay of hyperpolarization to ACh, despite the fact both stimuli induced similar local (initiating) hyperpolarization (length constants 1.2 and 1.9 mm, respectively; Emerson et al., 2002). Therefore, the intercellular spread of current *via* gap junctions appears in some way to be sustained. One ion channel that appears to be involved, at least in some cases, is K_{IR} (Goto et al., 2004, Jantzi et al., 2006, Rivers et al., 2001, Longden and Nelson, 2015). However, the K_{IR} channel may not necessarily represent a universal mechanism, as Ba^{2+} (to inhibit K_{IR}) did not alter conducted dilatation to either ACh or levcromakalim in rat mesenteric arteries (Takano et al., 2004). Possibly, K^+ release from vascular cells can serve as an amplification mechanism, with a distinct parallel to its role as an EDH(F), where it acts through K_{IR} and Na/K ATPase (Edwards et al.,

1998). Both K_{IR} and SK_{Ca} occur at the borders of endothelial cells, and it is also highly likely that an isoform of the ATPase is expressed within this space in the vicinity of inter-EC gap junctions (Dora et al., 2008, Winter and Dora, 2007, Kansui et al., 2004). So it remains to be seen if mechanisms such as hyperpolarization-mediated Ca^{2+} influx can activate K_{Ca} channels and serve to help sustain conducted hyperpolarization. The possibility that hyperpolarization can stimulate EC Ca^{2+} influx and spread between ECs to sustain CVD has been discussed recently, and the reader is referred to these reviews for more information on this aspect (Dora and Garland, 2013, Segal, 2015).

Relevance of EDH in vivo and translation

A number of studies indicate that the EDH mechanism is effective *in vivo*. Perhaps most directly, disruption of the gene for IK_{Ca} ($K_{Ca}3.1^{-/-}$) was found to impair EDHF-mediated hyperpolarization and also found to associate with a significant increase in mean arterial blood pressure and left ventricular hypertrophy (Si et al., 2006). Furthermore, the additional disruption of SK_{Ca} suggested that IK_{Ca} is the predominant component of the EDHF mediated hyperpolarization and vasodilation (Brähler et al., 2009), and also responsible for the spread of hyperpolarization that underlies CVD (Wolfle et al., 2009). More recent in vivo work in large mammals supports an important influence of EC K_{Ca} channels on blood pressure (Damkjaer et al., 2012), and is consistent with the suggestion that the EDH pathway operates in humans. This suggestion was based on indirect measurements, to assess forearm blood flow (Inokuchi et al., 2003). That EDH does operate in human resistance arteries was shown directly by microelectrode measurements from isolated coronary arterioles (Miura et al., 1999). In these vessels, endothelium-dependent hyperpolarization to

bradykinin persisted in the presence of nitric oxide synthase and cyclooxygenase inhibitors, and was sensitive to block with TEA (Miura et al., 1999). More recently, the mediator(s) of hyperpolarization in these coronary arterioles has been suggested to include metabolites of cytochrome P450 epoxygenase and H_2O_2 (Larsen et al., 2008). EDH linked vasodilation appears to be reduced in cardiovascular disease in both animal models and in humans (Hilgers and Webb, 2007, Dal-Ros et al., 2009, Weston et al., 2010, Luksha et al., 2012), and recent work is starting to reveal mechanistic changes, involving EC signalling microdomains and the scaffolding protein AKAP150, that may help to explain this disruption (Sonkusare et al., 2014, Bagher and Garland, 2014). So future research in this area may identify novel targets with potential to address or reverse reduced EDH-mediated vasodilation.

Conclusion

Since EDHF was first identified in the 1980s, there has been substantial progress in defining the fundamental mechanisms responsible for generating endothelial cell hyperpolarization, or EDH. Similarly, discovering the mechanisms responsible for transferring EDH radially into the adjacent smooth muscle layers and along the axis of arteries to affect distant vasodilatation, or CVD, has helped to shape our understanding of the importance of hyperpolarization in integrating changes in tissue blood flow. Along the way, probably the most important discoveries have been the recognition that hyperpolarization reflects the activity of two separate forms of K_{Ca} channel, the SK_{Ca} and IK_{Ca} channels, and that both are located in the endothelium. The reason for this suggestion is simply that without this knowledge it is unlikely that our understanding of EDH would have advanced much at all. However, the complex nature of the signalling pathway, including the more recent appreciation that EC K_{Ca}

channels are arranged discretely within signalling microdomains, means that future challenges are now to unravel both the complexity and local regulation of EC microdomains, and to discover to what extent they may be subject to disruption by disease. The latter offering exciting potential for developing ways to affect modulation, with the aim of improving or sustaining physiological function in the resistance vasculature.

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Conflicts of interest

The authors have no conflicts of interest

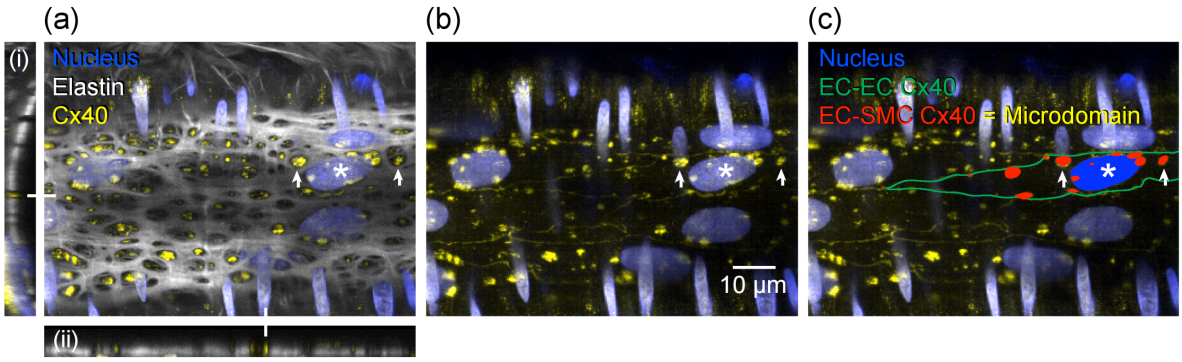

Figure 1

Figure 1. Microdomains in arterioles.

Immunolabelling and structure imaging using confocal microscopy in an isolated, cannulated and pressurized rat cremaster arteriole. Connexin 40 (Cx40) was apparent in high density within holes through the internal elastic lamina (IEL, compare a & b), defining the myoendothelial (EC-SMC) gap junctions and signalling microdomain (drawn in red, c, see expression of IK_{Ca} & TRPV4 channels in Bagher et al., 2012, and methods therein). The 3D structure through the radial and longitudinal lengths of the arteriole are shown in a(i) and (ii), respectively; the white bars indicate the positions of the cross-sections. Cx40 was also present between endothelial cells (EC-EC) (drawn in green, c). NB., a single EC has multiple sites where signalling can occur via the microdomain. The asterisk indicates the same nucleus, and arrows examples of Cx40 within holes through the IEL in panels a-c.

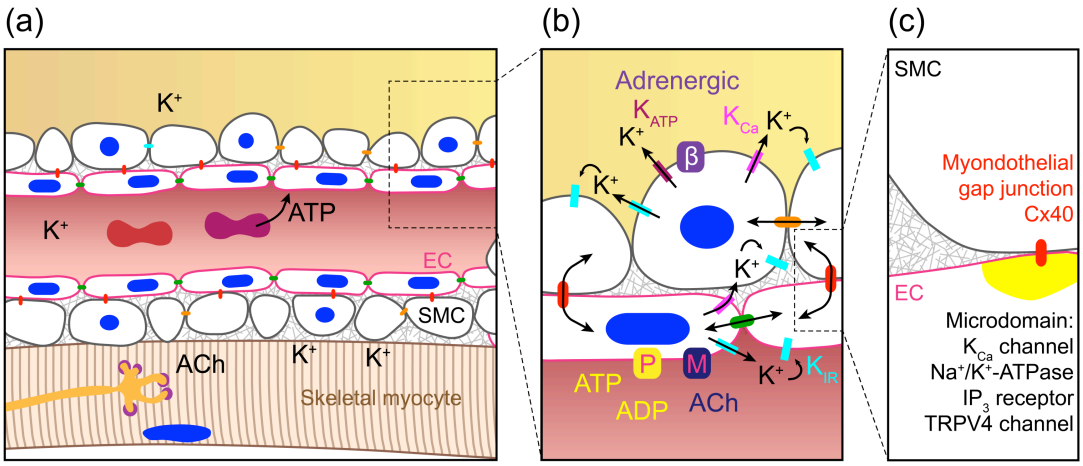

Figure 2

Figure 2. EDH stimulates local and conducted dilation.

Agonists released in an autocrine or paracrine manner activate endothelial cell (EC) receptors to stimulate endothelium-dependent hyperpolarization (EDH) Panel A & B. In the examples of ACh, ATP and ADP, each bind G-protein coupled receptors linked to the formation of IP_3 for Ca^{2+} release, and Ca^{2+} influx through transient receptor potential (TRP) and other Ca^{2+} channels. These activate K_{Ca} channels, in particular the IK_{Ca} channels aligned within the myoendothelial microdomain (drawn yellow in panel C, see also Figure 1). Passage of current via myogendothelial gap junctions or diffusion of a factor such as K^+, can hyperpolarize smooth muscle cells (SMCs), close voltage-dependent Ca^{2+} channels and relax the cells. Some agonists bind SMC receptors to stimulate hyperpolarization, for example catecholamines stimulate β-adrenoceptors which couple via PKA to K_{ATP} channels. EC- and SMC-dependent hyperpolarization can lead to conducted dilatation, in arteries and arterioles where homo- and heterocellular gap junctions are patent and the endothelium can serve as the conduit for passage of current Panel B. In all cases the K^+ released from cells can act on K_{IR} channels and stimulate the Na^+/K^+-ATPase, each of which stimulate

further hyperpolarization. Key: EC, endothelial cell. SMC, smooth muscle cell. K_{ATP}, ATP inhibited potassium channels. K_{Ca}, Calcium activated potassium channels. K_{IR},