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Themed Section: Vascular Endothelium in Health and Disease

REVIEW

EDHF: spreading the influence of the endothelium

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Our view of the endothelium was transformed around 30 years ago, from one of an inert barrier to that of a key endocrine organ central to cardiovascular function. This dramatic change followed the discoveries that endothelial cells (ECs) elaborate the vasodilators prostacyclin and nitric oxide. The key to these discoveries was the use of the quintessentially pharmacological technique of bioassay. Bioassay also revealed endothelium-derived hyperpolarizing factor (EDHF), particularly important in small arteries and influencing blood pressure and flow distribution. The basic idea of EDHF as a diffusible factor causing smooth muscle hyperpolarization (and thus vasodilatation) has evolved into one of a complex pathway activated by endothelial Ca^{2+} opening two Ca^{2+}-sensitive K^+-channels, $K_{Ca}2.3$ and $K_{Ca}3.1$. Combined application of apamin and charybdotoxin blocked EDHF responses, revealing the critical role of these channels as iberiotoxin was unable to substitute for charybdotoxin. We showed these channels are arranged in endothelial microdomains, particularly within projections towards the adjacent smooth muscle, and close to interendothelial gap junctions. Activation of K_{Ca} channels hyperpolarizes ECs, and K^+ efflux through them can act as a diffusible 'EDHF' stimulating Na^+/K^+-ATPase and inwardly rectifying K-channels. In parallel, hyperpolarizing current can spread from the endothelium to the smooth muscle through myoendothelial gap junctions upon endothelial projections. The resulting radial hyperpolarization mobilized by EDHF is complemented by spread of hyperpolarization along arteries and arterioles, effecting distant dilatation dependent on the endothelium. So the complexity of the endothelium still continues to amaze and, as knowledge evolves, provides considerable potential for novel approaches to modulate blood pressure.

LINKED ARTICLES

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Abbreviations

BK_{Ca}, large conductance Ca^{2+}-sensitive K^+-channels; CaSR, calcium-sensing receptors; Cx, connexin; CyPPA, N-cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine; EDH, endothelium-dependent hyperpolarization; EDRF, endothelium-derived relaxing factor; EET, epoxyeicosatetraenoic acid; 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; PGI_2, prostaglandin I_2; SK_{Ca}, small conductance Ca^{2+}-sensitive K^+-channels; TRP, transient receptor potential

This review was inspired by the 2009 JR Vane Lecture, delivered by the first author in the Summer of that year at the Edinburgh BPS meeting. It tracks our perspective of the key discoveries and the development of understanding of the pathway commonly referred to as endothelium-derived hyperpolarizing factor or EDHF. As such, it is probably not as all encompassing as many reviews, but provides more of a personal view of the evolution of this field over the last 20 or so years.

After 30 years, it still seems quite remarkable to think that until circa 1980, the endothelium was considered to be merely an inert lining, covering the luminal surface of blood

vessels. Much like Teflon®, endothelial cells (ECs) were assumed simply to present a non-stick surface, enabling them to separate the flowing blood from the pro-aggregatory constituents of the sub-intimal layers of the vascular wall. This view changed dramatically with Robert Furchgott's discovery of endothelium-derived relaxing factor (EDRF), reported in a seminal paper (Furchgott and Zawadzki, 1980) that formed the basis for the later award of the 1998 Nobel Prize in Physiology or Medicine. The prize was awarded to Furchgott, along with Louis Ignarro and Ferid Murad, for 'their discoveries concerning nitric oxide (NO) as a signalling molecule in the cardiovascular system'. However, as we shall see, NO represents just one of several key processes by which the endothelium regulates the smooth muscle cells (SMCs) that form the major part of the wall of most blood vessels.

It is of course interesting to speculate how long it might have taken without this discovery before we appreciated the crucial role the endothelium plays in controlling both the distribution of blood and pressure within the circulation. In all likelihood, not very long at all, because an active role for the endothelium had already been recognized in the UK by John Vane, Salvador Moncada and colleagues. During the 1970s, they discovered that the anti-aggregating properties of this cellular monolayer reflected its ability to elaborate a novel arachidonic acid derivative that was also a potent vasodilator. They initially termed this short-lived derivative prostaglandin X (PGX), though we of course now know it as prostacyclin (PGI_2). The discovery played a significant part in securing the 1982 Nobel Prize in Physiology or Medicine for Vane, together with Bergström and Samuelsson. So as such, 1982 could well be considered to represent the first Nobel Prize for the discovery of an endothelium-derived mediator. Indeed, by 1990, the secretory activity of the endothelium was sufficiently appreciated for Anggard (1990) to propose that it should be considered 'the largest endocrine gland in the body'. Vane showed clearly that this was an appropriate description in his Croonian Lecture delivered to the Royal Society a few years later (Vane, 1994). Vane's very significant contributions, including the discovery of PGI_2, have been elegantly reviewed recently by Rod Flower, one of his distinguished colleagues during that exciting period of pharmacological discovery in the 1970s (Flower, 2006).

Of great interest to pharmacologists is of course the fact that the discovery of both PGI_2 and EDRF, and thus the award subsequently of two Nobel prizes, relied on the classic pharmacological approach of bioassay elegantly developed by Sir John Gaddum. He once famously remarked 'The pharmacologist has been a "jack of all trades", borrowing from physiology, biochemistry, pathology, microbiology, and statistics – but he has developed one technique of his own and that is the technique of bioassay . . .' (Gaddum, 1964). Vane very cleverly adapted Gaddum's 'superfusion' bioassay to provide the cascade bioassay. Carefully selected tissues were arranged in two series, to allow the parallel assay of injected samples and/or the active components in fluid taken directly from the outflow of a perfused organ (Vane, 1964). This approach enabled first the discovery of thromboxane A_2 and, subsequently, the demonstration that arterial microsomes can metabolise PGG_2 to a labile product with both vasodilator and platelet anti-aggregatory properties. This was PGX, which once the structure was elucidated was given the trivial name prostacyclin (Whittaker *et al*., 1976). Shortly afterwards, it was realized that the site of vascular synthesis was in fact the endothelium (Bunting *et al*., 1977; Moncada *et al*., 1977; Weksler *et al*., 1977). A more detailed overview of these major discoveries can be obtained in Moncada *et al*. (1976), Gryglewski *et al*. (1976) and Flower (2006).

Of course, Furchgott's discovery of EDRF also depended entirely on the use of bioassay, or perhaps more accurately the judicious interpretation of what was his rather unexpected data. Having designed an experimental protocol to investigate β-adrenoceptor-evoked relaxation in isolated aortic rings, the story is that his technician mixed up the protocol. The result was that a robust relaxation was obtained to muscarinic agonists. At the time this was a very surprising outcome, because although muscarinic agonists were known as potent vasodilators *in vivo*, *in vitro* they either did not affect vascular preparations or caused vasoconstriction. Furchgott quickly realized that an absence of dilator ability *in vitro* correlated with damage to the endothelium during tissue isolation. Like Vane, he then modified the bioassay technique, this time sandwiching together artery strips, one with and one without endothelium, to show that smooth muscle relaxation to muscarinic agonists was due to the release of a diffusible dilator from the endothelium, which he named EDRF. The rest, as they say, is history. By 1988, EDRF had been identified as NO and the fact that NO serves as a fundamental biological signalling molecule throughout the body was widely recognized.

A third endothelium-derived dilator: the discovery of EDHF

Kuriyama's group in Japan had shown, rather oddly, that muscarinic stimulation of rabbit and guinea pig arteries could evoke vascular hyperpolarization associated with vasoconstriction (Kuriyama and Suzuki, 1978; Kitamura and Kuriyama, 1979). After Furchgott's discovery of the endothelium as the primary target for muscarinic agonists, Bolton showed that smooth muscle hyperpolarization to carbachol also depended on the integrity of this monolayer of cells, and suggested that muscarinic stimulation might cause hyperpolarization by releasing a factor from the ECs (Bolton *et al*., 1984). The coincident contractile action reported by Kuriyama *et al*. could then be explained simply by the fact that, in the species they studied, the muscarinic receptors were present on endothelial *and* vascular SMCs, with stimulation of both receptor populations generating a predominantly contractile response.

Two absolutely fundamental questions arose from considering these observations together. First, as both EDRF and hyperpolarization originated in the endothelium, was the latter simply a facet of the former? Second, how important was the smooth muscle hyperpolarization in terms of vascular physiology; was it simply a pharmacological curiosity?

Both questions were cleverly addressed by Chen *et al*., and presented first at a meeting in Melbourne in 1987, then in a classic paper that truly marked the beginning of the EDHF field (Chen *et al*., 1988). Why was this paper so seminal? Because through the use of microelectrodes, they were able to provide the first direct demonstration indicating endothelium-dependent hyperpolarization to be distinct from EDRF. By 1987, it was clear that EDRF was in fact NO or

an NO-releasing entity (see Furchgott, 1998). At that time, the pharmacological tools available to probe the effects of NO were fairly limited, not least by the fact that NO synthase had yet to be identified (see Schmidt *et al.*, 1988a,b; Bredt and Snyder, 1990; Forstermann *et al.*, 1990), so of course inhibitors for this enzyme remained unavailable for a few years (Moore *et al.*, 1990; Rees *et al.*, 1990). However, methylene blue (which blocks guanylyl cyclase and scavenges NO) and haemoglobin (which binds NO) were available (Martin *et al.*, 1985), and neither altered endothelium-dependent hyperpolarization in the rat aorta and pulmonary artery. Nor did they depress ^{86}Rb efflux, a marker of K^+ movement. In contrast, smooth muscle relaxation (of tone induced with noradrenaline) was inhibited by well over 50% while cGMP accumulation was effectively abolished. Furthermore, none of these responses were altered by indomethacin. Not surprisingly then, the major conclusion was '*the properties of this [hyperpolarizing] agent suggest it should be designated endothelium-derived hyperpolarizing factor (EDHF).*' Although this work did not provide any evidence for a discrete factor *per se*, the use of bioassay again proved crucial. Félétou and Vanhoutte (1988) reported smooth muscle hyperpolarization in endothelium-denuded small coronary arteries, but only when these arteries were co-incubated with femoral artery segments with an intact endothelium. Furthermore, although this study did not suggest that hyperpolarization might be due to anything other than NO/EDRF, it did reveal that hyperpolarization could be blocked with ouabain. As we discuss later, it transpires in some arteries that Na^+/K^+-ATPase is a target for K^+ acting as an EDHF.

While Chen *et al.* (1988) clearly showed that endothelium-dependent hyperpolarization was distinct from the action of EDRF, the strong and predominant vasodilator influences of the latter seemed to argue against the possibility that EDHF was actually an important physiological entity. The concept of a new endogenous relaxing agent elaborated by the endothelium had been developed in a 'Current Awareness' article in *Trends in Pharmacological Sciences* by Taylor and Weston (1988), and they speculated that as hyperpolarization presumably caused vasodilatation by closing voltage-dependent Ca^{2+} channels, '*Such a process is likely to be very important in small arterioles which are very dependent on Ca^{2+} influx during contraction*'. This proved to be a very insightful suggestion. In small resistance arteries, neither hyperpolarization nor relaxation to ACh were reduced by oxyhaemoglobin, indomethacin or nitro-L-arginine (Garland and McPherson, 1992). So hyperpolarization could completely relax pre-constricted resistance arteries. By this time, the use of the Mulvany–Halpern wire myograph made it possible to attempt simultaneous recording of smooth muscle membrane potential and tension, so data could be generated from a single artery segment under the same experimental conditions, avoiding any differences due to the stretch applied to the muscle cells, a concern when these parameters were recorded independently.

So the EDHF phenomenon came to be 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. Given the technical difficulties associated with microelectrode recording in vascular tissue, it is not really surprising that, in spite of the quite considerable literature generated in this area, the vast majority of studies simply assess EDHF indirectly, by measuring vascular relaxation in the presence of a contractile agent (such as an α-adrenoceptor or thromboxane receptor agonist). Thus, although many papers refer to EDHF, they overlook the fundamental importance of recording the membrane potential changes that defined the pathway. So in effect, most studies were not investigating the consequences of hyperpolarization but of *repolarization*. This distinction has actually proved to be crucial when coming to understand the complexity of the mechanisms that are responsible for initiating and spreading endothelium-dependent hyperpolarization. Although the acronym EDHF has been used most widely to denote the phenomenon of endothelium-dependent hyperpolarization (EDH), it is perhaps pertinent at this stage to point out that, especially in smaller vessels, there is very good evidence a factor may not necessarily be involved. As such, EDH is really a more accurate description of what is a key pathway for vasodilatation.

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

The next major step in defining the EDH pathway was the discovery that hyperpolarization reflected the activation of two types of K^+-channel, the small and intermediate conductance Ca^{2+}-sensitive K^+ channels (K_{Ca}) – SK_{Ca} ($K_{Ca}2.3$) and IK_{Ca} ($K_{Ca}3.1$)(nomenclature follows Alexander *et al.*, 2009). Furthermore, and probably equally as important, these channels were shown to be located on the endothelium, rather than serving as a smooth muscle target for a diffusible endothelium-derived factor or EDHF.

Although K^+ efflux was clearly a fundamental, defining feature of the EDHF response (Chen *et al.*, 1988), it took quite some time to identify the underlying K^+-channels. With hindsight, it is clear that in large part this was because two types of K^+-channel are involved. EDHF-mediated responses were quickly shown to be insensitive to glibenclamide, excluding a role for ATP-sensitive K^+-channels (K_{ATP}) (Garland and McPherson, 1992; Plane and Garland, 1993). A key role for voltage-gated K^+-channels (K_V) also seemed extremely unlikely, as the EDHF response relied on pronounced and sustained hyperpolarization. This was confirmed when EDHF-like relaxation was shown to be insensitive to K_V block (Adeagbo and Triggle, 1993). However, K_{Ca} did seem to have some role, as relaxation was reduced by either the non-selective blocker tetrabutylammonium or the specific SK_{Ca} ($K_{Ca}2.3$) channel blocker apamin (Adeagbo and Triggle, 1993; Holzmann *et al.*, 1994). Taking this further, by recording both hyperpolarization and relaxation simultaneously, we found not only that apamin but also that charybdotoxin slightly reduced both responses. We assumed at that stage charybdotoxin must be blocking the large-conductance K_{Ca} (BK_{Ca}: $K_{Ca}1.1$) channels known to be present on the SMCs, and reasoned that these data indicated a role for both SK_{Ca} and BK_{Ca} channels. Suspecting parallel signalling, we applied both of these toxins in combination, and for the first time obtained a block of EDHF hyperpolarization and the associated relaxation (Waldron and Garland, 1994). This proved to be a seminal observation. It was confirmed soon after by Zygmunt and Hogestatt (1996), who crucially extended the observation to show that iberiotoxin was not able to substi-

tute for charybdotoxin. As well as BK_{Ca}, charybdotoxin was known to block the intermediate form of K_{Ca} (IK_{Ca}: $K_{Ca}3.1$) channels, thus it appeared to be that these channels, along with the SK_{Ca} channels, were involved in the EDHF response.

It was generally assumed, wrongly as it turns out, that both types of K^+-channel must be located on the smooth muscle, and thus subject to activation by a diffusible EDHF released from the endothelium. At that time, it was clear from the work of Mark Nelson and others, that vascular smooth muscle contained BK_{Ca} channels, and we were able to demonstrate this with high concentrations of NO, which activated BK_{Ca} channels in mesenteric artery SMCs. However, we could find no evidence for SK_{Ca} channels (Mistry and Garland, 1998a,b). Around this time, Gillian Edwards and Arthur Weston in Manchester published a critical appraisal of the EDHF field, and with the benefit of hindsight it is really quite remarkable how accurate the insights provided in this article have proved to be. Not least was the suggestion that perhaps the K_{Ca}-channels might actually reside on the endothelium rather than the SMCs. They also suggested that K^+ ion efflux through these channels may serve to activate muscle Na^+/K^+-ATPase and/or inwardly rectifying K^+-channels (K_{IR}) to cause hyperpolarization, making K^+ ion the elusive EDHF (Edwards and Weston, 1998). This period marked the start of an exciting collaboration between two of us (CJG and KAD) and the Manchester group, with a number of major outcomes. First, intracellular recording directly from *endothelial* cells *in situ* revealed that ACh evoked hyperpolarization in these cells, and this response could be blocked by apamin and charybdotoxin in combination. The simplest explanation was that the K_{Ca} resides on the ECs. This of course made sense when one remembers that M_3 muscarinic receptors signal via inositol trisphosphate ($InsP_3$), so are able to provide the signal for activation of the Ca^{2+}-sensitive, but voltage-insensitive, K_{Ca}. Thus, we concluded that the SK_{Ca} and IK_{Ca} channels were present on the endothelium not the SMCs, a finding which was subsequently confirmed by us and others. The second major outcome of our joint studies was to provide direct evidence suggesting that, after efflux from these channels, K^+ acts as an EDHF (Edwards *et al.*, 1998).

K^+ – a humoral EDHF

The concept of a local increase in extracellular K^+ serving to evoke vasodilatation was not in itself novel. Small increases in $[K^+]_o$ had been shown directly to cause vasodilatation by Dawes (1941) and, in the cerebral circulation, similar increases were suggested to underlie, at least in part, the close coupling between nerve activity and blood flow that had first been defined in 1890 by Roy and Sherrington (Roy and Sherrington, 1890; Kuschinsky *et al.*, 1972). The mechanism responsible is explained by observations in pressurized coronary and cerebral arteries, where increases in extracellular K^+ of <20 mM (in total) stimulated smooth muscle hyperpolarization and relaxation by increasing inwardly rectifying K^+-channel (K_{IR}) conductance and activating Na^+/K^+-ATPase (McCarron and Halpern, 1990; Knot *et al.*, 1996). Completely novel now was the discovery that K^+ released from the ECs into the artery wall could evoke hyperpolarization, and thus relaxation, of the adjacent SMCs. Using isolated mesenteric and hepatic arteries from the rat, we observed that both smooth muscle hyperpolarization and relaxation to either exogenous K^+ or EDHF (activated by stimulating selectively the endothelium with ACh) were of similar magnitude and sensitivity to inhibition with a combination of Ba^{2+} and ouabain, employed to block K_{IR} channels and Na^+/K^+-ATPase. However, in contrast to the SMCs, the EC hyperpolarization to ACh was not sensitive to Ba^{2+} and ouabain (Figure 1). This observation suggested that the release of K^+ through SK_{Ca} and IK_{Ca} channels was activating K_{IR} channels and Na^+/K^+-ATPase to evoke hyperpolarization. Furthermore, during EC stimulation with ACh, a K^+-selective electrode inserted into the proximity of the endothelial-smooth muscle interface recorded an increase in $[K^+]_o$ of around 10 mM. The increase was sufficient to explain the measured EDHF hyperpolarization and relaxation (Edwards *et al.*, 1998). However, despite the importance of these observations, everything was not completely clear-cut. The small arteries used in this study were the rat hepatic and mesenteric arteries, and while the EDHF response in the former was blocked with Ba^{2+} and ouabain, in the latter a significant component persisted. At the time, we concluded that the persistent relaxation must reflect an additional pathway, and suggested that myoendothelial gap junctions (MEGJs) between endothelial and SMCs may be responsible. We subsequently proved this is, in fact, the case and this work is discussed next (Mather *et al.*, 2005).

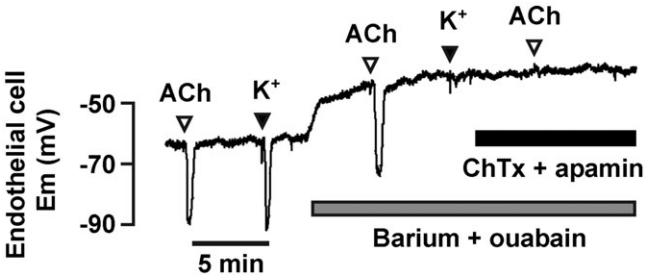

Figure 1

Hyperpolarization of impaled endothelial cells to bolus additions of ACh (10 μM) and K^+ (5 mM) in isolated, pinned and superfused rat hepatic arteries. The combination of barium (30 μM, to block K_{IR} channels) and ouabain (1 mM, to inhibit the Na^+/K^+-ATPase) fully blocked hyperpolarization to K^+, but not to ACh. In separate experiments, this combination blocked smooth muscle cell hyperpolarization to ACh (not shown). The K_{Ca} channel blockers charybdotoxin (ChTx; 100 nM) and apamin (100 nM), abolished both endothelial cell (shown) and smooth muscle cell (not shown) hyperpolarization to ACh. First published in Edwards *et al.* (1998).

The concept of endothelium-derived K^+ acting as a hyperpolarizing factor has been subsequently supported by studies in arteries from a range of species and locations, and including human arteries (Chrissobolis *et al.*, 2000; McGuire *et al.*, 2002; Bussemaker *et al.*, 2003; Nelli *et al.*, 2003; McNeish *et al.*, 2005). However, this was not immediately the case. Following the initial suggestion in Edwards *et al.* (1998), a number of laboratories were unable to replicate arterial relaxation by raising $[K^+]_o$, which of course is an absolute requirement if this ion really is an endogenous hyperpolarizing factor (Edwards *et al.*, 1999; 2000; Quignard *et al.*, 1999). Critically, this failure to evoke vasorelaxation to $[K^+]_o$ included the rat mesenteric resistance artery (Doughty *et al.*, 2000; Lacy *et al.*, 2000), one of the vessels used in Edwards

et al. (1998), and the internal carotid and small coronary arteries. In the latter, the EDHF response appears mediated predominately through MEGJs, while in the studies using the mesenteric artery, the important concept of functional or physiological antagonism was overlooked in the experimental design.

The latter point was demonstrated by examining the ability of elevated $[K^+]_o$ to relax and repolarize rat small mesenteric arteries during either submaximal or maximal stimulation with phenylephrine. The mesenteric arteries used did not spontaneously develop tone, so to study both hyperpolarization (or more accurately repolarization) and the resulting relaxation, the α_1-adrenoceptor agonist was applied before K^+, to evoke smooth muscle depolarization and contraction. As the phenylephrine concentration increased towards maximal, repolarization and relaxation to $[K^+]_o$ was blocked (Dora and Garland, 2001). The explanation was simply that stimulation of the muscle α_1-adrenoceptors was increasing the efflux of K^+ through iberiotoxin-sensitive BK_{Ca} channels, with the result that K^+ accumulated in the extracellular space and saturated the Na^+/K^+-ATPase activity. This saturation then effectively blocked responses to any additional (exogenous) K^+ ion. When efflux from the smooth muscle was reduced, in the presence of iberiotoxin, then the functional antagonism was removed and exogenous K^+ ion was again able to evoke repolarization/hyperpolarization and relaxation, or in pressurized arteries increase diameter (Dora *et al*., 2002). The extracellular accumulation of K^+ that follows smooth muscle stimulation, and underlies the physiological antagonism, was termed the 'K^+-cloud' by Edwards and Weston (Weston *et al*., 2002).

Gap junctions – a physical link between muscle and endothelium

At the same time as the K^+ story was developing, Tudor Griffith's group in Cardiff proposed that electrical coupling through MEGJs could explain NO-independent, endothelium-dependent, relaxations in rabbit iliac artery (Taylor *et al*., 1998). This was despite earlier suggestions that the gap junction inhibitors, halothane and 1-heptanol, had no effect on responses ascribed to EDHF (Zygmunt and Hogestatt, 1996). The presence of gap junctions between cells in the vascular wall had been recognized for many years (Rhodin, 1967), and a key role for gap junctions as a means to enable conducted vasodilatation to ACh had been clearly demonstrated in arterioles of the microcirculation by Segal and Duling (1989). This group then used intracellular microelectrodes to demonstrate directly that close electrical coupling existed between the smooth muscle and the ECs, suggesting that this coupling enabled hyperpolarization to spread along arterioles and affect dilatation (Xia *et al*., 1995). Furthermore, they identified connexin 40 and 43 (Cx40 and Cx43) as integral components of the gap junctions (Little *et al*., 1995).

That gap junctions could underpin EDHF responses was supported by whole-cell patch clamp experiments in arterioles. These showed that hyperpolarization generated by ACh in the endothelium of intact vessels was, to all intents and purposes, identical in these cells and the adjacent SMCs (Yamamoto *et al*., 1999). However, after blocking gap junctions with 18β-glycyrrhetinic acid, ACh only induced an outward current in the ECs. Furthermore, removing extracellular Ca^{2+} or adding charybdotoxin reduced the EC hyperpolarization, supporting the suggestion that hyperpolarization of the smooth muscle was purely indirect. However, the functional significance of gap junctions, just like K^+, seemed to vary between arteries and was not apparent in every preparation (Bény and Schaad, 2000). One possibility was that the functional role of MEGJs might increase in importance as vessels decreased in size. If this were true, it might then explain the fact that EDHF-evoked relaxation increases in importance as artery diameter decreases (Garland *et al*., 1995; Shimokawa *et al*., 1996).

This hypothesis gained considerable support from a morphological study that attempted to quantify muscle–endothelial and endothelial–endothelial gap junctions in the rat mesenteric arterial bed. MEGJs were indeed present in this artery and, at <100 nm in diameter, small compared with the homocellular gap junctions between the ECs. Importantly, they also appeared to be present in larger numbers in the distal compared with the proximal regions of the mesenteric arterial bed. These observations were of course consistent with a functional role of increasing importance as artery size decreased (Sandow and Hill, 2000). The larger gap junctions between the ECs were clearly pentalaminar and distributed extensively throughout the mesenteric arterial bed. These observations have not been extended to other beds to allow confirmation, or not, of the general principle, presumably in large part because of the extremely laborious nature of this type of research. However, Sandow *et al*. (2004) did show that the incidence of MEGJs decreased in the saphenous artery of rats as they matured to adulthood, and that this decrease was accompanied by a loss of EDHF-dependent relaxation to ACh. Again, evidence consistent with an important functional role for MEGJs.

MEGJs appear as thickenings of the membranes of the two adjacent cell types, where they come into close apposition (Rhodin, 1967). In the rat, they occur on projections of the ECs which pass through the internal elastic lamina (IEL; Figure 2), although not all projections seem to possess a junction (Sandow and Hill, 2000). Incidentally, it is presumably this anchoring that had made it so difficult to remove endothelium from perfused rat arterial beds in early studies of EDRF, effective removal requiring the use of either detergent (Randall and Hiley, 1988), an air bubble (Atkinson *et al*., 1994) or *Staphylococcus aureus* α-toxin (Laher *et al*., 1995) physically to destroy the cells, rather than collagenase simply to dislodge them from the vessel wall, as in small rabbit vessels (Furchgott *et al*., 1987). The gap junctions are formed by connexins located in the membrane of the 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 (see Johnstone *et al*., 2009). In the vasculature, connexons can be formed from Cx37, Cx40, Cx43 and/or Cx45, the number indicating the molecular mass of the protein. For a detailed overview of gap junctions in EDH, readers are referred to a recent review (de Wit and Griffith, 2010).

The involvement by MEGJs in the EDHF response, minor or not, of course removes any absolute reliance on a diffusible factor as *the* underlying mechanism. So, as mentioned earlier, because hyperpolarization is initiated in the endothelium, a

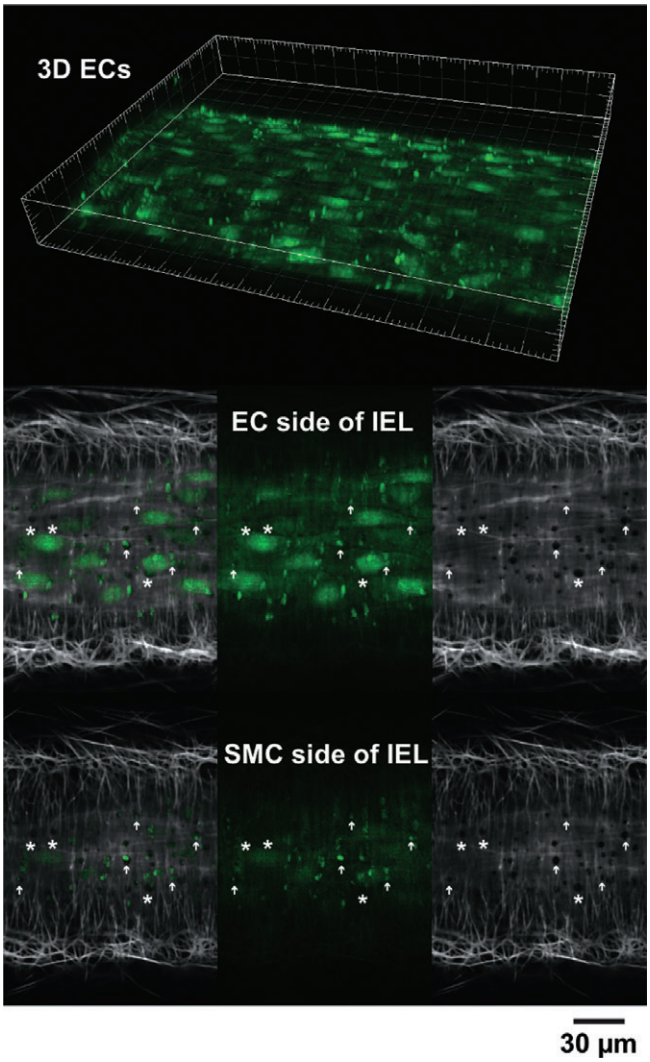

Figure 2

Confocal micrographs of endothelial cells (ECs) in an artery. The fluorescent Ca^{2+} indicator Oregon Green 488 BAPTA-1 was loaded into ECs (green) of a pressurized rat mesenteric artery. Serial images through the *z*-axis allowed 3D reconstruction of the artery wall (top) where bright EC projections were clearly visible. Frame major ticks 20 µm. Following visualization of the internal elastic lamina (IEL, grey scale) with Alexa 633 hydrazide, single image planes collected 2 µm apart at the EC and smooth muscle cell (SMC) side of the IEL (lower panels) showed the projections passed towards the SMCs (arrows). These projections are sites where myoendothelial gap junctions may form. Note that not all holes were associated with EC staining (asterisks) (P. Yarova, unpubl. data).

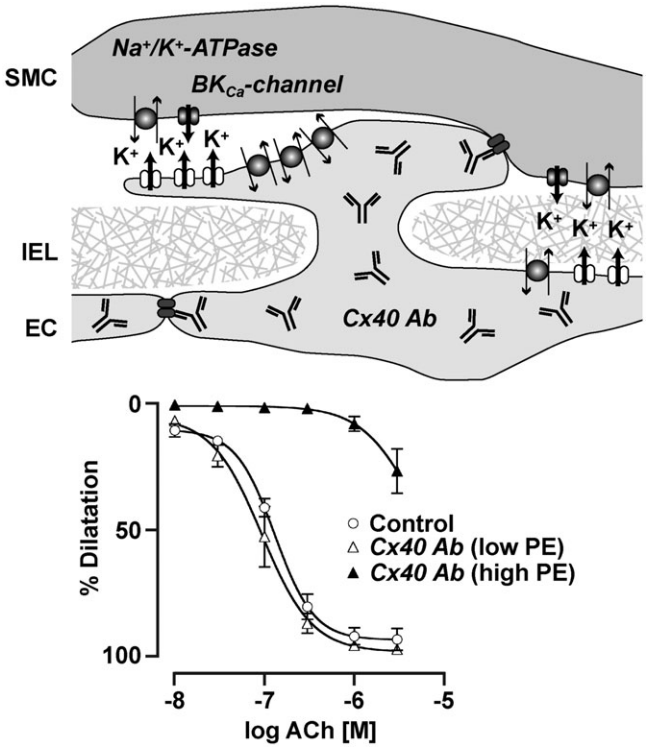

Figure 3

Antibodies targeted to connexin 40 (Cx40 Ab) can inhibit endothelium-dependent hyperpolarization-mediated dilatation responses. Cx40 Abs were loaded into endothelial cells (ECs) of pressurized rat mesenteric arteries and inhibited cell–cell coupling via Cx40 expressed at both EC–EC and myoendothelial gap junctions. An effect against ACh was only observed when the concentration of phenylephrine (PE) used to generate tone was high. This may be due to an ability of a true endothelium-dependent hyperpolarizing factor such as K^+ to independently act of electrical coupling via the smooth muscle cell (SMC) Na^+/K^+-ATPase. At high concentrations, PE activates BK_{Ca}-channels which can prevent further activation of the Na^+/K^+-ATPase, possibly due to a 'K^+ cloud'. Modified from Mather *et al.* (2005). IEL, internal elastic lamina.

more accurate and up-to-date descriptor for this route to vasodilatation is EDH rather than EDHF. Unraveling the functional contribution of gap junctions in EDH dilatation, and identifying if individual connexins are interchangeable, or if they are restricted in any contribution they may make, has not been at all easy. This is largely because the pharmacological tools available to uncouple gap junctions invariably have other effects. As already mentioned, Cx40 appears important in vascular gap junctions and was hypothesized to play a role in the MEGJs underlying EDHF-mediated responses. To probe a potential functional role, we developed a technique selectively to load the endothelium of pressurized resistance arteries intracellularly with antibodies (by pinocytosis). Using this approach, we found that anti-Cx40 antibody blocked EDHF dilatation but only in arteries undergoing a robust preconstriction to phenylephrine sufficient to block the parallel action of K^+ as an EDHF (Mather *et al.*, 2005). Under similar conditions, antibodies to the intracellular carboxy-terminal region of Cx40 (residues 340–358) or a mimetic peptide directed at the cytoplasmic loop (Cx40; residues 130–140) were each effective in this regard (Figure 3). However, antibodies against intracellular regions of Cx37 or Cx43, or mimetic peptide for the intracellular loop region of Cx37, were inactive. Simultaneous intra- and extraluminal incubation of arteries with inhibitory peptides targeted to extracellular regions of EC connexins (43Gap 26, 40Gap 27, and 37,43Gap 27) also failed to modify the EDHF response. The functional significance of Cx40 was strengthened by the fact

that it could actually be visualized (by immunohistochemistry) within the end of EC projections.

Overall, our data described a mechanism for EDH-mediated dilatation whereby hyperpolarization generated in the ECs diverged along two paths, one mediated by K^+ efflux and the other by hyperpolarization conducted to the muscle through MEGJs and critically involving Cx40. In arteries undergoing submaximal constriction, both pathways contribute to dilatation, but as constriction intensifies so does the 'density' of the extracellular K^+ cloud. The result is block of the ability of K^+ released by the endothelium to serve as an EDHF, by saturating its target effector. At this point, MEGJs alone sustain EDH vasodilatation. Therefore, together these two pathways explain the 'EDHF' response in the mesenteric artery.

Differential activation of SK_{Ca} and IK_{Ca} and the development of a concept of endothelial membrane microdomains

Interestingly, and perhaps very importantly, the individual contributions that EC SK_{Ca} and IK_{Ca} channels make to EDH can vary. Although both of these K^+-channels, are of course, central to EDH, in isolated mesenteric arteries simultaneous measurements of tension and membrane potential showed that SK_{Ca} channels alone were responsible for hyperpolarization (from circa –55 mV) (Figure 4). However, input from IK_{Ca} channels also became apparent once arteries were stimulated with phenylephrine, used to develop the (depolarization and) contraction necessary to evoke endothelium-dependent relaxation (Crane *et al.*, 2003). Selective, differential activation of K_{Ca} channels might be explained by the intracellular Ca^{2+} sensitivity of the two channel types and/or access to cytoplasmic Ca^{2+}, or perhaps reflect a separate and distinct subcellular localization in each case. Clearly, there is some division of signalling within ECs, because, for example, although both NO synthase and K_{Ca} channels are activated by Ca^{2+}, not all agents inducing endothelium-dependent relaxation activate both NO- and EDHF-mediated relaxation: these agents include abnormal cannabidiol and virodhamine which act at a receptor for cannabinoid-like agents in the endothelium (Ho and Hiley, 2003; 2004).

As far as EDH in the rat small mesenteric artery is concerned, it turns out to be the case that the differential activation correlates with the restriction of IK_{Ca} ($K_{Ca}3.1$) channels to the endothelial projections, the projections passing through holes in the IEL towards the smooth muscle. In marked contrast to $K_{Ca}3.1$, SK_{Ca} ($K_{Ca}2.3$) channels are distributed throughout the EC membrane, but are observed in particularly high concentration close to the relatively large EC–EC gap junctions (Sandow *et al.*, 2006; Dora *et al.*, 2008) (Figure 4). Also, in marked contrast to $K_{Ca}3.1$, the $K_{Ca}2.3$ channels are located in caveolae (Absi *et al.*, 2007). The concentration of $K_{Ca}3.1$ channels in the endothelial projections seems to be part of a complex signalling microdomain, as the projections and their immediate vicinity also contain high levels of both Na^+/K^+-ATPase and the Cx37 and Cx40 that are integral to the MEGJs (Mather *et al.*, 2005; Dora *et al.*, 2008). But the true level of complexity may well be far greater. For example, endothelial projections within cerebral arteries contain transient receptor potential (TRP) A1, a

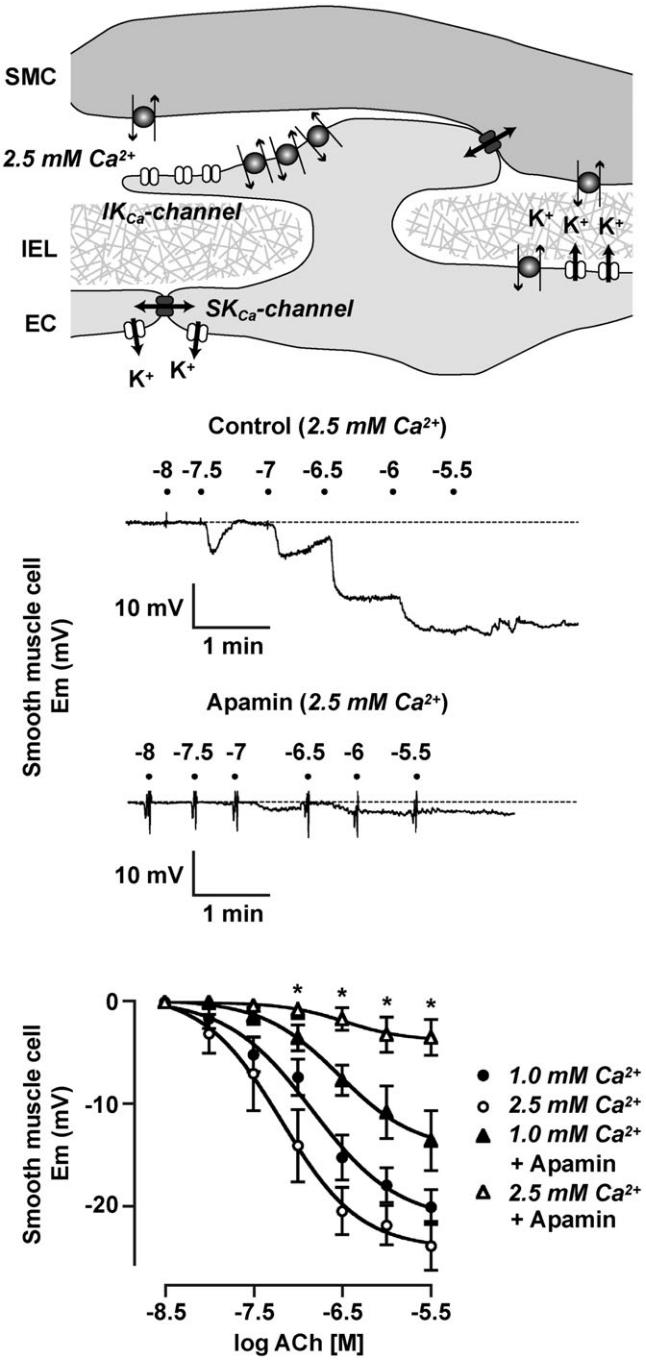

Figure 4

Role for SK_{Ca} channels in endothelium-dependent hyperpolarization responses depends on the extracellular Ca^{2+} concentration. In rat mesenteric arteries, SK_{Ca} ($K_{Ca}2.3$) channels are highly expressed at endothelial cell (EC) borders and to a limited extent, in the EC projections through the internal elastic lamina (IEL). In contrast, IK_{Ca} ($K_{Ca}3.1$) channels appear confined to the EC projection microdomain (top panel). The hyperpolarization to ACh when extracellular [Ca^{2+}] is held at 2.5 mM is fully blocked by apamin (middle panels), but not so when extracellular Ca^{2+} is reduced to 1 mM (bottom panel). Modified from Crane *et al.* (2003); Dora *et al.* (2008). SMC, smooth muscle cell.

Ca^{2+}-permeable, non-selective, cation channel which is activated to generate EDH by electrophilic compounds such as the irritants acrolein and mustard oil (Earley *et al.*, 2009). On the other hand, in mesenteric arteries, calcium-sensing receptors (CaSR) co-localize with $K_{Ca}3.1$ channels, outside the caveolae (Absi *et al.*, 2007; Saliez *et al.*, 2008).

Caveolae are flask-shaped indentations (or caves) covering the entire cell surface. They serve to anchor proteins, including both G protein-coupled and other receptors (such as $InsP_3$, receptors), with proteins such as $K_{Ca}2.3$, TRPC1 and TRPV4 channels (Lockwich *et al.*, 2000), thus forming highly organized, signalling microdomains (Rath *et al.*, 2009; Chidlow and Sessa, 2010). They seem crucial for EDH-mediated vasodilatation, as dilatation is absent in mice with the caveolin-1 gene deleted. The deficiency has been linked with TRPV4 activity, rather than to any direct effect on K_{Ca} channels, although the latter was not assessed (Saliez *et al.*, 2008). Interestingly, this particular gene knockout also reduced Cx40, Cx43 and Cx37 expression, suggesting that gap junction formation may be compromised, and thus the integration of electrical events within the blood vessel wall (see next).

Restriction of $K_{Ca}3.1$ channels to the relatively narrow EC projections might hinder the access of EC cytoplasmic Ca^{2+} necessary for channel activation, while perhaps facilitating a signal for activation derived from the smooth muscle. To investigate this possibility, we imaged $[Ca^{2+}]_i$ changes within the EC projections in mesenteric arteries mounted in a wire myograph, but we were unable to resolve any difference in $[Ca^{2+}]_i$ increases following EC stimulation with ACh and during simultaneous smooth muscle stimulation with phenylephrine (Dora *et al.*, 2008). The ECs display spontaneous Ca^{2+} events, and these have been termed 'pulsars'. Interestingly, they appear to align with EC projections, and $K_{Ca}3.1$ channels are basally activated by these spontaneous events, suppressing smooth muscle membrane potential by around 8 mV. As well as $K_{Ca}3.1$ channels, $InsP_3$-sensitive Ca^{2+} stores are also concentrated in the projections and ACh stimulates a circa 2.5-fold increase in pulsar frequency (Ledoux *et al.*, 2008).

Although there appeared to be no difference in the evoked $[Ca^{2+}]_i$ during stimulation with ACh in the absence or presence of phenylephrine, IK_{Ca}-mediated hyperpolarization could be detected alongside that due to SK_{Ca}, if extracellular $[Ca^{2+}]_o$ was reduced from 2.5 mM to 1 mM (Figure 4). Furthermore, the normal recruitment of IK_{Ca} channels during stimulation of the endothelium with ACh, while phenylephrine was stimulating the smooth muscle, was lost if Ca^{2+} entry into the muscle was reduced with voltage-dependent Ca^{2+} channel blockers (Dora *et al.*, 2008). So, the localized dynamics of $[Ca^{2+}]_o$ in the intercellular space, around EC projections, may be a fundamental physiological controlling mechanism in both the elaboration and transmission of EDH. This mechanism may well involve the CaSR shown by Arthur Weston and colleagues to be localized within the endothelium and, most importantly, outside caveolae and in close association with $K_{Ca}3.1$ channels (Weston *et al.*, 2005). These receptors respond to changes in $[Ca^{2+}]_o$ within the physiological range, and link to activate IK_{Ca} channels (Weston *et al.*, 2005) and cause vasorelaxation (Ohanian *et al.*, 2005). Finally, IK_{Ca} channels are also subject to control by cAMP, as forskolin suppressed hyperpolarization through these channels (Dora *et al.*, 2008).

Does EC K_{Ca} preferentially link to separate vasodilatation mechanisms?

The fact that EDH is generated by the activity of two types of K_{Ca} channels, and that in the rat small mesenteric artery, $K_{Ca}3.1$ channels are only found on the EC projections, raises the possibility that the transmission of EDH to the muscle via either K^+ or MEGJs may be linked to one type of K_{Ca} channel. This seems to be the case. Uncoupling gap junctions with carbenoxolone caused IK_{Ca} channels alone to underlie EDH. Furthermore, the ensuing vasorelaxation could be inhibited with ouabain, suggesting that IK_{Ca} channel activity is linked, presumably through changes in extracellular K^+ concentration, to the activation of smooth muscle Na^+/K^+-ATPase. On the other hand, SK_{Ca}-mediated hyperpolarization appeared *mainly* to affect relaxation through MEGJs and involve preferential activation of K_{IR} channels. Using a selective activator for SK_{Ca} channels, N-cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine, Weston *et al.* (2010) demonstrated that the hyperpolarization evoked by this agent was blocked with either apamin or Ba^{2+} and was therefore consistent with this suggestion.

Other endothelium-dependent pathways involving hyperpolarization

In the years following the discovery of 'EDHF', a number of candidate molecules have been proposed as diffusible EDHFs. A recent review (Edwards *et al.*, 2010) summarizes the key candidates and highlights a critical point: the majority of these agents do not show sensitivity to block with a combination of apamin and charybdotoxin (or TRAM-34) and are invariably sensitive to agents targeting smooth muscle K^+-channels. For example, epoxyeicosatetraenoic acids (EETs) (Campbell *et al.*, 1996) activate smooth muscle BK_{Ca} channels and NO acts principally via K_{ATP} channels (Tare *et al.*, 1990; Garland and McPherson, 1992). The role of EETs in EDHF vasorelaxation has, in particular, been the subject of quite considerable investigation and the reader is directed to an extensive and recent review of this literature by Campbell and Fleming (2010). The 2010 review by G. Edwards, M. Félétou and A. Weston proposes a helpful classification of hyperpolarizing pathways, or more usually EDH-like relaxation, defining them as either classical or non-classical. The former show sensitivity to block with apamin and TRAM-34, while being resistant to block with apamin and iberiotoxin. The non-classical group describes agents that are EDHFs, and play a role in evoking or facilitating smooth muscle hyperpolarization in/under specified circumstances (Edwards *et al.*, 2010). The non-classical group does not preclude signalling via EC projection microdomains and, for some of the more hydrophobic EDHFs, this may actually be crucial in terms of reducing diffusion distance.

Spreading the influence: conducted vasodilatation

So far we have focused on that hyperpolarization (or EDH) originating in the endothelium that passes radially into the artery wall to evoke vasodilatation. However, hyperpolariza-

tion can also move more generally through the artery wall, to affect a response first described by August Krogh (Krogh, 1920) as spreading or conducted vasodilatation. As well as the endothelium, hyperpolarization can of course be initiated directly in smooth muscle by mechanisms independent of either SK_{Ca} or IK_{Ca}. channels Whether originating in the smooth muscle or the endothelium, hyperpolarization can then spread axially to evoke vasodilatation. As such, it is able to translate focal dilatation into the more widespread drop in vascular resistance that is necessary to increase blood flow. For agents to achieve significant increases in tissue blood flow, any increase in artery diameter must significantly reduce vascular resistance. If dilatation only occurred focally, it would be unlikely to do this, because resistance is in part a function of the vessel length. However, if dilatation can also spread upstream, significant blood flow increases will follow (Kurjiaka and Segal, 1995; Dora *et al.*, 2000).

Conducted vasodilatation has been most extensively studied in the microcirculation, where there appears to be a correlation between agonists that open K^+-channels and an ability to reliably evoke spreading dilatation (Delashaw and Duling, 1991). Thus, the signal for spreading dilatation is thought simply to reflect the passage of current to adjacent cells, that is, cells not directly stimulated by the agonist (Dora *et al.*, 2000; Emerson and Segal, 2000). Passage can occur bi-directionally along the length of the vessel and is intrinsic to the wall of the artery. Furthermore, it relies on the endothelium and is not dependent on either nerves or a change in blood flow (Delashaw and Duling, 1991; Rivers, 1997; Dora *et al.*, 2000; Emerson and Segal, 2000; Segal, 2005; Winter and Dora, 2007). Studies in exteriorized microvascular beds *in situ* (Dora *et al.*, 2000), and arterioles isolated from those vascular beds, have used the endothelium-dependent agonist ACh to evoke robust responses following application to the outside of arterioles using micropipettes.

In skeletal muscle, spreading or ascending dilatation also occurs in larger arteries (Hilton, 1959) and we demonstrated recently that it can also be sustained in small resistance arteries, such as the rat mesenteric artery, which have three to five layers of muscle, suggesting that the phenomenon is of relevance across the vasculature (Takano *et al.*, 2004; Winter and Dora, 2007).

Importance of the endothelium in spreading dilatation

Orientation favours a role for ECs as the conduit for spread of hyperpolarization: they align along the principal axis of the vessel while the muscle cells are arranged circularly. This seems indeed to be the case, as activating the K_{ATP} channels, which are restricted to the SMCs in rat mesenteric arteries (White and Hiley, 2000; Takano *et al.*, 2004; Dora *et al.*, 2008) showed that the SMCs are not coupled sufficiently to enable dilatation to spread over long distances (Takano *et al.*, 2004) and, secondly, that the endothelium is the conduit for electrical coupling (Haas and Duling, 1997; Yamamoto *et al.*, 1999; Emerson and Segal, 2000; Winter and Dora, 2007). The way in which the hyperpolarization is elicited is not crucial in determining the subsequent spread, only in initiating cell–cell coupling, because activation of either endothelial or smooth muscle K^+-channels causes this response. However, some agonists may stimulate additional signalling pathways that may either augment (e.g. by cAMP; Popp *et al.*, 2002) or reduce (e.g. by α_1-adrenoceptor stimulation, protein kinase C; Haug *et al.*, 2003; Bao *et al.*, 2004) the ability of hyperpolarization to spread between the vascular cells.

Spreading dilatation following luminal perfusion of agonists

Circulating vasoactive agonists are crucially involved in the physiological control of regional blood flow, yet quite remarkably only very few studies attempt to apply agonists into the artery lumen when studying vascular reactivity. The consideration of extra- versus intra-luminal agonist application is of course crucial when probing the mechanism of action of agonists with opposing effects through the endothelium and on the SMCs, for example, purinergic (such as ATP) and adrenergic (such as adrenaline) agonists. We have addressed this issue by developing a novel approach with isolated and pressurized small arteries. By triple-cannulation of small arteries (the third cannula being inserted into a side branch), it is possible to infuse agonists luminally into a restricted downstream region. In this way, any spreading dilatation that is evoked can be quantified easily, as it spreads upstream against the direction of intraluminal fluid flow. Using this approach, luminal perfusion of either ATP or UTP was shown to stimulate both local and spreading dilatations (Winter and Dora, 2007). As all the evidence available is consistent with an essential role for the endothelium, in enabling such vascular responses to be coordinated effectively, any dysfunction in this monolayer, for example in conditions such as diabetes, obesity and hypertension, will markedly disrupt the ability of blood vessels to control local tissue perfusion.

What mechanisms sustain spreading dilatation: another signalling circuit?

As spreading dilatation appears to be completely reliant on membrane hyperpolarization, any agonist able to stimulate hyperpolarization will potentially evoke this response. Spread will rely on both homo- and hetero-cellular gap junctions to enable the necessary current spread. So a key question is whether current passing between cells of the arterial wall decays passively, or does an amplification mechanism exist to sustain dilatation. The available evidence in isolated, pressurized arteries supports a role for an undefined amplification process, as current appears to spread further than predicted by passive decay. This was elegantly demonstrated in cannulated hamster retractor small arteries (one to two layers of smooth muscle), where the decay of injected current was faster than the decay of hyperpolarization to ACh, despite the fact both induced a 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 central in this response is K_{IR} (Rivers *et al.*, 2001; Goto *et al.*, 2004; Jantzi *et al.*, 2006). However, a widespread role for this channel does not seem likely, as Ba^{2+} (which inhibits K_{IR} channels) had no effect on spreading dilatation to either ACh or levcromakalim in rat mesenteric arteries (Takano *et al.*, 2004). However, the Na^+/K^+-ATPase activated by modest increases in extracellular $[K^+]$ (Edwards

et al., 1998) may play some 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 role as an EDHF (Edwards *et al.*, 1998). This is supported by the expression of both K_{IR} and $K_{Ca}2.3$ channels at the borders of ECs (Dora *et al.*, 2008), at sites resembling that of inter- EC gap junctions (Kansui *et al.*, 2004; Winter and Dora, 2007). 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 serve to 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 might play a role (Budel *et al.*, 2003; Uhrenholt *et al.*, 2007) although inhibition of NO synthase does not appear to reduce spreading dilatation (Oishi *et al.*, 2001; Domeier and Segal, 2007; Winter and Dora, 2007). We have previously shown that EC $[Ca^{2+}]_o$ does not detectably increase in response to local (McSherry *et al.*, 2005; 2006) or spreading (Takano *et al.*, 2004) hyperpolarization in either rat mesenteric or cremasteric arteries. However, recent evidence shows that the presence of tone can unmask a detectable, slow, conducted intercellular Ca^{2+} wave, both *in vitro* (Uhrenholt *et al.*, 2007) and *in vivo* (Tallini *et al.*, 2007), although it is important to note that this wave is not a requirement for the rapid spread of hyperpolarization and associated dilatation (Takano *et al.*, 2004; Tallini *et al.*, 2007).

EDHF in disease

It is clear that EDH is a prominent regulatory mechanism in the vasculature and, as EC dysfunction appears to be an early feature in cardiovascular disease (Vanhoutte *et al.*, 2009), the question arises as to the extent of involvement of hyperpolarizing responses. Although the role of NO signalling has been extensively studied in disease, fewer data are available with regard to the effects of cardiovascular disease or vascular injury on EDH. However, modifying the expression levels of EC SK_{Ca} channels in a transgenic model (SK3T/T) clearly demonstrated that decreasing or increasing these endothelial cell channels caused commensurate changes in blood pressure (Taylor *et al.*, 2003). These observations were extended by studies involving targeted deletion of $K_{Ca}3.1$ channels, and also $K_{Ca}3.1$/SK3 channel double knock out animals, to show significant rises in mean arterial blood pressure (Si *et al.*, 2006; Brahler *et al.*, 2009). Thus, openers of these channels may represent a novel therapeutic approach to hypertension (Grgic *et al.*, 2009). Most recently, electrophysiological studies have revealed that hyperpolarization through caveolae-based SK_{Ca} channel activation was compromised in mesenteric arteries from 12 to 16-week-old animals spontaneously hypertensive rats, while IK_{Ca} channel pathways were unaltered (Weston *et al.*, 2010). However, although the hyperpolarization linked to IK_{Ca} channel activation was not reduced at this age, it appeared that the channel protein level was decreasing. So perhaps, in the longer term, hyperpolarization through this EC channel is also disrupted. Clearly, much more research is needed to define the extent and means by which EDH is altered by cardiovascular disease, and in a range of disease models.

Conclusion

Since the discovery of EDHF in the 1980s, clear and sustained progress has been made in defining the fundamental mechanisms responsible for generating EC hyperpolarization, or EDH. Our understanding of how EDH then transfers radially into the adjacent smooth muscle, and axially along arteries to evoke distant vasodilatation, has similarly increased. These responses together serve to integrate changes in tissue blood flow. Perhaps the most important key steps along the way have been to recognize that hyperpolarization arises from activation of SK_{Ca} and IK_{Ca} channels, and that both channel types reside in the endothelium, but within different microdomains. Future challenges will be to unravel the complexity and local regulation of these membrane microdomains, and to show how they may be disrupted by disease and possibly modulated to improve or sustain physiological function.

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Conflict of interest

The authors have no conflicts of interest.

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