EDHF: bringing the concepts together

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Endothelial cells synthesize and release vasoactive mediators in response to various neurohumoural substances (e.g. bradykinin or acetylcholine) and physical stimuli (e.g. cyclic stretch or fluid shear stress). The best-characterized endothelium-derived relaxing factors are nitric oxide and prostacyclin. However, an additional relaxant pathway associated with smooth muscle hyperpolarization also exists. This hyperpolarization was originally attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF) that diffuses to and activates smooth muscle K^+ channels. More recent evidence suggests that endothelial cell receptor activation by these neurohumoural substances opens endothelial cell K^+ channels. Several mechanisms have been proposed to link this pivotal step to the subsequent smooth muscle hyperpolarization. The main concepts are considered in detail in this review.

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In various blood vessels, endothelium-dependent relaxations can be accompanied by the endothelium-dependent hyperpolarization of smooth muscle cells [1–5]. These endothelium-dependent relaxations and hyperpolarizations can be partially or totally resistant to inhibitors of cyclooxygenases and nitric oxide (NO) synthases [6,7] and can occur without an increase in intracellular levels of cyclic nucleotides in the smooth muscle cells [3,8,9]. Therefore, the existence of an additional pathway that involved smooth muscle hyperpolarization was suggested and attributed to a non-characterized endothelial factor called endothelium-derived hyperpolarizing factor (EDHF) [10].

In general, hyperpolarization of smooth muscle produces relaxation by reducing both the open probability of voltage-dependent Ca^{2+} channels and the turnover of intracellular phosphatidylinositides, thus decreasing the intracellular Ca^{2+} concentration {$[Ca^{2+}]_i$} [11]. The contribution of EDHF-mediated responses as a mechanism for endothelium-dependent relaxation increases as the vessel size decreases [12], apart from the coronary and renal vascular beds in which EDHF plays a major role even in conduit arteries [13,14]. In mice, EDHF-mediated responses in resistance vessels are at least as important as endothelium-derived NO in mediating agonist-induced, endothelium-dependent vasodilatation because neither deletion of the gene encoding endothelial NO synthase nor inhibition of NO synthase attenuates agonist-induced vasodilator responses *in vivo* and *in vitro* [15,16].

Pivotal role of Ca^{2+} and endothelial Ca^{2+}-activated K^+ channels in the EDHF pathway

EDHF-mediated responses, in response to agonists that stimulate G-protein-coupled receptors, are associated with an increase in $[Ca^{2+}]_i$ in the endothelial cell (Fig. 1) [17,18] and are also generated by substances that increase endothelial $[Ca^{2+}]_i$ in a receptor-independent manner (e.g. Ca^{2+} ionophores, and the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid) [19,20]. Conversely, a decrease in the extracellular Ca^{2+} concentration attenuates EDHF responses [21]. This implies that for EDHF-mediated responses, as for many other endothelial functions, the increase in endothelial $[Ca^{2+}]_i$ is a crucial step [22].

Initially, several lines of evidence suggested that the endothelium-dependent hyperpolarization of smooth muscle cells resulted from the opening of K^+ channels in the smooth muscle plasmalemma. First, the hyperpolarization was abolished by K^+ concentrations higher than 25 mM [23]. Second, agonists that produced endothelium-dependent hyperpolarizations also stimulated the efflux of ^{42}K (or ^{86}Rb) from pre-loaded arteries [3,4]. Third, a decrease in membrane resistance of the vascular smooth muscle cells was observed during endothelium-dependent hyperpolarizations [23], suggesting that the hyperpolarization was due to the opening of a K^+ channel rather than the closing of, for example, a Cl^- or a nonspecific cationic channel. Collectively, these observations suggested that the endothelium-dependent hyperpolarization of the smooth muscle cells involved an increase in K^+ conductance and that EDHF was an endothelium-derived K^+ channel opener. However, this interpretation has to be modified in light of more recent experimental observations.

Identification of the K^+ channels involved in the EDHF pathway

Endothelium-dependent hyperpolarizations (in the presence of inhibitors of NO synthases and cyclooxygenases) are not prevented by glibenclamide [an inhibitor of ATP-dependent K^+ (K_{ATP}) channels] but are blocked by specific toxins that inhibit Ca^{2+}-sensitive (K_{Ca}) K^+ channels. Indeed, a hallmark of the EDHF-mediated response, first observed by Garland's group, is its abolition by the combination of apamin [a specific inhibitor of K_{Ca} channels of small conductance (SK_{Ca} channels)] plus charybdotoxin [a nonselective inhibitor of large-conductance (BK_{Ca}) and intermediate-conductance (IK_{Ca}) channels, and of some voltage-dependent K^+-channels] [24–26], although either toxin can be effective alone in some circumstances [27,28].

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The effect of apamin can be mimicked by scyllatoxin, a structurally distinct SK_{Ca} channel inhibitor, indicating that SK_{Ca} channels are involved in endothelium-dependent hyperpolarizations [26]. However, although iberiotoxin (a specific inhibitor of BK_{Ca} channels) can abolish NO- and prostacyclin-independent vasodilatation in some vascular beds *in vivo* [29,30], in arteries in which the combination of charybdotoxin and apamin is essential to inhibit responses, iberiotoxin cannot substitute for charybdotoxin [25,31], which excludes a pivotal role for BK_{Ca} channels in many EDHF-mediated responses.

Which cell type expresses the K_{Ca} channels involved in the EDHF response?

There are no reports of smooth muscle K_{Ca} channels that are sensitive to charybdotoxin but not to iberiotoxin, which suggests that the EDHF-activated IK_{Ca} channel is not present on the smooth muscle cells. However, both IK_{Ca} and SK_{Ca} channels are expressed in endothelial cells [32–34] and endothelial K^+ channels are activated by stimuli that produce an increase in $[Ca^{2+}]_i$ [35]. The hyperpolarization of the endothelial cells elicited by acetylcholine or bradykinin is blocked by the combination of apamin and charybdotoxin [36,37]. In addition, these two toxins prevent EDHF-mediated smooth muscle relaxation when selectively applied to the endothelium [38]. Finally, 1-ethyl-2-benzimidazolinone (1-EBIO), an activator of IK_{Ca} channels (and possibly SK_{Ca}, but not BK_{Ca}, channels) induces endothelial cell hyperpolarization and the endothelium-dependent hyperpolarization of smooth muscle cells, responses that are sensitive to charybdotoxin but insensitive to iberiotoxin [39,40]. Collectively, this evidence suggests that the toxin combination targets two types of K_{Ca} channels (IK_{Ca} and SK_{Ca} channels) expressed on endothelial cells (i.e. prevents endothelial cell hyperpolarization) rather than K^+ channels activated by an EDHF and located on smooth muscle cells.

Beyond endothelial hyperpolarization

Why have so many vastly different hypotheses been proposed concerning the mechanism of EDHF-mediated responses? Increasing the $[Ca^{2+}]_i$ in endothelial cells opens not only SK_{Ca} and IK_{Ca} channels, which results in the efflux and accumulation of K^+ in the myo-endothelial space, but also leads to the activation of various enzymes including phospholipases and the subsequent metabolism of arachidonic acid by cytochrome P450 epoxygenases. Currently, experimental evidence favours three explanations for the EDHF phenomenon: (1) the increase in endothelial $[Ca^{2+}]_i$ triggers the synthesis of a cytochrome P450 metabolite that is essential for the subsequent EDHF-mediated responses; (2) endothelial cell hyperpolarization is transmitted to the vascular smooth muscle via gap junctions; and (3) K^+, released from the endothelial cells via K_{Ca} channels, induces smooth muscle hyperpolarization by activating K^+ channels and/or Na^+–K^+-ATPase on vascular smooth muscle cells.

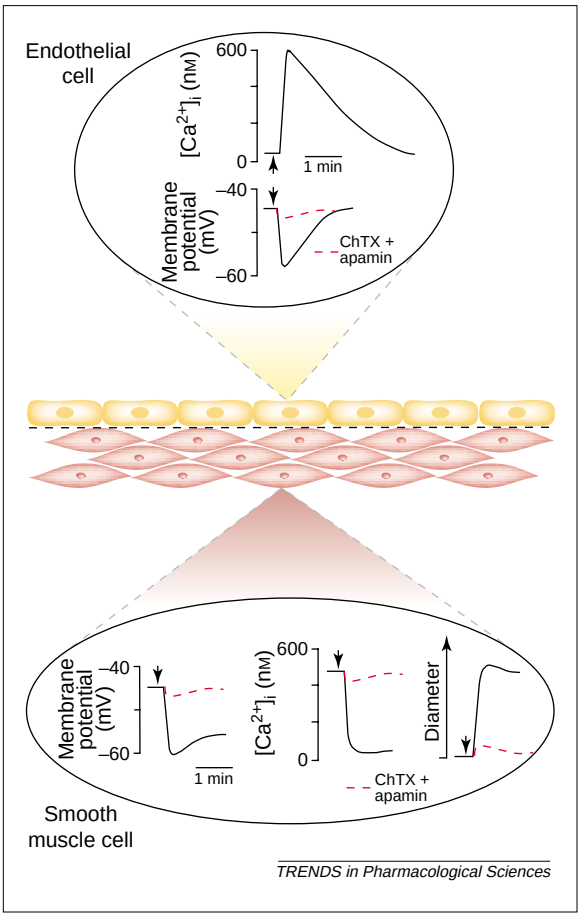

Fig. 1. The endothelium-derived hyperpolarizing factor (EDHF) phenomenon: correlation between the intracellular concentration of Ca^{2+} {$[Ca^{2+}]_i$} and membrane hyperpolarization in endothelial cells and vascular smooth muscle cells. Following stimulation of endothelial cells with an agonist such as acetylcholine or bradykinin (arrow), there is a transient increase in the $[Ca^{2+}]_i$, which leads to the activation of Ca^{2+}-dependent K^+ channels and membrane hyperpolarization. The term 'EDHF-mediated responses' reflects the mechanism by which this endothelial cell hyperpolarization is transferred to vascular smooth muscle cells. In the smooth muscle the consequence of membrane hyperpolarization is a decrease in $[Ca^{2+}]_i$ and vessel dilatation (increase in diameter). Preventing endothelial cell hyperpolarization by including the combination of charybdotoxin (ChTX) [a nonselective inhibitor of large-conductance (BK_{Ca}) and intermediate-conductance (IK_{Ca}) channels, and of some voltage-dependent K^+ channels] and apamin [a specific inhibitor of K_{Ca} channels of small conductance (SK_{Ca})] blocks the subsequent alterations in smooth muscle membrane potential, $[Ca^{2+}]_i$ and tone (in the presence of inhibitors of cyclooxygenase and nitric oxide synthase).

Other explanations for the EDHF phenomenon have been reviewed elsewhere [10].

A role for epoxyeicosatrienoic acids?

Epoxyeicosatrienoic acids (EETs) are arachidonic-acid-derived products of cytochrome P450 epoxygenases that appear to play an important role in the regulation of vascular homeostasis [41]. Most of the evidence in favour of EDHF being a short-lived metabolite of arachidonic acid, produced

via the cytochrome P450 epoxygenase pathway, has been obtained using bovine [42,43], porcine [42,44], canine [45] and human coronary arteries [28,46].

In these blood vessels, EDHF-mediated responses are blocked by inhibitors of cytochrome P450. Although classical cytochrome P450 inhibitors were nonspecific, new selective and structurally different compounds do inhibit EDHF-mediated responses in some arteries [47]. Moreover, EETs relax coronary arteries and hyperpolarize smooth muscle cells by increasing the open-state probability of K_{Ca} channels that are sensitive to tetraethylammonium, charybdotoxin or iberiotoxin [43,48–50]. In porcine coronary arteries, endothelial cells express several cytochrome P450 epoxygenases and in this artery as well as in hamster resistance arteries, endothelium-dependent hyperpolarization and relaxation can be inhibited by antisense oligonucleotides against cytochrome P450 2C [50,51]. Furthermore, EDHF-mediated responses are increased by agents that enhance the endothelial expression of cytochrome P450 [44,50]. Taken together, such data strongly suggest that the activation of a cytochrome P450 epoxygenase is a prerequisite for the generation of EDHF-mediated relaxation in the porcine coronary artery.

However, although EETs are generated by endothelial cells both under resting conditions and in response to cell stimulation with agonists, there is little direct evidence demonstrating that these cytochrome P450 metabolites can be released from endothelial cells in amounts sufficient to elicit smooth muscle cell hyperpolarization and relaxation. Whether or not this is due to current technical limitations in measuring the concentration of these metabolites remains to be determined. Supramaximal concentrations of receptor-dependent or -independent agonists, and haemodynamic stimuli such as pulsatile stretch are however able to stimulate the release of EETs from isolated bovine and porcine coronary arteries in addition to from cultured endothelial cells [44,50,52,53]. Under such circumstances, EETs are endothelium-derived hyperpolarizing factors that elicit smooth muscle relaxation by opening BK_{Ca} channels. However, the fact that exogenously applied EETs activate BK_{Ca} channels, rather than IK_{Ca} and SK_{Ca} channels (the channels activated in EDHF responses), suggests that a diffusible EET is unlikely to account for the majority of EDHF-mediated responses.

One possibility that might reconcile many of the experimental observations is that hyperpolarization of endothelial cells might be partly regulated by activation of the cytochrome P450. For example, EETs might modulate endothelial Ca^{2+} influx in response to Ca^{2+}-store depletion [54] and might facilitate the activation of endothelial K^+ channels by increasing their sensitivity to Ca^{2+} [55,56]. Additionally, EETs also exert a biphasic change in gap junctional communication between endothelial cells [57] and their effect on myo-endothelial gap junctions is currently under investigation. Therefore, in coronary arteries, EETs and other products of cytochrome P450 could be reclassified as intracellular second messengers crucial to the initiation and transmission of endothelial cell hyperpolarization, and, as a consequence, to EDHF-mediated hyperpolarization and relaxation of vascular smooth muscle cells.

A role for gap junctions?

Gap junctions not only couple endothelial cells to endothelial cells, and smooth muscle cells to smooth muscle cells, but also couple smooth muscle cells to endothelial cells, thus providing a low-resistance electrical pathway between these two cell layers [58]. One study has suggested that the number of these heterocellular myo-endothelial gap junctions increases with the diminution in the size of the artery [59], a finding that would parallel the changing importance of the contribution of EDHF to the control of vascular tone [12]. Substances that produce endothelium-dependent hyperpolarization of vascular smooth muscle cells also hyperpolarize endothelial cells with the same time-course [60]. In various microcirculatory beds, the link between the EDHF phenomenon and the spread of an electric current through myo-endothelial gap junction has been established. For example, in submucosal arterioles of the guinea-pig small intestine, acetylcholine induces outward currents in endothelial cells and smooth muscle cells that are blocked by the combination of charybdotoxin plus apamin. After the administration of blockers of gap junctions, acetylcholine elicits an outward current in endothelial cells but no longer in the smooth muscle cells, which suggests that the two cell types are connected electrically and form a functional syncytium [61–63]. In arterioles and feed arteries from the retractor muscle of the hamster, simultaneous measurements of the membrane potential in endothelial cells and smooth muscle cells show that electrical signals travel freely and bidirectionally between the two layers. These electrical signals are associated with vasomotor responses, and hyperpolarization originating in a single endothelial cell can drive the relaxation of smooth muscle cells throughout an entire arteriolar segment [64,65]. Cell activation by an agonist facilitates myo-endothelial gap junctional communication because a more pronounced myo-endothelial coupling is observed in acetylcholine-stimulated arteries than in arteries in which either smooth muscle or endothelial cells were stimulated by injection of a negative current [64].

In larger blood vessels, the suggestion that EDHF-mediated responses require functional myo-endothelial gap junctional communication is more controversial. The specificity of gap junction inhibitors (carbenoxolone, glycyrrhetinic acid, halothane and heptanol) is often questionable. By contrast, peptides with sequences similar to a portion of the extracellular

loop of the connexon proteins [the so-called gap peptides (e.g. GAP27)] can inhibit gap junctions [66,67]. Although such peptides are assumed to inhibit myo-endothelial gap junctions, this has merely been inferred from their ability to reduce dye coupling in cultured cells that express only connexin 43 and 40 [67]. However, electrical coupling between endothelial cells and smooth muscle cells can be observed in the absence of dye coupling [63]. Furthermore, the assumption that gap junction inhibitors are acting at the level of myo-endothelial junctions could be mistaken. For example, in the porcine coronary artery, endothelium-dependent hyperpolarizations to substance P can be recorded in the smooth muscle cells situated close to the intimal layer or close to the adventitia. GAP27 inhibits the hyperpolarization in the outer layer while the response remains unaffected in the intimal layer, suggesting that smooth muscle rather than myo-endothelial gap junctions were targeted by the peptide [37]. Clearly, there is need for further investigation of the function of gap junctions and for a better understanding of the mechanism of action of these gap junction inhibitors.

Are gap junctions a privileged site for the transfer of electrical charges or do they transfer a messenger molecule (e.g. EDHF)? In some microcirculatory beds, such as the retractor muscle of the hamster, an electrotonic current spread from endothelial cells to smooth muscle cells seems to be the most likely explanation of the EDHF phenomenon [65]. The possibility that gap junctions facilitate the diffusion of an 'hyperpolarizing factor', such as cAMP [67], from the endothelium to the smooth muscle raises other questions. First, if cAMP were transferred from endothelial cells to smooth muscle cells the overall response would be similar to that elicited by prostacyclin and should be sensitive to an inhibitor of K_{ATP} channels. Second, any 'factor' transferred via gap junctions would have to be hydrophilic (to allow its passage through the aqueous pore) and should also be actively regenerated because a significant dilution of the factor would occur from the site of its production during its rapid distribution throughout the smooth muscle layers of the vascular wall. Therefore, should cAMP play a role in the EDHF response, it is more likely that it is involved in the dynamic regulation of gap junctional communication than acting as an EDHF *per se* [57].

A role for K^+ ions?

One consequence of the opening of the endothelial cell K_{Ca} channel is the accumulation of K^+ ions in the intercellular space between endothelial cells and smooth muscle cells. A moderate increase in the myo-endothelial K^+ concentration, such as that which occurs on stimulation of endothelial cells in rat hepatic artery [36], can induce the hyperpolarization of vascular smooth muscle cells by activating the inwardly rectifying K^+ (K_{IR}) channels and the Na^+–K^+-ATPase [68,69]. In the rat hepatic and mesenteric arteries, the endothelium-dependent smooth muscle hyperpolarization is partially inhibited by ouabain (an inhibitor of Na^+–K^+-ATPase) and barium (at a concentration that selectively inhibits K_{IR} channels), and is abolished by the application of both inhibitors together [36]. The maintenance of the endothelial cell hyperpolarization despite the abolition of smooth muscle hyperpolarization in the presence of barium plus ouabain indicates that these inhibitors act on the smooth muscle [36]. The ouabain sensitivity of the EDHF response suggests that the type of Na^+–K^+-ATPase involved is likely to contain a type 2 or 3 α-subunit [39]. At this point, it is important to note that submicromolar concentrations of ouabain selectively target the Na^+–K^+-ATPase and inhibit EDHF-mediated responses without affecting the resting membrane potential or gap junctional communication [70,71].

Nevertheless, the proposal that EDHF might simply be endothelium-derived K^+ has been refuted [72–75] and indeed K^+-induced relaxations and hyperpolarizations are not observed in some blood vessels that exhibit EDHF-mediated responses [76]. However, in many vessels, these apparent discrepancies can be explained. The hypothesis of K^+ as an EDHF has been established on the basis of electrophysiological experiments, whereas the criticisms were based on experiments measuring vascular tone. In comparison to electrophysiological studies, tension recordings are more difficult to interpret because of the obligatory presence of a contractile agonist to induce tone. The elevation of smooth muscle $[Ca^{2+}]_i$ might result in the diffusion of Ca^{2+} through myo-endothelial gap junctions and thus influence endothelial cell Ca^{2+}-dependent processes [77]. Experiments in which tension and membrane potential were simultaneously measured clearly demonstrated that the ability of K^+ to cause hyperpolarization, and thus act as an EDHF, decreases as the level of agonist-dependent stimulation increases [78]. The depolarization produced by the agonist increases the $[Ca^{2+}]_i$ that stimulates K^+ efflux from smooth muscle. This efflux might form a 'K^+ cloud' that surrounds the myocytes and prevents any effect of additionally added K^+ or any component of the EDHF response that could have resulted from a direct effect of K^+ on the smooth muscle [79]. Under those circumstances, the contribution of gap junctions to the EDHF phenomenon dominates. Almost certainly, the obligatory use of contractile agonists in the studies involving the measurement of tension in rat arteries could account for observations that gap junctions have essentially no role (electrophysiological measurements) [76] as opposed to a major role (tension study) [67] in EDHF-mediated responses and that K^+ is (electrophysiological investigations) [36,76] or is not (tension experiments) [72–75] an EDHF.

In vivo most arteries are contracted to some extent depending on the level of sympathetic activation or

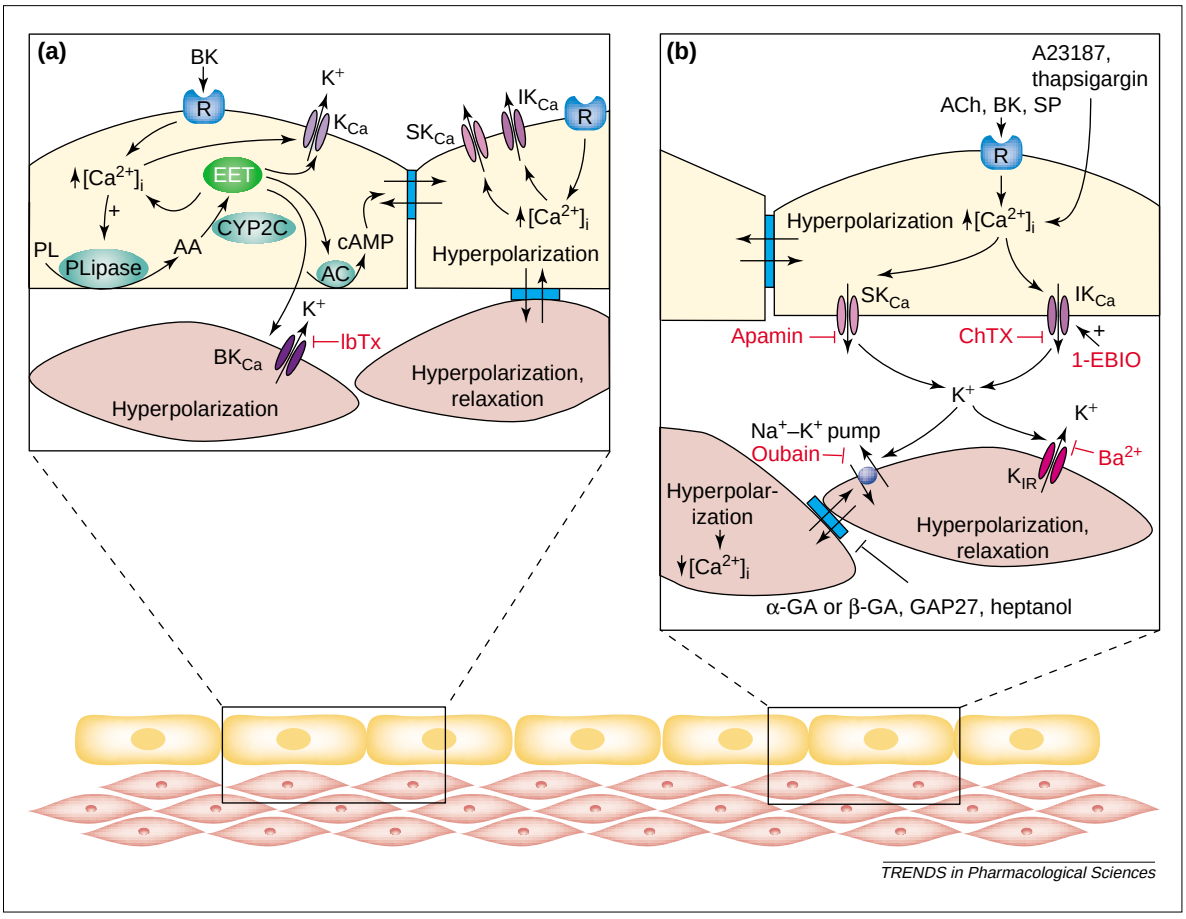

Fig. 2. Bringing the concepts together. (a) Epoxyeicosatrienoic acids (EETs) might act as both intracellular and extracellular messengers. Following endothelial cell stimulation with bradykinin (BK), the intracellular concentration of Ca^{2+} {$[Ca^{2+}]_i$} increases and leads to the activation of a phospholipase (PLipase), which liberates arachidonic acid (AA) from membrane phospholipids (PLs). The subsequent activation of a cytochrome P450 epoxygenase (CYP2C) results in the generation of EETs, which in turn affect Ca^{2+} signalling, the Ca^{2+} sensitivity of Ca^{2+}-dependent K^+ (K_{Ca}) channels and the generation of cAMP by adenylyl cyclase (AC), as well as gap junctional coupling in the endothelial cells. EETs and/or their metabolites can also diffuse to smooth muscle cells and activate large-conductance K_{Ca} channels (BK_{Ca} channels), which are sensitive to iberiotoxin (IbTX). (b) The role of K^+ ions. Endothelial cell stimulation by receptor-dependent [e.g. acetylcholine (ACh), BK and substance P (SP)] and receptor-independent Ca^{2+}-elevating agonists (e.g. the Ca^{2+} ionophore A23187 and the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin) initiates endothelial cell hyperpolarization by activating small- and intermediate-conductance K_{Ca} channels [SK_{Ca} and IK_{Ca} channels, sensitive to apamin and charybdotoxin (ChTX), respectively]. Endothelial cell hyperpolarization leads to the accumulation of K^+ ions in the sub-endothelial space in concentrations sufficient to activate inwardly rectifying K^+ (K_{IR}) channels [which are blocked by barium (Ba^{2+})] and/or the Na^+–K^+ pump (blocked by low concentrations of ouabain). 1-Ethyl-2-benzimidazolinone (1-EBIO) is an activator of IK_{Ca} channels whereas α- and β-glycyrrhetinic acid (α- and β-GA), GAP27 and heptanol block gap junctional communication. (a and b) The role of gap junctions. The hyperpolarization of the endothelial cells, following K_{Ca} channel activation, can be transmitted along the monolayer of endothelial cells or towards the smooth muscle cells through gap junctions.

of circulating hormones. However, in healthy men infusion of barium, ouabain or their combination reduce brachial artery blood flow and K^+ infusion produces vasodilatation sensitive to these inhibitors [80], suggesting that K^+ as an EDHF could play a physiological role.

Conclusion: bringing the concepts together

There is now good evidence that EDHF-mediated responses are initiated by an increase in the endothelial $[Ca^{2+}]_i$ and the consequent activation of endothelial SK_{Ca} and IK_{Ca} channels, which elicits the hyperpolarization of the endothelial cells (Fig. 2). In some tissues, the hyperpolarization of the endothelial cells might be regulated by the activation of cytochrome P450 and the resulting generation of EETs. The endothelial hyperpolarization could then spread to the adjacent smooth muscle cells through myo-endothelial gap junctions (which might also be modulated by EETs) or the efflux of K^+ through the endothelial SK_{Ca} and IK_{Ca} channels could elicit the hyperpolarization of the surrounding myocytes by activating K_{IR} channels and/or the Na^+–K^+-ATPase. These mechanisms are not necessarily mutually exclusive and, in a given blood vessel, they could occur simultaneously or sequentially and might act synergistically. The relative proportion of each mechanism almost certainly depends on numerous parameters including the state of activation of the vascular smooth muscle, the density of myo-endothelial gap junctions and the level of the expression of cytochrome P450 and the appropriate isoforms of Na^+–K^+-ATPase and/or K_{IR} channels.

Note added in proof

Recently, a specific antagonist of EETs was synthesized and was shown to further substantiate, in bovine coronary artery, the involvement of cytochrome P450 epoxygenase metabolites in EDHF-mediated responses [81]. However, it is not yet known whether this antagonist targets the endothelial cells or the smooth muscle cells.

A transient increase in the level of cAMP might play a permissive role in the EDHF-mediated responses by enhancing the electrotonic spread of endothelial hyperpolarization through the vessel wall [82]. This study further suggests that cAMP might act as a modulating factor at the level of myo-endothelial gap junctions and gap junctions coupling smooth muscle cells.

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