EDHF is not K⁺ but may be due to spread of current from the endothelium in guinea pig arterioles

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Coleman, H. A., Marianne Tare, and Helena C. Parkington. EDHF is not K⁺ but may be due to spread of current from the endothelium in guinea pig arterioles. Am J Physiol Heart Circ Physiol 280: H2478–H2483, 2001.—Endothelium-derived hyperpolarizing factor (EDHF)-attributed hyperpolarizations and relaxations were recorded simultaneously from submucosal arterioles of guinea pigs with the use of intracellular microelectrodes and a video-based system, respectively. Membrane currents were recorded from electrically short segments of arterioles under single-electrode voltage clamp. Substance P evoked an outward current with a current-voltage relationship that was well described by the Goldman-Hodgkin-Katz equation for a K⁺ current, consistent with the involvement of intermediate- and small-conductance Ca²⁺-activated K⁺ channels. 1-Ethyl-2-benzimidazolinone relaxed the arterioles and evoked hyperpolarizations that were blocked by charybdotoxin, but not by iberiotoxin. Application of K⁺-induced depolarization under conditions in which EDHF evoked hyperpolarization. The Ba²⁺-sensitive component of the K⁺-induced current was inwardly rectifying, in contrast to the outwardly rectifying current evoked by substance P. EDHF-attributed hyperpolarizations in dye-identified smooth muscle cells were indistinguishable from those recorded from dye-identified endothelial cells in the same arterioles. These results provide evidence that EDHF is not K⁺ but may involve electrotonic spread of hyperpolarization from the endothelial cells to the smooth muscle cells.

calcium-activated potassium current; endothelium; gap junction; voltage clamp; 1-ethyl-2-benzimidazolinone

DESPITE VARIOUS ATTEMPTS to identify the processes underlying the phenomenon of endothelium-derived hyperpolarizing factor (EDHF), the nature of EDHF remains enigmatic. The main contenders that are the focus of many studies include a product of the cytochrome P-450 pathway (2, 6–8, 14, 19, 21, 23, 32, 34, 39), release of K⁺ from the endothelial cells (6, 14, 16, 26, 33), and electrotonic spread of hyperpolarization generated in endothelial cells to the underlying smooth muscle (4, 5, 9, 20, 22, 27, 42, 43).

The ultimate identification of EDHF as a chemical entity or some other process requires a detailed knowledge of its mechanisms of action, including the ionic mechanisms underlying the hyperpolarization. To this end, we recently used the single-electrode voltage-clamp technique to describe the ionic currents that underlie the EDHF-attributed hyperpolarization evoked by ACh in submucosal arterioles of the guinea pig (10). The current had characteristics consistent with the involvement of Ca²⁺-activated K⁺ (IKCa) channels of intermediate and small conductance (IKCa and SKCa channels, respectively) (10). The presence of IKCa channels in blood vessels has been suggested by the effects of 1-ethyl-2-benzimidazolinone (1-EBIO), an activator of IKCa channels, which has been shown to relax (1, 38) and hyperpolarize (17, 18) some vessels. In the present study, we tested the effect of 1-EBIO on membrane potential in dye-identified endothelial and smooth muscle cells to gain further evidence of IKCa channels in these arterioles and to provide information as to the site of generation of EDHF.

The likelihood of electrical coupling between the endothelial and smooth muscle cells of at least some blood vessels (4, 9, 20, 27, 28, 35, 41), including submucosal arterioles (10, 11), invites additional study, since it could explain EDHF. Definitive evidence of this mechanism could be provided by agents that disrupt electrical conductance of gap junctions. Unfortunately, the putative gap junction inhibitors based on glycyrrhetinic acid and its derivatives do not appear to uncouple the cells electrically to any great extent, but they possess a range of nonspecific effects that severely limit their usefulness in the assessment of the role of gap junctions in mediating the EDHF-attributed hyperpolarization (10, 37). In the present study, we provide evidence that the EDHF-attributed hyperpolarization is not due to K⁺, but recordings made from dye-identified smooth muscle and endothelial cells in the same segments of arteriole are consistent with electrotonic spread of current from the endothelial cells to the smooth muscle cells in arterioles.

METHODS

Guinea pigs were killed by cervical dislocation and exanguination with approval of the Monash University Animal Ethics Committees. A section of the small intestine was removed from each animal, the muscle and mucosal layers were peeled away, and the thin sheet of connective tissue containing the submucosal arterioles was pinned to the floor of the recording chamber (10). The tissue was continuously

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superfused at 3 ml/min and 35°C with physiological saline solution consisting of (in mM) 120 NaCl, 5 KCl, 25 NaHCO3, 1 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, and 11 glucose and bubbled with 95% O2-5% CO2. 

Arterioles (20–50 μm OD) were studied by recording membrane potential with intracellular microelectrodes simultaneously with contractile activity recorded as diameter by computer-based analysis of video images (Diamtrak) (30). The cells from which the recordings were made were identified by loading the cells with the fluorescent dye Lucifer yellow (2%), which was included in the tips of the microelectrodes (10). In some experiments, the arterioles were cut into electrically short segments, and membrane currents were recorded with single intracellular microelectrodes under voltage-clamp mode using a switching amplifier (AxoClamp-2, Axon Instruments) (10). Membrane currents were normalized to the input capacitance of the arteriole segment. Periodic voltage ramp commands, generated with pClamp 6 software (Axon Instruments), were used to determine current-voltage relationships. Responses to voltage ramps recorded before drug application were subtracted from the ramp responses recorded during drug application to determine the current-voltage relationships of the current activated or inhibited by the drug.

ACh, substance P, l-NAME, indomethacin, ouabain,iberiotoxin (IbTx), and dilithium Lucifer yellow CH were obtained from Sigma Chemical, and 1-EBIO was obtained from Tocris Cookson. Charybdotoxin (ChTx) was synthesized by Auspep (Australia).

Data were compared using Student’s t-test and the software package InStat 3 (GraphPad). Values are means ± SE; n refers to the number of animals. P < 0.05 were considered statistically significant.

RESULTS

The EDHF-attributed hyperpolarizations recorded from dye-identified smooth muscle cells were indistinguishable from the hyperpolarizations evoked by ACh in dye-identified endothelial cells in the same section of arteriole (n = 3). This is illustrated in Fig. 1, in which the membrane had been depolarized using a low concentration of Ba2+ to partially block the inwardly rectifying K+ (Kir) channel. With the very large resting membrane potentials of both cell types in these tissues, the EDHF-attributed hyperpolarization was small (~2 mV) in the absence of Ba2+ (10).

In the presence of l-NAME and indomethacin, substance P (1 μM) evoked hyperpolarization of the arterioles (n = 6). Under voltage-clamp condition, it evoked an outward current that inactivated during the continued presence of substance P (Fig. 2A; n = 3). The current activated by substance P, and attributed to EDHF, reversed at −77 ± 5 mV (n = 3), which is not different from the likely K+ equilibrium potential of −85 mV (P = 0.27), was outwardly rectifying, and was well described by the Goldman-Hodgkin-Katz equation for a K+ current (Fig. 2B). The hyperpolarization evoked by substance P was blocked by the combined presence of 30 nM ChTx + 0.25 μM apamin (n = 3).

The presence of IKCa channels in the submucosal arterioles was tested by applying an activator of these channels, 1-EBIO. 1-EBIO evoked hyperpolarization in 11 of 12 dye-identified endothelial cells, and hyperpolarizations of similar amplitudes were recorded in 3 of 3 dye-identified smooth muscle cells. Figure 3A shows that 1-EBIO (600 μM) evoked hyperpolarization that was insensitive to IbTx (45 nM) but was blocked by ChTx (40 nM, n = 3), properties that indicate that IKCa, but not SKCa or large-conductance Ca2+-activated K+ (BKCa) channels were activated by 1-EBIO. The functional significance of 1-EBIO in terms of evoking relaxation of the arterioles is demonstrated in Fig. 3B (n = 8).

It has been reported that the EDHF-attributed hyperpolarization in rat hepatic arteries is due to the release of K+ from the endothelial cells, since the EDHF-attributed hyperpolarization could be blocked by a combination of Ba2+ and ouabain (16). This possibility was assessed in submucosal arterioles by comparing the effect of ACh with the effect of 5 mM KCl in solutions containing normal physiological saline solution, ouabain, and ouabain + Ba2+. As shown in Fig. 4A, ACh evoked hyperpolarization under conditions in which KCl evoked depolarization. In the presence of ouabain and ouabain + Ba2+, ACh still evoked substantial hyperpolarization that was associated with relaxation of the arteriole (Fig. 4A). The currents activated by the addition of 5 mM KCl were recorded under...
the voltage-clamp condition, and the Ba$^{2+}$-sensitive component is shown in Fig. 4Bd. Of the total current resulting from the application of K$^+$, $87.1 \pm 7.8\%$ ($n = 3$, at a membrane potential of $-80$ mV) was sensitive to blockade by Ba$^{2+}$. This current was inwardly rectifying, typical of K$_{IR}$ channels (Fig. 4Bb), and thus had a shape different from that of the outwardly rectifying current activated by substance P and attributed to EDHF (Fig. 2). Furthermore, the Ba$^{2+}$-sensitive current activated by raised K$^+$ reversed at a membrane potential appreciably more positive than the EDHF current activated by substance P (cf. Figs. 4Bd and 2B).

DISCUSSION

The results of this study show that substance P activates an EDHF-attributed K$^+$ current that is well described by the Goldman-Hodgkin-Katz equation and, therefore, displays little, if any, voltage-dependent channel gating. This property is consistent with the activity of IK$_{Ca}$ and SK$_{Ca}$ channels and with the recent detailed study of the currents activated by ACh and attributed to EDHF in these arterioles (10). We have gone on to show that 1-EBIO can also evoke hyperpolarization in these arterioles. Although ChTx abolished the hyperpolarization due to 1-EBIO, it did not abolish the hyperpolarization evoked by ACh. We previously showed that the outward current evoked by ACh is reduced by ChTx but requires ChTx combined with apamin to abolish the outward current (10). Thus, whereas the actions of 1-EBIO are consistent with its activation of IK$_{Ca}$ channels, ACh is likely to additionally activate SK$_{Ca}$ channels, which are blocked by apamin.

The location of the K$^+$ channels underlying the EDHF hyperpolarization is a fundamental question. We now show that the EDHF-attributed hyperpolarization recorded from dye-identified smooth muscle...
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Fig. 4. Effects of raised KCl. A: in an arteriole at rest, ACh (1 μM for 30 s) evoked hyperpolarization in contrast to the depolarization evoked by addition of 5 mM KCl (30 s). Ouabain (20 μM) and ouabain + 30 μM Ba²⁺ depolarized the membrane. Once threshold was reached, initiation of action potentials (top trace) resulted in constriction of the arteriole (bottom trace). ACh still evoked hyperpolarization and relaxation, while KCl evoked depolarization. B: in a voltage-clamped segment of arteriole, 30 μM Ba²⁺ inhibited a resting conductance (a) that had an inwardly rectifying current-voltage (I-V) relationship in physiological salt solution (PSS, b). Addition of 5 mM KCl resulted in a Ba²⁺-sensitive current that was large and inward (c), had an inwardly rectifying current-voltage relationship, which reversed at potentials close to the new K⁺ equilibrium potential, and was more positive than the original zero-current potential (d). Iₘ, membrane current.

Cells are indistinguishable from the response recorded from dye-identified endothelial cells in the same segment of arteriole. This, together with our previous work showing that the endothelial and smooth muscle cells are tightly coupled electrically (10), suggests that the two layers function as a single electrical syncytium, in general agreement with other results (43) including a mathematical model of coupling in these arterioles (11), as well as the results obtained from some other

blood vessels (4, 9, 20, 27, 28, 35, 41). Furthermore, 1-EBIO evoked IKCa-dependent hyperpolarizations in the endothelial and smooth muscle cells. IKCa channels occur in tissues rich in epithelia (24), including endothelial cells (25, 29, 40), but there is little, if any, evidence for their presence in noncultured vascular smooth muscle cells. Consistent with an endothelial location of IKCa channels, 1-EBIO activated a current with IKCa-like properties in cultured endothelial cells but not in isolated smooth muscle cells of the rat hepatic artery (18). Our observations are therefore consistent with the EDHF-attributed hyperpolarization involving the activation of IKCa channels in the endothelial cells and the current then spreading with negligible attenuation via myoendothelial gap junctions to the smooth muscle cells. In addition, sensitivity of a component of the EDHF current to block by apamin indicates that SKCa channels are also involved in the EDHF-attributed hyperpolarization of submucosal arterioles (10).

An alternative explanation for our observations is that EDHF may be a diffusible factor that hyperpolarizes the smooth muscle and that hyperpolarization could then spread electrotonically to the endothelial cells. One way to resolve the issue would be to inhibit the myoendothelial gap junctions. Although glycyrrhetinic acid and its derivatives have been used to inhibit gap junctions, these triterpenoid saponins have a number of effects in these arterioles, as well as in larger blood vessels, that include the inhibition of action potentials, inhibition of hyperpolarization in identified endothelial cells, and considerable depolarization (10, 37). Thus the nonspecific effects of glycyrrhetinic acid and its derivatives preclude the usefulness of these compounds in determining the role of gap junction communication in the actions of EDHF, similar to other putative gap junction inhibitors. Peptides based on the extracellular structure of connexins may provide a means of inhibiting gap junction communication. However, putative peptide gap junction inhibitors such as Gap 27 have not been shown to inhibit current flow through gap junctions. Inhibition of dye coupling is not necessarily a good indicator of inhibition of electrical coupling, since we observe good electrical coupling between endothelial and smooth muscle cells in the absence of dye coupling (Fig. 1).

The possible involvement of K⁺ as EDHF was explored further in this study. The application of additional K⁺ resulted in depolarization, in contrast to the hyperpolarization evoked by ACh under similar conditions. Furthermore, the hyperpolarization evoked by ACh was not inhibited by ouabain with or without low concentrations of Ba²⁺, providing further evidence that EDHF does not involve activation of the Na⁺-K⁺ pump and/or Kᵢᵣ channels, in agreement with the effects of added K⁺ recorded under voltage-clamp condition from short segments of these arterioles (10). This is despite the observations that the addition of K⁺ was capable of activating Kᵢᵣ channels and that these channels carried the majority of the K⁺-induced current. The Ba²⁺-sensitive component of the current activated by raised...
K+ was inwardly rectifying, in contrast to the outwardly rectifying current attributed to EDHF (Fig. 2B). The more positive reversal potential reflects the new K+ equilibrium potential in raised extracellular K+. Thus the EDHF-attributed hyperpolarization does not involve the activation of K\textsubscript{IR} channels. The ouabain-sensitive current in these arterioles has a relatively flat current-voltage relationship with a likely reversal potential of around −134 mV (10), which is also very different from the current-voltage relationship of the EDHF-attributed current; this means that the Na\textsuperscript{+}–K\textsuperscript{+} pump is not involved in the EDHF-attributed hyperpolarization.

Apart from K+, metabolites of arachidonic acid catalyzed by cytochrome P-450 have been suggested to be EDHF(s) and have, therefore, been the focus of many studies (see the introduction). We are not aware of any reports of these agents activating IK\textsubscript{Ca} or SK\textsubscript{Ca} channels, but epoxyeicosatrienoic acids, which are products of the cytochrome P-450 pathway, have been reported to activate BK\textsubscript{Ca} channels (3, 15, 19, 44). These observations do not necessarily exclude the involvement of the cytochrome P-450 pathway in the production of EDHF(s), since the actions of these metabolites have not been thoroughly worked out. Furthermore, because BK\textsubscript{Ca}, IK\textsubscript{Ca}, and SK\textsubscript{Ca} channels are activated by raised cytoplasmic free Ca\textsuperscript{2+}, heterogeneity in the distributions of these channels between different blood vessels could result in some differences in the channels that underlie the EDHF-attributed hyperpolarization.

In conclusion, the results presented here are consistent with the idea that the EDHF-attributed hyperpolarization results from the activation of IK\textsubscript{Ca} and SK\textsubscript{Ca} channels and not from the activation of K\textsubscript{IR} channels or the Na\textsuperscript{+}–K\textsuperscript{+} pump (10). Endothelial cells in the aortic valve, and therefore not in contact with smooth muscle cells, respond to stimulation with ACb by hyperpolarizing (31) with a time course that is strikingly similar to that recorded from submucosal arterioles (this study), which is consistent with the generation of EDHF in the endothelial cells. Thus, although there is still no definitive evidence, the most likely explanation for EDHF is that the EDHF-attributed hyperpolarization of these arterioles involves electrotransient spread of hyperpolarizing current from the endothelial cells, via myoendothelial gap junctions, to the smooth muscle cells.

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REFERENCES