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Modulation of Endothelial Cell $K_{Ca}3.1$ Channels During Endothelium-Derived Hyperpolarizing Factor Signaling in Mesenteric Resistance Arteries

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Abstract—Arterial hyperpolarization to acetylcholine (ACh) reflects coactivation of $K_{Ca}3.1$ (IK_{Ca}) channels and $K_{Ca}2.3$ (SK_{Ca}) channels in the endothelium that transfers through myoendothelial gap junctions and diffusible factor(s) to affect smooth muscle relaxation (endothelium-derived hyperpolarizing factor [EDHF] response). However, ACh can differentially activate $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, and we investigated the mechanisms responsible in rat mesenteric arteries. $K_{Ca}3.1$ channel input to EDHF hyperpolarization was enhanced by reducing external $[Ca^{2+}]_o$ but blocked either with forskolin to activate protein kinase A or by limiting smooth muscle $[Ca^{2+}]_i$ increases stimulated by phenylephrine depolarization. Imaging $[Ca^{2+}]_i$ within the endothelial cell projections forming myoendothelial gap junctions revealed increases in cytoplasmic $[Ca^{2+}]_i$ during endothelial stimulation with ACh that were unaffected by simultaneous increases in muscle $[Ca^{2+}]_i$ evoked by phenylephrine. If gap junctions were uncoupled, $K_{Ca}3.1$ channels became the predominant input to EDHF hyperpolarization, and relaxation was inhibited with ouabain, implicating a crucial link through Na^+/K^+-ATPase. There was no evidence for an equivalent link through $K_{Ca}2.3$ channels nor between these channels and the putative EDHF pathway involving natriuretic peptide receptor-C. Reconstruction of confocal z-stack images from pressurized arteries revealed $K_{Ca}2.3$ immunostain at endothelial cell borders, including endothelial cell projections, whereas $K_{Ca}3.1$ channels and Na^+/K^+-ATPase α_2/α_3 subunits were highly concentrated in endothelial cell projections and adjacent to myoendothelial gap junctions. Thus, extracellular $[Ca^{2+}]_o$ appears to modify $K_{Ca}3.1$ channel activity through a protein kinase A–dependent mechanism independent of changes in endothelial $[Ca^{2+}]_i$. The resulting hyperpolarization links to arterial relaxation largely through Na^+/K^+-ATPase, possibly reflecting K^+ acting as an EDHF. In contrast, $K_{Ca}2.3$ hyperpolarization appears mainly to affect relaxation through myoendothelial gap junctions. Overall, these data suggest that K^+ and myoendothelial coupling evoke EDHF-mediated relaxation through distinct, definable pathways. (***Circ Res*. 2008;102:1247-1255.**)

Key Words: potassium channel ■ endothelial cells ■ hyperpolarization ■ membrane potential ■ electrophysiology ■ vasodilation

The importance of the arterial endothelium for relaxation of the subjacent smooth muscle is well established. Whatever the final endothelium-derived effector, a key event is an initial increase in endothelial cell $[Ca^{2+}]_i$. In the case of endothelium-derived hyperpolarizing factor (EDHF) (the NO- and prostanoid-independent pathway), this increase crucially activates endothelial $K_{Ca}2.3$ and $K_{Ca}3.1$ channels. Activation of these K_{Ca} channels leads on to arterial hyperpolarization and dilation (see elsewhere for review[1]), and a changing role for each subtype has been implicated in pathological responses within blood vessels.[2,3]

Although activation of both $K_{Ca}2.3$ and $K_{Ca}3.1$ channels leads ultimately to vascular dilation, each can provide a variable contribution to EDHF hyperpolarization. Individual input is influenced by the extent of ongoing arterial (background) constriction. Increasing endothelial cell $[Ca^{2+}]_i$ (with acetylcholine [ACh]) in quiescent rat mesenteric arteries hyperpolarizes the resting membrane potential through $K_{Ca}2.3$ channels alone, but, during smooth muscle depolarization and the associated contraction necessary to observe EDHF relaxation, $K_{Ca}3.1$ channels are also activated.[4] This differential activation correlates with a distinct subcellular distribution of the channels. $K_{Ca}3.1$ channels are localized within the endothelial cell projections through the internal elastic lamina (IEL) that form myoendothelial gap junction (MEGJs) with the adjacent smooth muscle. In contrast, $K_{Ca}2.3$ channels are diffusely distributed throughout the plasmalemma of endothelial cells.[5] How the different channel types are activated independently is not known, but concentrating $K_{Ca}3.1$ channels within the narrow endothelial cell projections may hinder access of activating Ca^{2+} from the main body of the endothelial cells, while, perhaps, facilitating activation by a signal

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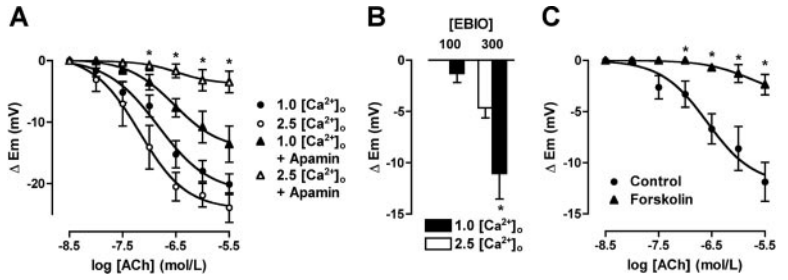

Figure 1. Summarized data showing the average change in membrane potential (ΔEm) to cumulative increases in [ACh] applied to evoke EDHF hyperpolarization in mesenteric arteries. A, Reducing $[Ca^{2+}]_o$ from 2.5 to 1.0 mmol/L depressed the steady-state hyperpolarization to ACh (n=7 and 12, respectively). In 1.0 mmol/L Ca^{2+} and with 50 nmol/L apamin present, ≈50% hyperpolarization remained (n=4), whereas in 2.5 mmol/L $[Ca^{2+}]_o$, apamin fully blocked hyperpolarization (n=13). B, Histogram summarizing the increase in smooth muscle membrane potential following endothelial cell stimulation with 1-EBIO in the presence of 50 nmol/L apamin. In 1.0 mmol/L $[Ca^{2+}]_o$, hyperpolarization to both 100 and 300 μmol/L 1-EBIO was enhanced (n=4 and 5, respectively). *$P<0.05$ relative to control (2.5 mmol/L $[Ca^{2+}]_o$). C, In 1.0 mmol/L $[Ca^{2+}]_o$ Krebs with 50 nmol/L apamin present, EDHF hyperpolarization evoked with ACh was significantly inhibited in the additional presence of 1 μmol/L forskolin (n=8, $P<0.05$).

derived from or associated with the contracting smooth muscle. In small arteries and arterioles, Ca^{2+} and/or a Ca^{2+} signal has been shown to spread from the muscle to the endothelium.[6–8] In the mesenteric artery, this process relies on Ca^{2+} signaling via MEGJs.[7,8] Extracellular Ca^{2+} levels ($[Ca^{2+}]_o$) may also have a significant influence on endothelial cell signaling. In the mesenteric artery, an extracellular calcium-sensing receptor (CaSR) on the endothelium links to activate $K_{Ca}3.1$ channels selectively and appears to colocalize with this channel (but not with $K_{Ca}2.3$ channels).[9] This raises the possibility that alterations in extracellular Ca^{2+} concentration may help determine the relative contribution from $K_{Ca}3.1/2.3$ channels. In addition, $K_{Ca}3.1$ but not $K_{Ca}2.3$ channel activity is inhibited by protein kinase A phosphorylation in enteric neurons, indicating regulation may occur independently of intracellular $[Ca^{2+}]$ change.[10–12]

In the rat mesenteric artery, transfer of endothelial hyperpolarization to the muscle, in part, reflects K^+ ion efflux through endothelial K_{Ca} channels and, in part, spread of hyperpolarization via MEGJs.[13–16] Extracellular K^+, mimicking its action as a diffusible EDHF, stimulates smooth muscle hyperpolarization and relaxation primarily by activating Na^+/K^+-ATPase.[17] However, in situ, the ability of K^+ to act as an EDHF is inversely related to ongoing arterial contraction because, as contraction increases, Na^+/K^+-ATPase activity is swamped by K^+ leaving the smooth muscle through BK_{Ca} channels.[17–19] In contrast, MEGJs operating in parallel to K^+ enable a constant spread of hyperpolarization, and this route to relaxation thus becomes predominant as arterial tone approaches maximum.[16] Therefore, 1 possibility is that $K_{Ca}2.3$ and $K_{Ca}3.1$ channels may each separately underlie 1 of these routes to vasodilation. In support of this possibility, recent evidence from rat mesenteric artery has suggested that $K_{Ca}2.3$ channel activation may be intimately linked to the release of C-type natriuretic peptide, which acts as a diffusible EDHF through natriuretic peptide receptor-C.[20]

The aims of the present study were, therefore: (1) to investigate how $K_{Ca}3.1$ channels are independently activated; and (2) to show whether the subcellular distribution of $K_{Ca}2.3/K_{Ca}3.1$ channels within rat mesenteric endothelial cells can be correlated with discrete EDHF pathways, with functional consequences for relaxation and of direct relevance to endothelial changes associated with vascular disease.

Materials and Methods

An expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org, gives full details regarding the experimental techniques used. These included intracellular sharp microelectrode recording and simultaneous tension measurements, Ca^{2+} imaging from both smooth muscle and endothelial cells, and immunohistochemistry of rat isolated small mesenteric arteries.

Results

Reducing External $[Ca^{2+}]_o$ Recruits $K_{Ca}3.1$ Channel Input to EDHF Hyperpolarization

Reducing external $[Ca^{2+}]_o$ from 2.5 to 1.0 mmol/L slightly reduced smooth muscle resting membrane potential (from −51.7±1.6 to −47.8±0.9 mV, n=7 and 12) and depressed the cumulative EDHF hyperpolarization to ACh (maximal increase 23.9±2.4 mV [n=7] and 20.1±1.7 mV [n=12], respectively; Figure 1A). In contrast to the situation in 2.5 mmol/L Ca^{2+} (and see also Crane et al[4]), apamin failed to block EDHF hyperpolarization in 1 mmol/L Ca^{2+} (n=4). Furthermore, low $[Ca^{2+}]_o$ facilitated the ability of the $K_{Ca}3.1$ channel activator 1-EBIO (100 and 300 μmol/L) to evoke smooth muscle hyperpolarization (Figure 1B). In separate experiments using 1.0 mmol/L $[Ca^{2+}]_o$ with apamin present (resting membrane potential: −48.6±1.8 mV, n=8), 1 μmol/L forskolin significantly reduced EDHF hyperpolarization by 80% to 100% (Figure 1C) and hyperpolarization to 300 μmol/L 1-EBIO from 8.5±1.9 mV to 2.9±0.9 mV (n=5).

Alteration in $[Ca^{2+}]_o$ also modified arterial tone (Figure I in the online data supplement). Increasing $[Ca^{2+}]_o$ from 1.0 mmol/L relaxed precontracted arteries via endothelial cell $K_{Ca}3.1$ channels, with complete relaxation obtained in the presence of 2.5 mmol/L $[Ca^{2+}]_o$.

Limiting Smooth Muscle Ca^{2+} Increase Modifies $K_{Ca}2.3$ and $K_{Ca}3.1$ Channel Input to EDHF Dilation in Rat Mesenteric Arteries

In the presence of the voltage-gated Ca^{2+} channel (VGCC) inhibitor nifedipine (10 μmol/L) and during depolarization to phenylephrine (PE) (1 μmol/L: 7.2±1.8 mV, n=12), apamin (50 nmol/L) alone effectively abolished EDHF hyperpolarization (23.4±1.3 mV hyperpolarization to 3 μmol/L ACh reduced to 1.9±1.3 mV, n=12) (Figure 2A through 2C), whereas TRAM-34 (1 μmol/L) had little effect (hyperpolarization to 3 μmol/L ACh 19.6±1.4 mV, n=12) (Figure 2B). Hyperpolarization to the direct $K_{Ca}3.1$ channel activator 1-EBIO (100 or 300 μmol/L) was not altered by nifedipine (control: 3.9±1.0 and 8.8±0.8 mV, n=4; versus

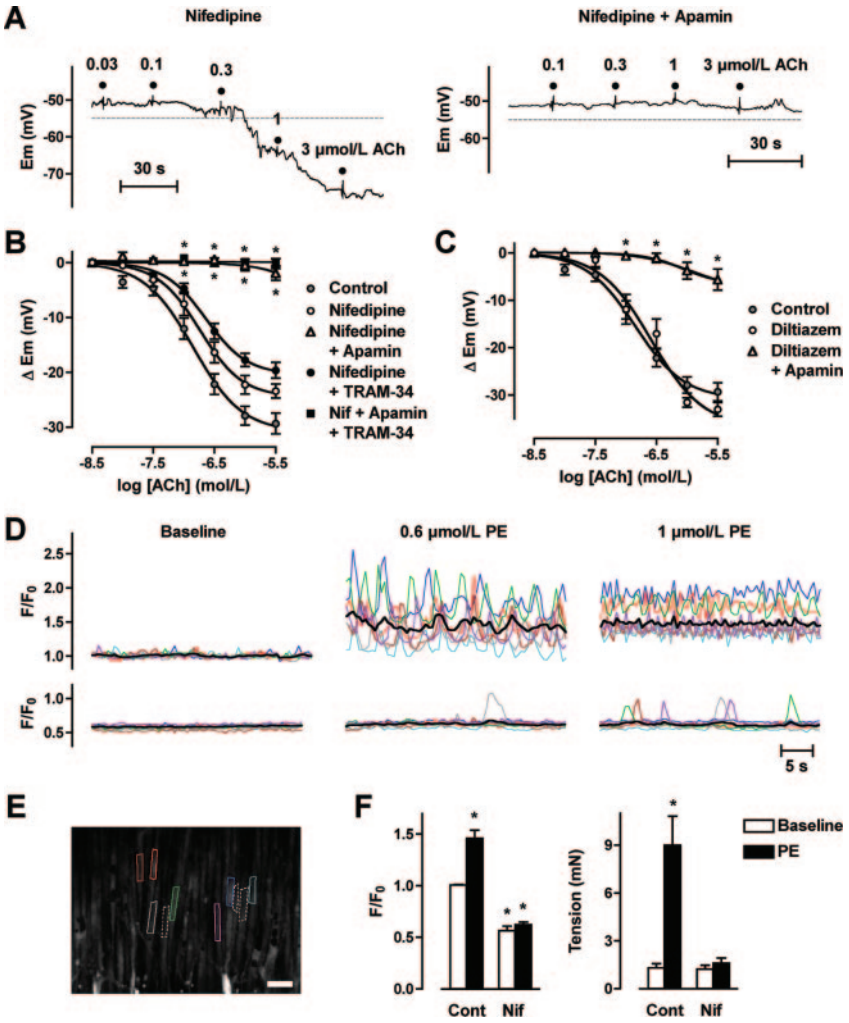

Figure 2. ACh-mediated stimulation of $K_{Ca}3.1$ channels is prevented by blocking VGCC in rat mesenteric arteries. A, Original traces demonstrating concentration-dependent EDHF-induced smooth muscle hyperpolarization evoked by ACh in the presence of nifedipine (10 μmol/L) and prior depolarization to PE. Hyperpolarization overshot the resting membrane potential (dashed line) and was abolished by apamin (50 nmol/L). B, Summarized data showing the average change in membrane potential (ΔEm) to cumulative increases in [ACh] in arteries stimulated with PE in the presence of nifedipine (10 μmol/L, n=4 to 10) or in the additional presence of either apamin (50 nmol/L, n=4 to 12) or TRAM-34 (1 μmol/L, n=7 to 14) or both inhibitors together (n=5 to 8). C, Summarized data showing the average change in membrane potential (ΔEm) to cumulative increases in [ACh] in arteries stimulated with PE in the presence of diltiazem (10 μmol/L, n=4 to 6) or in the additional presence of apamin (50 nmol/L, n=5 to 7). D, Smooth muscle Ca^{2+} signals in the absence (upper traces) and presence of nifedipine (10 μmol/L, lower traces) color-coded to the field of interest shown in the image in E. Applying 0.6 or 1 μmol/L PE stimulated a marked increase in the global average level of [Ca^{2+}] (black line), representing asynchronous Ca^{2+} increases within individual muscle cells. Both the average increase and the majority of the oscillations were abolished in the presence of nifedipine (see supplemental Video 1 for the movie corresponding to these data). F, Summarized data showing inhibition of the increase in $[Ca^{2+}]_i$ stimulated by 1 μmol/L PE (control [Cont] n=6, nifedipine [Nif] n=3) (left) and the associated block of arterial contraction (paired data) (right). *$P<0.05$ relative to control baseline.

nifedipine: 3.5±0.7 and 6.2±2.2 mV, n=5, respectively). In the presence of another VGCC inhibitor, diltiazem (10 μmol/L), and PE depolarization (7.0±0.9 mV, n=6), apamin again effectively abolished EDHF hyperpolarization (33.0±1.5 mV hyperpolarization to 3 μmol/L ACh reduced to 5.6±2.2 mV, n=6) (Figure 2C), whereas subsequent addition of the ATP-sensitive K^+ (K_{ATP}) channel opener levcromakalim (3 μmol/L) evoked a robust hyperpolarization of 29.4±3.9 mV (n=6). Nifedipine reduced the smooth muscle cell $[Ca^{2+}]_i$ and tension increase to PE (Figure 2D through 2F).

Imaging both global and subcellular increases in endothelial cell [Ca^{2+}] to ACh failed to reveal any significant difference between ACh applied under resting conditions (baseline) compared with during smooth muscle stimulation with PE (Figure 3A and 3B). In Figure 3A, the measurement regions could be limited to the holes through the IEL, where a Ca^+ increase was clearly observed within endothelial cell projections.

$K_{Ca}3.1$ Channel or Na^+/K^+-ATPase Block Abolishes EDHF Dilation When a Gap Junction Uncoupler Is Present

In unstimulated arteries, 30 to 60 minutes of exposure to the gap junction uncoupler carbenoxolone (100 μmol/L) did not alter either the smooth muscle (−50±1.7 and −50.6±0.9 mV, n=11 and 6, respectively) or the endothelial cell resting potential (−52.4±1.3 and −51.9±1.3 mV, n=11 and 6). With ACh, carbenoxolone reduced smooth muscle (EDHF) hyperpolarization from a maximum of 20.9±1.2 mV (to −69.7±1.7 mV, n=9) to 9.9±3 mV (to −60.5±2.7 mV, n=6) (Figure 4A and 4B) but did not reduce (direct) endothelial cell hyperpolarization to 1 μmol/L ACh (8.9±1.4 and 8.1±1.7 mV, n=5) (Figure 4C). However, it reversibly blocked the indirect hyperpolarization recorded from endothelial cells, where hyperpolarization was induced by stimulating K_{ATP} channels in the smooth muscle (5 μmol/L levcromakalim: control 19.8±2.1 mV, n=5; plus carbenoxolone, 0.4±0.4 mV, n=3; after washout, 19.7±2.0 mV, n=3) (Figure 4C).

Carbenoxolone (100 μmol/L) did not alter contraction to either submaximal or maximal concentrations of PE or the subsequent EDHF relaxation to ACh during submaximal contraction. However, in arteries maximally contracted with PE, the EDHF-mediated relaxation, which under these stimulation conditions relies solely on MEGJs,[16] was abolished even though levcromakalim (1 μmol/L) was able to stimulate maximal relaxation (97.1±1.0%, n=6, Table).

In arteries submaximally stimulated with PE, depolarization and contraction did not differ from control (control:

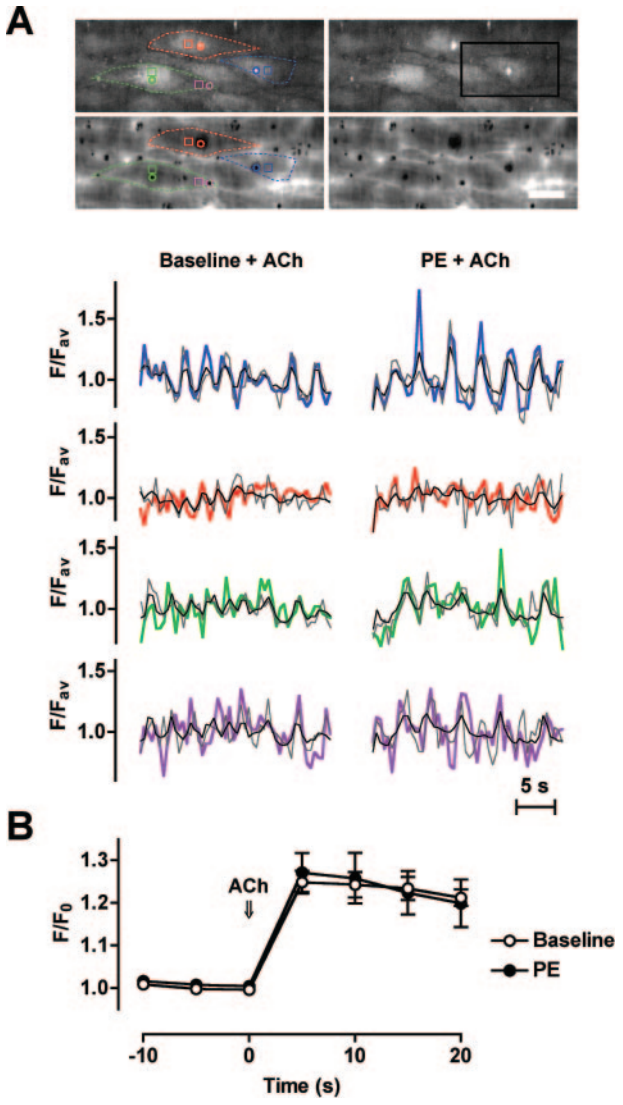

Figure 3. Similar increases in endothelial cell $[Ca^{2+}]_i$ evoked with ACh alone and applied during smooth muscle stimulation with PE. A, Mesenteric arteries were mounted in a pressure myograph and changes in endothelial cell $[Ca^{2+}]_i$ assessed at the interface between the endothelium and the IEL. Upper micrographs show loaded endothelial cells (average of 20 seconds). Note bright spots (endothelial cell projections) that correspond to holes in the IEL (lower micrographs). Bar=20 μm. Lower micrographs show the time course of fluorescence changes in individual endothelial cells in response to ACh (1 μmol/L) added under baseline conditions (left) and in the presence of PE (0. 3 to 0.6 μmol/L) (right). Regions were placed over (color) and adjacent to (gray) the endothelial cell projections and around the whole cell (black). The colors relate to subcellular regions within the micrographs (see supplemental Video 2 for the movie corresponding to these data from the region indicated by the black box). Representative of 3 experiments. B, Mesenteric arteries were mounted in a wire myograph, and changes in (whole) endothelial cell $[Ca^{2+}]_i$ were measured before and during ACh (1 μmol/L), either from baseline or in the presence of PE (0.3 to 0.6 μmol/L, n=3, paired data). Note that endothelial cell projections were not loaded in these experiments. (See the expanded Materials and Methods section in the online data supplement for details.)

9.2±1.0 mV, 6.3±0.4 mN; with carbenoxolone: 9.2±1.6 mV, 6.9±0.6 mN; n=8), but the ACh-evoked increase in membrane potential was reduced by ≈10 mV (Figure 4B), increasing to −63.4±4.5 mV (n=7) compared with −70.9±2.4 mV (n=8) without carbenoxolone. However, this reduction in maximum hyperpolarization was not sufficient to modify the associated relaxation. In contrast, in the additional presence of 1 μmol/L TRAM-34, relaxation to ACh was almost abolished (92.5±2.8% [n=5] reduced to 26.1±6.7% [n=5]; Figure 4B). Ouabain (100 μmol/L) had a similar inhibitory effect to TRAM-34, depressing relaxation to ACh (from 98.9±1.0%) to only 23.5±5.7% in the presence of carbenoxolone (n=3). In contrast, under similar conditions, apamin had only a slight inhibitory effect (Figure 4B).

Possible Link Between $K_{Ca}2.3$ Channels and C-Type Natriuretic Peptide?

Use of the selective natriuretic peptide receptor-C antagonist M372049 (100 nmol/L) and comparison of EDHF responses (to ACh) with the action of exogenous C-type natriuretic peptide (CNP) did not suggest any link between $K_{Ca}2.3$ channel activation and the subsequent activation of natriuretic peptide receptor-C leading to hyperpolarization and relaxation (supplemental Figure II).

Dimensional Localization of Na^+/K^+-ATPase and K Channels Within the Rat Mesenteric Artery

Immunofluorescence indicating Na^+/K^+-ATPase α_2 (and similarly for α_3) subunits was distributed throughout the cells within pressurized rat mesenteric arteries (Figure 5). A particularly intense, punctate fluorescence signal was discretely aligned with holes through the IEL and above the plane of focus of the endothelium, at the interface between the IEL and the smooth muscle (noted to be an endothelial cell projection[21]). This corresponds with regions of MEGJ formation where we have previously demonstrated colocalization of $K_{Ca}3.1$ channels, connexin (Cx)40, and Cx37.[5,16] In contrast, staining for $K_{ir}2.1$ channels was homogeneous within endothelial cells, and that for $K_{Ca}2.3$ channels was very clear at interendothelial cell borders, similar to staining for Cx37 and Cx40.[5,22] There was also strong staining for K_{ir}, $K_{Ca}2.3$, and $K_{Ca}3.1$ channels in endothelial cell projections, whereas staining for $K_{Ca}3.1$ channels and Na^+/K^+-ATPase was not evident (or at least markedly less) in other regions of the endothelial cells. No immunostaining was observed in arteries where the primary antibody was omitted during preparation or following prior incubation with blocking peptide.

Discussion

These data show that the ability of $K_{Ca}3.1$ channels to contribute to EDHF hyperpolarization depends on extracellular $[Ca^{2+}]_o$, possibly linked to the extent of basal stimulation of the extracellular CaSRs known to colocalize with these channels in the mesenteric artery endothelium. Recruitment of $K_{Ca}3.1$ channels to EDHF hyperpolarization (and relaxation) did not appear to reflect Ca^{2+} or a Ca^{2+} release signal passing from the smooth muscle into the endothelial projections that form MEGJs. However, it may reflect a local depletion of extracellular $[Ca^{2+}]_o$ around the endothelial projections containing $K_{Ca}3.1$ as $[Ca^{2+}]_o$ enters the smooth muscle through VGCC, because recruitment was reduced in the presence of VGCC blockers to limit the ability of PE to

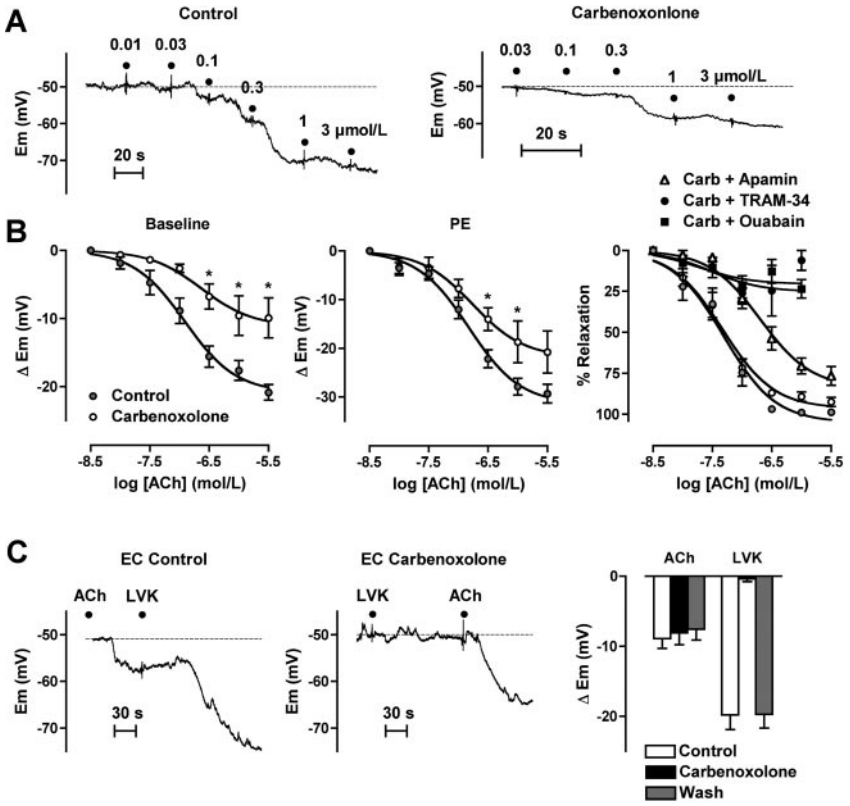

Figure 4. Gap junctions facilitate the contribution of $K_{Ca}2.3$ channels to EDHF hyperpolarization in rat mesenteric artery. A, Original traces demonstrating ACh-mediated concentration-dependent increases in smooth muscle cell membrane potential from the resting membrane potential (dashed lines). Carbenoxolone (100 μmol/L) did not alter the resting membrane potential but reduced the hyperpolarization to ACh. B, Left, Summarized data showing the average change in membrane potential (ΔEm) to cumulative increases in [ACh] to evoke EDHF hyperpolarization in arteries from resting membrane potential (n=6 to 11). Middle, In the presence of PE, carbenoxolone (100 μmol/L) depressed EDHF hyperpolarization (n=5 to 9) but not relaxation to ACh (n=8 to 14). Right, The EDHF-mediated relaxation was reduced by the addition of apamin (50 nmol/L, n=9 to 13) and almost abolished by TRAM-34 (1 μmol/L, n=5 to 12) or ouabain (100 μmol/L, n=3 to 9), each applied when carbenoxolone was present. *$P<0.05$ relative to control. C, Original traces demonstrating, Left, endothelial cell hyperpolarization to ACh (1 μmol/L) and levcromakalim (5 μmol/L). Middle, carbenoxolone (100 μmol/L) blocked hyperpolarization to levcromakalim not ACh. Right, average increases in endothelial cell membrane potential before, during application, and after washout of carbenoxlone, demonstrating reversible block of hyperpolarization to levcromakalim. ACh, n=4, 5, 3; levcromakalim, n=5, 3, 3, respectively.

raise muscle $[Ca^{2+}]_i$. Finally, $K_{Ca}3.1$ channel input may be enhanced by the K^+ ions acting on the same, or closely adjacent, cells via the Na^+/K^+-ATPase (and possibly K_{ir} channels). This suggests that K^+ efflux through K_{Ca} channels discretely localized in endothelial projections may underlie its action as a "diffusible" EDHF, whereas hyperpolarization attributable to K_{Ca} channels expressed near interendothelial cell borders ($K_{Ca}2.3$) relies primarily on extant MEGJs for effective spread into the media.

In the rat mesenteric artery, we have recently shown that $K_{Ca}3.1$ channels are concentrated in the head region of endothelial cell projections protruding through the IEL to form MEGJs.[5] In the endothelial cells, strong punctate signal for $K_{Ca}3.1$ channels colocalized with Cx37 and Cx40 at the interface with the smooth muscle. Furthermore, at an ultrastructural level $K_{Ca}3.1$ channels and both Cx37 and Cx40 conjugated gold label was associated with endothelial cell projections forming MEGJs. In contrast, although $K_{Ca}2.3$ channels were localized in close proximity to Cx37, Cx40, and Cx43, they appeared to be mainly adjacent to endothelial–endothelial cell gap junctions.[5,16] These observations correlate with electrophysiological and immunoprecipitation data. In the former, the contribution from $K_{Ca}2.3$ and $K_{Ca}3.1$ channels to ACh-mediated hyperpolarization could be separated. Endothelial $K_{Ca}3.1$ channel activity was only apparent when the arterial smooth muscle was depolarized and contracted (leading to EDHF repolarization to close to resting potential).[4] In the latter, $K_{Ca}2.3$ and $K_{Ca}3.1$ channels were associated with caveolin-rich and -poor fractions of endothelial cell membrane, respectively.[9] The present experiments subtly extend

Table. Carbenoxolone Only Blocks EDHF Relaxation to ACh in Mesenteric Arteries Maximally Contracted With PE

	Tension (mN)	[PE] (μmol/L)	Relaxation (%)	pD_2	n
Low contraction					
Control	10.8±0.6	0.4±0.1	96.4±1.2	7.2±0.1	5
+Carb	8.9±0.7	0.6±0.1	92.9±3.2	6.9±0.1	5
High contraction					
Control	19.0±2.5	10.0±0.0	95.4±0.6	7.0±0.1	6
+Carb	18.6±1.8	10.0±0.0	10.3±7.7*	7.2±0.5	6

The concentration of carbenoxolone (Carb) was 100 μmol/L. Average relaxation to 1 μmol/L ACh obtained in arteries undergoing submaximal (low) contraction or maximal (high) contraction to PE. *$P<0.05$ relative to control; paired data for each level of tension.

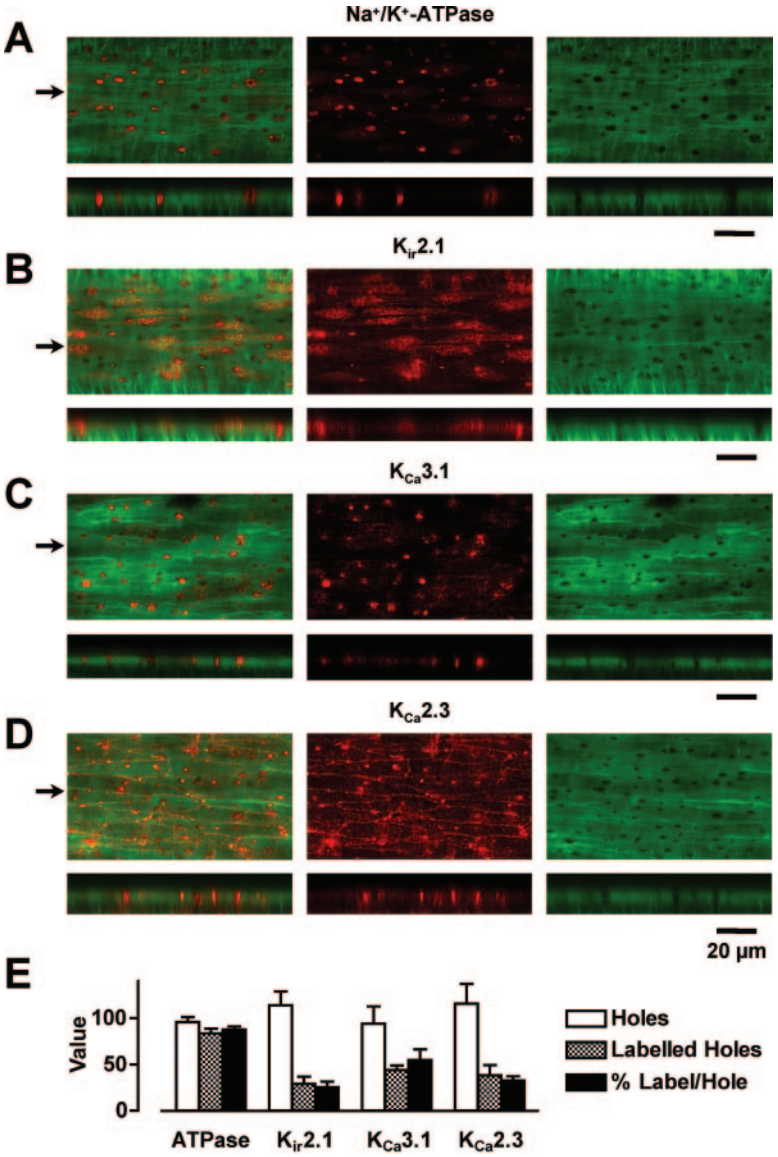

Figure 5. Endothelial cell immunohistochemical expression pattern for Na^+/K^+-ATPase, $K_{ir}2.1$, $K_{Ca}3.1$, and $K_{Ca}2.3$ channels in rat mesenteric arteries. A through D, Top, Confocal images of the wall of pressurized arteries showing a single z-axis plane through the IEL (green) and simultaneously acquired corresponding expression of protein (red). A through D, Bottom, Reconstruction of the confocal z-axis multiplane stack through the wall of the artery corresponding to a line drawn through the images in the upper panels at the positions indicated by arrows. Note the presence of protein staining within the holes through the IEL in both the top and bottom images. Both the Na^+/K^+-ATPase α_2 subunit and $K_{Ca}3.1$ channels are highly expressed within the holes, whereas $K_{ir}2.1$ and $K_{Ca}2.3$ channels are also highly expressed within endothelial cells and at endothelial cell borders, respectively. In these images, the Alexa Fluor 488 secondary antibody was only applied to the luminal side of the artery, and the confocal laser and PMT settings were identical for each z-stack. Bar indicates 20 μm in the x-axis in all images. Representative of at least 3 arteries. E, Quantification of protein expression within the holes through the IEL (n=3 to 5). [Holes], number of holes in the IEL/100 μm^2.

our morphological data and provide novel insight into the control of endothelial $K_{Ca}3.1$ channel activation and therefore their contribution to EDHF responses. Reconstruction of confocal z-stack images from the wall of mesenteric arteries, obtained in a physiological orientation (as opposed to perfusion fixation and flat mounting for microscopy),[5] confirmed the restriction of $K_{Ca}3.1$ channels within endothelial cell projections but also revealed a colocalization of $K_{Ca}2.3$.

With regard to the mechanism responsible for recruiting the $K_{Ca}3.1$ channels within endothelial cell projections, this was shown to be sensitive to $[Ca^{2+}]_o$ around the outside of the projections. Reducing $[Ca^{2+}]_o$ recruited $K_{Ca}3.1$ channels to EDHF hyperpolarization. These observations are consistent with recent evidence in lean Zucker rats, in which both $K_{Ca}2.3$ and $K_{Ca}3.1$ channels contributed to EDHF hyperpolarization in arteries bathed in Krebs containing 1.6 mmol/L $[Ca^{2+}]$.[2] A reasonable explanation in both studies is that lowering $[Ca^{2+}]_o$ reduces basal stimulation of an extracellular G protein–linked CaSR, receptors known to colocalize with $K_{Ca}3.1$ channels in "noncaveolin" membrane fractions of mesenteric endothelial cells.[9] Thus, lowering $[Ca^+]_o$ from 2.5 to 1 mmol/L reduced extracellular CaSR saturation and effectively increased availability of membrane $K_{Ca}3.1$ channels for activation by increases in $[Ca^{2+}]_i$ within the endothelial projections.

Limiting increases in smooth muscle $[Ca^{2+}]_i$ was also linked to the ability of endothelial $K_{Ca}3.1$ channels to provide significant input to EDHF hyperpolarization. Nifedipine or diltiazem reduced voltage-dependent Ca^{2+} entry into the smooth muscle, associated with a loss of $K_{Ca}3.1$ channel input to the EDHF response (evoked by ACh) and, as a consequence, sole reliance on the activation of apamin-sensitive $K_{Ca}2.3$ channels. One possible explanation is that during "normal" increases in smooth muscle $[Ca^{2+}]_i$, $K_{Ca}3.1$ channel input to the EDHF response is enabled because the increase in smooth muscle $[Ca^{2+}]_i$ following depolarization indirectly facilitates Ca^{2+} signaling in the endothelial cell projections and activates the channels. Direct measurement of endothelial cell $[Ca^{2+}]_i$ levels in both resistance arteries and arterioles has shown that smooth muscle stimulation with PE leads to $[Ca^{2+}]_i$ increases in the endothelium.[6–8] In isolated mesenteric

arteries from the rat, endothelial cell subpopulations displayed an increase in Ca^{2+} during muscle stimulation with PE, apparently reflecting spread of inositol-1,4,5-trisphosphate from the muscle to the endothelium.[7] However, the additional movement of Ca^{2+} into endothelial cell projections (and throughout the cells)[8,23] could not be discounted by these experiments. However, signaling via this route does not appear to explain our data. We were unable to detect any difference in $[Ca^{2+}]_i$ increases to ACh within the endothelial cell projection (or whole cell) during simultaneous muscle stimulation with PE. The caveat here is that it remains possible the resolution and limitations of our system prevented detection of very small and localized changes within endothelial cell projections or discrete changes in $[Ca^{2+}]_i$ within the projections during changes in diameter.

Alternatively, Ca^{2+} entry into the contracting muscle itself may be sufficient normally to reduce the $[Ca^{2+}]_o$ in the restricted extracellular space round the endothelial cell projection. As such, this would reduce "basal" CaSR stimulation and facilitate linked $K_{Ca}3.1$ channel availability, enabling activation of the latter by the increased $[Ca^{2+}]_i$ in the projections evoked by ACh. In support of this scenario, during synchronous neuronal activity the opening of VGCCs is known to substantially deplete (≈1 mmol/L) extracellular $[Ca^{2+}]_o$ in the limited volume of fluid surrounding the cells.[24–26] Whatever the precise explanation, within endothelial cell projections $K_{Ca}3.1$ channel activity may be suppressed through the action of protein kinase A. In enteric neurons, protein kinase A appears to maintain the $K_{Ca}3.1$ currents underlying slow, postspike, after-hyperpolarization in a closed state.[10] In our experiments, the ability of forskolin to inhibit EDHF hyperpolarization attributable to $K_{Ca}3.1$ channel activity suggests a similar mechanism operates in mesenteric endothelial cells.

One possible confounding influence in our experiments with VGCC blockers is the potential for nifedipine directly to block $K_{Ca}3.1$ channels. Dihydropyridines were initially shown to block the Gardos channel in erythrocytes,[27,28] and, more recently, Jiang et al[29] reported block of EDHF hyperpolarization in guinea pig cochlear artery, in which, in contrast to the mesenteric artery, hyperpolarization appears to depend on $K_{Ca}3.1$ channels alone. However, this did not appear to be a significant consideration in our experiments because: (1) diltiazem, which unlike the dihydropyridines does not block $K_{Ca}3.1$ channels,[27,29] had a similar effect to nifedipine; and (2) nifedipine did not alter hyperpolarization to the $K_{Ca}3.1$ channel activator 1-EBIO. The apparent inability of nifedipine to block $K_{Ca}3.1$ channels may relate to the experimental conditions. For example, block of the Gardos channel with nifedipine decreases with increasing extracellular K^+, a situation that would be predicted in our experiments in the presence of PE,[27] or it may simply be that although $K_{Ca}3.1$ channel activation is reduced, the reduction is not sufficient to suppress the overall EDHF hyperpolarization in intact arteries.

Another important observation in our study was the ability of either ouabain or TRAM-34 to block EDHF responses in the presence of the gap junction uncoupler carbenoxolone. Although carbenoxolone did reduce EDHF hyperpolarization, this did not modify the associated arterial relaxation. However, once ouabain was added along with carbenoxolone, EDHF relaxation was abolished. Although this concentration of ouabain blocks smooth muscle hyperpolarization and relaxation to exogenous K^+,[17] it has only a small inhibitory effect against the overall EDHF hyperpolarization and relaxation in submaximally contracted arteries, because the EDHF response is sustained by MEGJs, as explained in the introduction.[13,16] However, once the MEGJ pathway is compromised, in this case with carbenoxolone, blocking the action of endogenous K^+ significantly impacts on EDHF relaxation. The fact that a gap junction uncoupler (carbenoxolone) did not alter EDHF responses in submaximally contracted arteries, but abolished them in maximally contracted arteries, although not altering relaxation to levcromakalim, supports this scheme. Interestingly, with carbenoxolone present in submaximally contracted arteries, TRAM-34 (but not apamin) caused similar block to ouabain. This suggests that endogenous K^+ originating from the $K_{Ca}3.1$ channels in close proximity to focused clusters of Na^+/K^+-ATPase underlies the ability of K^+ to act as EDHF. In the absence of carbenoxolone, block of both $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, with TRAM-34 and apamin together, is necessary to block EDHF effects.[4] The concentration of carbenoxolone we used has been shown not to alter the $[Ca^{2+}]_i$ increase evoked in the endothelium with ACh,[16] and it blocks the spread of calcein from the endothelium into the smooth muscle.[8] Furthermore, we now show that carbenoxolone does not modify the resting potential in either the endothelial or smooth muscle cells nor the endothelial cell hyperpolarization evoked with ACh. However, notably, it did block hyperpolarization to levcromakalim from spreading through MEGJs to the endothelium. In this vessel, K_{ATP} channels are found only on the smooth muscle cells and not the endothelium.[30] Therefore, although it remains possible carbenoxolone has effects in addition to uncoupling gap junctions, this agent clearly and effectively blocks the spread of EDHF hyperpolarization through MEGJs and without disrupting the key events responsible for initiating an EDHF response via Ca^{2+} handling and K_{Ca} channel activation within the endothelium.

Finally, in rat mesenteric arteries, $K_{Ca}2.3$ channel activation has recently been suggested to be somehow linked to the release of CNP, with CNP then acting as an EDHF to hyperpolarize and relax the adjacent smooth muscle.[20] Furthermore, in this study, the natriuretic peptide receptor-C–selective antagonist M372049 was found to act synergistically with ouabain to block EDHF responses, leading to the suggestion that CNP acts alongside a $K_{Ca}3.1$-dependent signal, the 2 "arms" underpinning the EDHF response in mesenteric arteries. Although a link between $K_{Ca}3.1$ channels and Na^+/K^+-ATPase activation is consistent with our data, we were unable to provide any evidence to suggest that CNP plays a significant role in the EDHF response. Although M372049 clearly blocked arterial relaxation to exogenous CNP, it did not increase the inhibitory action of ouabain against EDHF hyperpolarization and relaxation, and it failed to inhibit the apamin-sensitive ($K_{Ca}2.3$ channel) component of EDHF hyperpolarization. The fact that exogenous CNP only evoked a modest relaxation without any hyperpolarization,

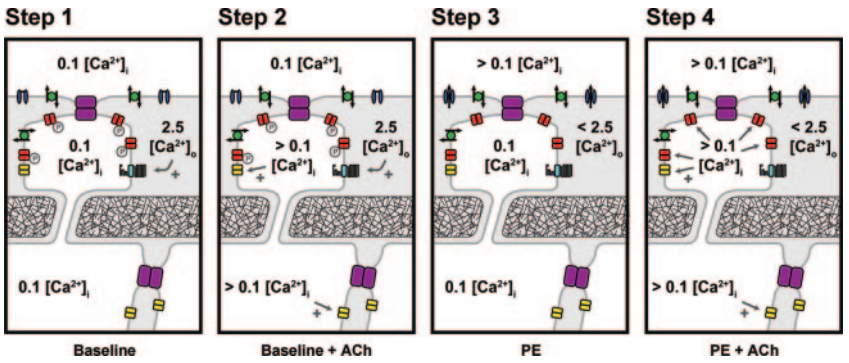

Figure 6. Suggested key steps underlying endothelial cell K_{Ca} channel activation and subsequent transfer of hyperpolarization to the mesenteric smooth muscle. Step 1: At rest in 2.5 mmol/L $[Ca^{2+}]_o$ Krebs solution, the endothelial cell CaSR is maximally stimulated and $K_{Ca}3.1$ channels are in some way inactivated, possibly via protein kinase A phosphorylation. Step 2: Stimulation of the endothelium with ACh raises cytoplasmic $[Ca^{2+}]_i$, activating apamin-sensitive $K_{Ca}2.1$ channels causing hyperpolarization, which can spread through homocellular (endothelial–endothelial) and heterocellular (myoendothelial, MEGJs) gap junctions. $K_{Ca}3.1$ channels are not available for agonist-induced $[Ca^{2+}]_i$ activation. Step 3: Activation of smooth muscle VDCC (with PE) causes a local "sink" of $[Ca^{2+}]_o$ in the vicinity of the endothelial projections. This reduction reduces stimulation of the CaSR and phosphorylation of $K_{Ca}3.1$ channels in the projections. Step 4: At this point, ACh stimulation of the endothelium raises cytoplasmic $[Ca^{2+}]_i$, now activating "available" $K_{Ca}3.1$, as well as $K_{Ca}2.3$. As in Step 2, the resulting hyperpolarization spreads through endothelial–endothelial and MEGJs gap junctions but now reflects input from both $K_{Ca}2.3$ and $K_{Ca}3.1$. Facilitated by ongoing muscle depolarization, endothelial K^+ efflux through K_{Ca} channels is sufficient to stimulate adjacent Na^+/K^+-ATPase–enhancing hyperpolarization. Further amplification may also occur through K_{IR} channel activation within this microdomain. Because smooth muscle cells repolarize to resting levels, VGCC open probability decreases, removing the Ca^{2+} sink; local $[Ca^{2+}]_o$ increases toward 2.5 mmol/L; and $K_{Ca}3.1$ channel activity is again removed from the control of intracellular $[Ca^{2+}]_i$. Gray indicates intercellular space; purple, gap junctions; red, $K_{Ca}3.1$ channels; yellow, $K_{Ca}2.3$ channels; blue, VGCCs; green, Na^+/K^+-ATPase; cyan, CaSR.

and that M372049 did not modify EDHF responses to ACh, also argues strongly against a significant role for CNP in the EDHF response.

Overall, our data indicate a clear correlation between the discrete localization of $K_{Ca}3.1$ channels within the endothelial cell projections that form MEGJs and their respective activation during smooth muscle stimulation. A suggested outline mechanism is presented in Figure 6. The complex dynamics of $[Ca^{2+}]_o$ change within the very restricted intercellular space between muscle and endothelial cell projections may provide the key to explaining why $K_{Ca}3.1$ channels are able to contribute to EDHF hyperpolarization and relaxation during muscle contraction, dropping out as membrane potential and tension (reflecting smooth muscle $[Ca^{2+}]_i$) return to resting levels, while $K_{Ca}2.3$ channels still continue to increase membrane potential to close to E_K. In addition, spatial clustering of $K_{Ca}3.1$ channels and Na^+/K^+-ATPase in a concentrated microdomain within the endothelial cell projections may serve to focus K^+ efflux for optimal stimulation of the pump, thus amplifying and/or initiating EDHF hyperpolarization in the smooth muscle cells.

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Disclosures

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