Modulation of Endothelial Cell $K_{Ca3.1}$ Channels During Endothelium-Derived Hyperpolarizing Factor Signaling in Mesenteric Resistance Arteries

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Abstract—Arterial hyperpolarization to acetylcholine (ACh) reflects coactivation of $K_{Ca3.1}$ ($IK_{Ca}$) channels and $K_{Ca2.3}$ ($SKCa$) 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 $K_{Ca3.1}$ and $K_{Ca2.3}$ channels, and we investigated the mechanisms responsible in rat mesenteric arteries. $K_{Ca3.1}$ channel input to EDHF hyperpolarization was enhanced by reducing external $[Ca^{2+}]$, but blocked either with forskolin to activate protein kinase A or by limiting smooth muscle $[Ca^{2+}]$, increases stimulated by phenylephrine depolarization. Imaging $[Ca^{2+}]$, within the endothelial cell projections forming myoendothelial gap junctions revealed increases in cytoplasmic $[Ca^{2+}]$, during endothelial stimulation with ACh that were unaffected by simultaneous increases in muscle $[Ca^{2+}]$, evoked by phenylephrine. If gap junctions were uncoupled, $K_{Ca3.1}$ 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 $K_{Ca3.1}$ 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 $K_{Ca2.3}$ immunostain at endothelial cell borders, including endothelial cell projections, whereas $K_{Ca3.1}$ channels and Na$^+$/K$^+$-ATPase $\alpha_1/\alpha_2$ subunits were highly concentrated in endothelial cell projections and adjacent to myoendothelial gap junctions. Thus, extracellular $[Ca^{2+}]$, appears to modify $K_{Ca3.1}$ channel activity through a protein kinase A-dependent mechanism independent of changes in endothelial $[Ca^{2+}]$. The resulting hyperpolarization links to arterial relaxation largely through Na$^+$/K$^+$-ATPase, possibly reflecting $K^+$ acting as an EDHF. In contrast, $K_{Ca2.3}$ 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 $[Ca^{2+}]$. In the case of endothelium-derived hyperpolarizing factor (EDHF) (the NO- and prostanoid-independent pathway), this increase crucially activates endothelial $K_{Ca2.3}$ and $K_{Ca3.1}$ channels. Activation of these $K_{Ca}$ channels leads on to arterial hyperpolarization and dilation (see elsewhere for review), and a changing role for each subtype has been implicated in pathological responses within blood vessels.

Although activation of both $K_{Ca2.3}$ and $K_{Ca3.1}$ 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 $[Ca^{2+}]$ (with acetylcholine [ACh]) in quiescent rat mesenteric arteries hyperpolarizes the resting membrane potential through $K_{Ca2.3}$ channels alone, but, during smooth muscle depolarization and the associated contraction necessary to observe EDHF relaxation, $K_{Ca3.1}$ channels are also activated. This differential activation correlates with a distinct subcellular distribution of the channels. $K_{Ca3.1}$ 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, $K_{Ca2.3}$ channels are diffusely distributed throughout the plasmalemma of endothelial cells. How the different channel types are activated independently is not known, but concentrating $K_{Ca3.1}$ 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.
derived from or associated with the contracting smooth muscle. In small arteries and arterioles, Ca²⁺ and/or a Ca²⁺ signal has been shown to spread from the muscle to the endothelium. In the mesenteric artery, this process relies on Ca²⁺ signaling via MEGJs. Extracellular Ca²⁺ levels ([Ca²⁺]₀) 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 KCa3.1 channels selectively and appears to colocalize with this channel (but not with KCa2.3 channels). This raises the possibility that alterations in extracellular Ca²⁺ concentration may help determine the relative contribution from KCa3.1/2.3 channels. In addition, KCa3.1 but not KCa2.3 channel activity is inhibited by protein kinase A phosphorylation in enteric neurons, indicating regulation may occur independently of intracellular [Ca²⁺] change.

In the rat mesenteric artery, transfer of endothelial hyperpolarization to the muscle, in part, reflects K⁺ ion efflux through endothelial KCa3 channels and, in part, spread of hyperpolarization via MEGJs. Extracellular K⁺, mimicking its action as a diffusible EDHF, stimulates smooth muscle hyperpolarization and relaxation primarily by activating Na⁺/K⁺-ATPase. 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 KCa channels. 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. Therefore, 1 possibility is that KCa2.3 and KCa3.1 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 KCa2.3 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.

The aims of the present study were, therefore: (1) to investigate how KCa3.1 channels are independently activated; and (2) to show whether the subcellular distribution of KCa2.3/KCa3.1 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, Ca²⁺ imaging from both smooth muscle and endothelial cells, and immunohistochemistry of rat isolated small mesenteric arteries.

**Results**

**Reducing External [Ca²⁺]₀, Recruits KCa3.1 Channel Input to EDHF Hyperpolarization**

Reducing external [Ca²⁺]₀, from 2.5 to 1.0 mmol/L slightly reduced smooth muscle resting membrane potential (from −51.7±1.6 mV 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 Ca²⁺ (and see also Crane et al), apamin failed to block EDHF hyperpolarization in 1 mmol/L Ca²⁺ (n=4). Furthermore, low [Ca²⁺]₀, facilitated the ability of the KCa3.1 channel activator 1-EBIO (100 and 300 μmol/L) to evoke smooth muscle hyperpolarization (Figure 1B). In separate experiments using 1.0 mmol/L [Ca²⁺]₀, 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 [Ca²⁺]₀, also modified arterial tone (Figure 1 in the online data supplement). Increasing [Ca²⁺]₀, from 1.0 mmol/L relaxed precontracted arteries via endothelial cell KCa3.1 channels, with complete relaxation obtained in the presence of 2.5 mmol/L [Ca²⁺]₀.

**Limiting Smooth Muscle Ca²⁺ Increase Modifies KCa2.3 and KCa3.1 Channel Input to EDHF Dilation in Rat Mesenteric Arteries**

In the presence of the voltage-gated Ca²⁺ 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 mmol/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 KCa3.1 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...
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 [Ca²⁺], and tension increase to PE (Figure 2D through 2F).

Imaging both global and subcellular increases in endothelial cell [Ca²⁺] 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 Ca²⁺ 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, 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:...
Materials and Methods section in the online data supplement for sections were not loaded in these experiments. (See the expanded to 0.6 \( \text{mol/L} \), \( n = 8 \), without carbenoxolone. However, this reduction in maximum hyperpolarization was not sufficient to modify the associated relaxation. In contrast, in the additional presence of 1 \( \mu \text{mol/L} \) TRAM-34, relaxation to ACh was almost abolished (92.5\( \pm \)2.8\% \( n = 5 \)) to 26.1\( \pm \)6.7\% \( n = 5 \); Figure 4B). Ouabain (100 \( \mu \text{mol/L} \)) had a similar inhibitory effect to TRAM-34, depressing relaxation to ACh (from 98.9\( \pm \)1.0\%\) to only 23.5\( \pm \)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 \( K_{\text{Ca}} \), 2.3 Channels and C-Type Natriuretic Peptide?

Use of the selective natriuretic peptide receptor-C antagonist M372049 (100 \( \mu \text{mol/mL} \)) and comparison of EDHF responses (to ACh) with the action of exogenous C-type natriuretic peptide (CNP) did not suggest any link between \( K_{\text{Ca}} \), 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 \( \alpha \), (and similarly, \( \alpha \), ) 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 to regions of MEGJ formation where we have previously demonstrated colocalization of \( K_{\text{Ca}} \), 3.1 channels, connexin (Cx)40, and Cx37.\(^{5,16} \) In contrast, staining for \( K_{\text{Ca}} \), 2.1 channels was homogeneous within endothelial cells, and that for \( K_{\text{Ca}} \), 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 \( K_{\mu} \), \( K_{\text{Ca}} \), 2.3, and \( K_{\text{Ca}} \), 3.1 channels in endothelial cell projections, whereas staining for \( K_{\text{Ca}} \), 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 \( K_{\text{Ca}} \), 3.1 channels to contribute to EDHF hyperpolarization depends on extracellular \([\text{Ca}^{2+}]_o\), 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 \( K_{\text{Ca}} \), 3.1 channels to EDHF hyperpolarization (and relaxation) did not appear to reflect \( \text{Ca}^{2+} \) release signal passing from the smooth muscle into the endothelial projections that form MEGJs. However, it may reflect a local depletion of extracellular \([\text{Ca}^{2+}]_o\), around the endothelial projections containing \( K_{\text{Ca}} \), 3.1 as \([\text{Ca}^{2+}]_o\), enters the smooth muscle through VGCC, because recruitment was reduced in the presence of VGCC blockers to limit the ability of PE to

![Figure 3](https://example.com/image.png)

Figure 3. Similar increases in endothelial cell \([\text{Ca}^{2+}]_o\), 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 \([\text{Ca}^{2+}]_o\), 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 \( \mu \text{m} \). Lower micrographs show the time course of fluorescence changes in individual endothelial cells in response to ACh (1 \( \mu \text{mol/L} \)) added during baseline conditions (left) and in the presence of PE (0.3 to 0.6 \( \mu \text{mol/L} \) ) (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 \([\text{Ca}^{2+}]_o\), were measured before and during ACh (1 \( \mu \text{mol/L} \), either from baseline or in the presence of PE (0.3 to 0.6 \( \mu \text{mol/L} \)), \( n = 3 \), 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\( \pm \)1.0 \( \text{mV} \), 6.3\( \pm \)0.4 \( \text{mN} \); with carbenoxolone: 9.2\( \pm \)1.6 \( \text{mV} \), 6.9\( \pm \)0.6 \( \text{mN} \); \( n = 8 \), the ACh-evoked increase in membrane potential was reduced by \( \approx 10 \text{ mV} \) (Figure 4B), increasing to \( -63.4 \pm 4.5 \text{ mV} \) \( n = 7 \) compared with \( -70.9 \pm 2.4 \text{ mV} \) \( n = 8 \) without carbenoxolone. However, this reduction in maximum hyperpolarization was not sufficient to modify the associated relaxation. In contrast, in the additional presence of 1 \( \mu \text{mol/L} \) TRAM-34, relaxation to ACh was almost abolished (92.5\( \pm \)2.8\% \( n = 5 \)) to 26.1\( \pm \)6.7\% \( n = 5 \); Figure 4B). Ouabain (100 \( \mu \text{mol/L} \)) had a similar inhibitory effect to TRAM-34, depressing relaxation to ACh (from 98.9\( \pm \)1.0\%) to only 23.5\( \pm \)5.7\% in the presence of carbenoxolone \( n = 3 \). In contrast, under similar conditions, apamin had only a slight inhibitory effect (Figure 4B).
Finally, KCa3.1 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\(_{\text{ir}}\) channels). This suggests that K\(^{+}\) efflux through KCa channels discretely localized in endothelial projections may underlie its action as a “diffusible” EDHF, whereas hyperpolarization attributable to KCa channels expressed near interendothelial cell borders (KCa2.3) relies primarily on extant MEGJs for effective spread into the media.

In the rat mesenteric artery, we have recently shown that KCa3.1 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 KCa3.1 channels colocalized with Cx37 and Cx40 at the interface with the smooth muscle. Furthermore, at an ultrastructural level KCa3.1 channels and both Cx37 and Cx40 conjugated gold label was associated with endothelial cell projections forming MEGJs. In contrast, although KCa2.3 channels were localized in close proximity to Cx37, Cx40, and Cx43, they appeared to be mainly adjacent to endothelial–endothelial cell gap junctions.\(^5\) These observations correlate with electrophysiological and immunoprecipitation data. In the former, the contribution from KCa2.3 and KCa3.1 channels to ACh-mediated hyperpolarization could be separated. Endothelial KCa3.1 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, KCa2.3 and KCa3.1 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

<table>
<thead>
<tr>
<th>Tension (mN)</th>
<th>[PE] (µmol/L)</th>
<th>Relaxation (%)</th>
<th>pD(_2)</th>
<th>n</th>
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<tbody>
<tr>
<td><strong>Low contraction</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>10.8±0.6</td>
<td>0.4±0.1</td>
<td>96.4±1.2</td>
<td>7.2±0.1</td>
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<tr>
<td>+Carb</td>
<td>8.9±0.7</td>
<td>0.6±0.1</td>
<td>92.9±3.2</td>
<td>6.9±0.1</td>
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<tr>
<td><strong>High contraction</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>19.0±2.5</td>
<td>10.0±0.0</td>
<td>95.4±0.6</td>
<td>7.0±0.1</td>
</tr>
<tr>
<td>+Carb</td>
<td>18.6±1.8</td>
<td>10.0±0.0</td>
<td>10.3 ±7.7*</td>
<td>7.2±0.5</td>
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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.
our morphological data and provide novel insight into the
control of endothelial KCa3.1 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 perfu-
sion fixation and flat mounting for microscopy),5 confirmed
the restriction of KCa3.1 channels within endothelial cell
projections but also revealed a colocalization of KCa2.3.

With regard to the mechanism responsible for recruiting
the KCa3.1 channels within endothelial cell projections, this
was shown to be sensitive to [Ca2+]o, around the outside of the
projections. Reducing [Ca2+]o, recruited KCa3.1 channels to
EDHF hyperpolarization. These observations are consistent
with recent evidence in lean Zucker rats, in which both KCa2.3
and KCa3.1 channels contributed to EDHF hyperpolarization
in arteries bathed in Krebs containing 1.6 mmol/L [Ca2+]o.2 A
reasonable explanation in both studies is that lowering [Ca2+]o
reduces basal stimulation of an extracellular G protein–linked
CaSR, receptors known to colocalize with KCa3.1 channels in
"noncaveolin" membrane fractions of mesenteric endothelial
cells.9 Thus, lowering [Ca2+]o, from 2.5 to 1 mmol/L reduced
extracellular CaSR saturation and effectively increased avail-
ability of membrane KCa3.1 channels for activation by in-
creases in [Ca2+]i, within the endothelial projections.

Limiting increases in smooth muscle [Ca2+]i was also
linked to the ability of endothelial KCa3.1 channels to provide
significant input to EDHF hyperpolarization. Nifedipine or
diltiazem reduced voltage-dependent Ca2+ entry into the
smooth muscle, associated with a loss of KCa3.1 channel input
to the EDHF response (evoked by ACh) and, as a conse-
quence, sole reliance on the activation of apamin-sensitive
KCa2.3 channels. One possible explanation is that during
“normal” increases in smooth muscle [Ca2+]i, KCa3.1 channel
input to the EDHF response is enabled because the increase in
smooth muscle [Ca2+]i, following depolarization indirectly
facilitates Ca2+ signaling in the endothelial cell projections
and activates the channels. Direct measurement of endothelial
cell [Ca2+]i, levels in both resistance arteries and arterioles has
shown that smooth muscle stimulation with PE leads to
[Ca2+]i increases in the endothelium.6–8 In isolated mesenteric

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**Figure 5.** Endothelial cell immunohistochemical expression pattern for Na+/K+-ATPase, KCa2.1, KCa3.1, and KCa2.3 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 subunit and KCa3.1 channels are highly expressed within the holes, whereas KCa2.1 and KCa2.3 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 μm².
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. However, the additional movement of Ca\(^{2+}\) into endothelial cell projections (and throughout the cells) 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\(^{2+}\)], 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\(^{2+}\)], within the projections during changes in diameter.

Alternatively, Ca\(^{2+}\) entry into the contracting muscle itself may be sufficient normally to reduce the [Ca\(^{2+}\)] in the restricted extracellular space round the endothelial cell projection. As such, this would reduce "basal" CaSR stimulation and facilitate linked KCa3.1 channel availability, enabling activation of the latter by the increased [Ca\(^{2+}\)], 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\(^{2+}\)], in the limited volume of fluid surrounding the cells. Whatever the precise explanation, within endothelial cell projections KCa3.1 channel activity may be suppressed through the action of protein kinase A. In enteric neurons, protein kinase A appears to maintain the KCa3.1 currents underlying slow, after-hyperpolarization in a closed state. In our experiments, the ability of forskolin to inhibit EDHF hyperpolarization attributable to KCa3.1 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 KCa3.1 channels. Dihydropyridines were initially shown to block the Gardos channel in erythrocytes, and more recently, Jiang et al reported block of EDHF hyperpolarization in guinea pig cochlear artery, in which, in contrast to the mesenteric artery, hyperpolarization appears to depend on KCa3.1 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 KCa3.1 channels, had a similar effect to nifedipine; and (2) nifedipine did not alter hyperpolarization to the KCa3.1 channel activator 1-EBIO. The apparent inability of nifedipine to block KCa3.1 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, or it may simply be that although KCa3.1 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\(^{+}\), 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. 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 KCa3.1 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 KCa3.1 and KCa2.3 channels, with TRAM-34 and apamin together, is necessary to block EDHF effects. The concentration of carbenoxolone we used has been shown not to alter the [Ca\(^{2+}\)], increase evoked in the endothelium with ACh, and it blocks the spread of calcine from the endothelium into the smooth muscle. 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. 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, KCa2.3 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. 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 KCa3.1-dependent signal, the 2 “arms” underpinning the EDHF response in mesenteric arteries. Although a link between KCa3.1 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 (KCa2.3 channel) component of EDHF hyperpolarization. The fact that exogenous CNP only evoked a modest relaxation without any hyperpolarization,
agonist-induced [Ca^{2+}]_{i} activation. Step 3: Activation of smooth muscle VDCC (with PE) causes a local “sink” of [Ca^{2+}]_{i} in the vicinity of the endothelial projections. This reduction reduces stimulation of the CaSR and phosphorylation of K_{Ca3.1} channels in the projections. Step 4: At this point, ACh stimulation of the endothelium raises cytoplasmic [Ca^{2+}]_{i}, now activating “available” K_{Ca3.1}, as well as K_{Ca2.3}. As in Step 2, the resulting hyperpolarization spreads through endothelial–endothelial and MEGJs gap junctions but now reflects input from both K_{Ca2.3} and K_{Ca3.1}. 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_{Ca} 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+}]_{i} increases toward 2.5 mmol/L; and K_{Ca3.1} channel activity is again removed from the control of intracellular [Ca^{2+}]_{i}, now activating “available” KCa3.1, as well as KCa2.3.

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

Overall, our data indicate a clear correlation between the discrete localization of K_{Ca3.1} 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+}]_{i}, change within the very restricted intercellular space between muscle and endothelial cell projections may provide the key to explaining why K_{Ca3.1} 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_{Ca2.3} channels still continue to increase membrane potential to close to E_K. In addition, spatial clustering of K_{Ca3.1} 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

None.

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1. WIRE MYOGRAPHY

Measurement of smooth muscle cell tension and smooth muscle or endothelial cell membrane potential

Male Wistar rats (200-250g) were euthanized using procedures defined by the Animals (Scientific Procedures) Act 1986, UK (Schedule 1 procedure). Segments of mesenteric artery (2 mm long) from a third-order branch (~350 µm) were mounted in a Mulvany-Halpern myograph (model 400A, DMT Denmark) in Krebs solution containing (mmol/L): NaCl, 118.0, NaCO₃, 25; KCl, 3.6; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.2; glucose, 11.0; CaCl₂, 2.5; and gassed with 95 % O₂ and 5 % CO₂ at 37 ºC. Following equilibration, arteries were set to a resting tension equivalent to that generated at 0.9 times the diameter of the vessel at 70 mmHg. Unless otherwise stated, only arteries with functional endothelium were used: over 90% relaxation to 1 µmol/L ACh. All experiments were performed in the presence of the nitric oxide (NO) synthase inhibitor, Nω-nitro-L-arginine methyl ester (100 µmol/L L-NAME). In some experiments, the [Ca²⁺]₀ was reduced to 1.0 mmol/L and allowed to equilibrate for 20 min before continuing experiments.

Sharp glass microelectrodes were used to measure the membrane potential of individual smooth muscle cells or endothelial cells (filled with 2 M KCl, tip resistances 70-100 MΩ), as previously described.¹ ² Rapid deflections in membrane potential to near −50 mV were observed upon cell impalement. The membrane potential was recorded via a pre-amplifier (Neurolog system, Digitimer Ltd., U.K.) linked to a MacLab data acquisition system (AD Instruments Model 4e) at 100 Hz. Drugs were added directly to the 10 mL bath, and mixed by gassing.
In some experiments, phenylephrine (PE) was used to evoke smooth muscle depolarization and contraction. A concentration was used to stimulate a stable level of contraction (\textit{circa} 50% maximum) and depolarization. Simultaneous changes in tension and membrane potential were assessed to (cumulative) concentrations of each agonist. Inhibitors were incubated for at least 15 minutes before repeated agonist responses.

\textit{Experiments using carbenoxolone.} To optimize gap junction uncoupling and to limit non-specific actions, carbenoxolone (100 \(\mu\)mol/L) was only added to the bath for 90-120 min (washed and re-added every 30 min).\(^3\) Further, the effects of carbenoxolone were shown to be reversible following a washout period of \(>\) 1 hour.

\textit{Experiments using forskolin.} At the concentration used to activate PKA in these experiments, forskolin (1 \(\mu\)mol/L) stimulated smooth muscle cell hyperpolarization (~10 mV), which was sensitive to glibenclamide. Therefore in order to assess the effect of forskolin-mediated stimulation of PKA on the hyperpolarization to ACh, experiments were performed in the presence of glibenclamide (10 \(\mu\)mol/L). Glibenclamide had no effect on the resting membrane potential (Control, -47.8 \(\pm\) 0.9 mV, \(n=12\); Glibenclamide, -48.6 \(\pm\) 1.8 mV, \(n=8\)), or the control hyperpolarization to ACh (Figure 1),\(^1\) and fully blocked the hyperpolarization to forskolin (to -50.4 \(\pm\) 1.8 mV, \(n=7\)). Note that these experiments were performed using 1.0 mmol/L \([Ca^{2+}]_o\) and in the presence of apamin (50 nmol/L).

\textit{Measurement of smooth muscle cell tension and changes in smooth muscle cell \([Ca^{2+}]_i\) levels}

A segment of mesenteric artery was mounted as described above except in a Mulvany-Halpern myograph designed for use on a confocal microscope (Model 120CW, Danish Myotechnology) and in MOPS buffer containing (mmol/L): NaCl, 145; KCl, 4.7; CaCl\(_2\), 2.0; MgSO\(_4\), 1.17; MOPS, 2.0; NaH\(_2\)PO\(_4\), 1.2; glucose, 5.0; pyruvate, 2.0; EDTA, 0.02; NaOH, 2.75 (the pH of the solution was adjusted to 7.40 \(\pm\) 0.02 at 37°C). The arteries were loaded
with the calcium-sensitive fluorescent dye, Oregon Green 488 BAPTA-1 AM (dissolved in DMSO and 0.02 % (w/v) Pluronic F-127) for 1 hour (added to the bath for 2 x 30 min), washed, and allowed to de-esterify for > 45 min. After excitation at 488 nm, the fluorescence emission intensity at 515 nm (Ca\(^{2+}\)) was recorded using a confocal microscope (Olympus FV300) mounted on an Olympus IX71 inverted microscope (Olympus, Japan) using a water immersion objective (40x, numerical aperture (NA) 0.8, working distance (WD) 3.3 mm, Olympus, Japan) and images (356 x 260 pixels, 2 Hz) stored for offline analysis (MetaMorph version 6.1, Universal Imaging). Average, relative changes in smooth muscle cell \([Ca^{2+}]_i\) were calculated as changes in intensity of fluorescence divided by fluorescence at time = 0 s (F/F\(_0\)), within selected cell regions over individual smooth muscle cells. Summary data comprise the averaged changes in fluorescence (compared to the paired, control baseline before adding PE and/or nifedipine) within individual arteries for each n value.

*Measurement of smooth muscle cell tension and changes in endothelial cell \([Ca^{2+}]_i\) levels*

A segment of mesenteric artery was mounted as described above in a Mulvany-Halpern confocal myograph system (Model 120CW, Danish Myotechnology) and in MOPS buffer. The endothelium of arteries was selectively loaded with Oregon Green 488 BAPTA-1 AM (prepared as above) by positioning a polished, glass pipette gently into the lumen of the artery and infusing dye for 30-40 min (or until endothelial cells were loaded) with a syringe pump (BeeHive® syringe pump system, Bioanalytical systems, USA). The bath was continuously perfused to avoid loading of smooth muscle cells. The IEL was stained with 0.5 μmol/L Alexa Fluor 633 hydrazide. Fluorescence at 515 nm (Ca\(^{2+}\)) and 660 nm (IEL) was simultaneously imaged using a confocal scanning head (Olympus FV500) mounted on an Olympus IX70 inverted microscope (Olympus, Japan) using a water immersion objective (40x, NA 0.8, WD 3.3 mm, Olympus, Japan) and images (640 x 200 pixels, 2 Hz) stored for
offline analysis (MetaMorph version 6.1, Universal Imaging). Average, relative changes in endothelial cell $[\text{Ca}^{2+}]$, were calculated as changes in intensity of fluorescence divided by fluorescence at time $=0$ s ($F/F_0$), within individual, whole endothelial cells. Each value is the average $F/F_0$ over 5 s, and summary data comprise the averaged changes in fluorescence before and after addition of PE within individual arteries.

2. PRESSURE MYOGRAPHY

Preparation of arteries for pressure myography

A segment of mesenteric artery was cut and then cannulated at each end with glass pipettes (external diameter 150 µm) and positioned near the base of a 1.5 ml temperature-regulated chamber (Warner Instruments) seated on the stage of an inverted microscope (IX 70, Olympus, UK) with attached confocal scanning unit (FV500, Olympus) and in MOPS buffer, as previously.$^{3,4}$ After 30 min superfusion at 37°C, reactivity was assessed by contraction to 3 µM PE followed by endothelium-dependent relaxation to 1 µmol/L ACh. Only vessels relaxing 90%, reflecting undamaged endothelium, were subsequently used.

Selective measurement of changes in endothelial cell projection $[\text{Ca}^{2+}]$, change

We found it was not feasible to load and image changes in $\text{Ca}^{2+}$ levels in endothelial cell projections in the wire myograph for two main reasons. Firstly, accurate timing of endothelial cell loading was not possible (vessels were not cannulated, so flow through the lumen was not fully controlled), and more importantly, we were unable to use high NA objectives in the wire myograph (physical constraint of mounting feet position in the chamber). Therefore arteries pressurized to 70 mmHg were lowered near to the base of the chamber and a high-resolution objective used. Further, the pinhole diameter was reduced as much as possible (200 µm) to improve confocality from within the endothelial cell projection focal plane.
(assessed by the location of the IEL), but this had the disadvantage of reducing the fluorescence intensity emanating from cells.

Following equilibration of pressurized arteries, MOPS solution containing Oregon Green 488 BAPTA-1 AM (10 µM, prepared as above) and 0.5 µM Alexa 633 hydrazide were perfused through the artery lumen for 28-40 min to enable the selective loading of the dye into endothelial cells, with careful attention to loading into endothelial cell projections, and to stain the IEL. The dyes were then washed out by luminal perfusion with MOPS buffer, and allowed to equilibrate for at least 30 min (to allow de-esterification of the Ca^{2+}-dye). After excitation at 488 and 633 nm, the fluorescence emission intensity at 515 nm (Ca^{2+}; from endothelial cells at the bottom of the artery) and 660 nm (IEL) were simultaneously acquired using an oil immersion objective (60x, NA 1.4, WD 0.1 mm, Olympus, Japan), and visualized using an confocal microscope (FV500, Olympus). Images (800 x 172 pixels, 2 Hz) were stored for offline analysis (MetaMorph version 6.1, Universal Imaging). It was not possible to image Ca^{2+} levels during changes in diameter (due to motion and therefore changes in focus), so responses were only obtained during periods of constant diameter (25 s). Further, as the focal plane was changed before and after addition of ACh (i.e. responses could not be accurately compared to baseline), the individual fluorescence levels during this 25 s period (F) are represented relative to the average of the entire 25 s period (F_{av}). Regions were selected 1) within endothelial cell projections, 2) adjacent to endothelial cell projections (each determined by the position of the IEL), and 3) around whole endothelial cells.

**Immunohistochemistry**

Pressurized arteries (mounted as above) were fixed in a 2% paraformaldehyde solution for 10 min at 37°C, washed with Krebs and preincubated for 1h (at 37°C and 50 mmHg in blocking buffer containing 1% BSA and 0.1% Tween20 then overnight in primary antibody (luminally
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and abluminally) at 4°C. This was followed by 1-2 h incubation in Alexa Fluor 488 secondary antibody at room temperature, as previously$^4$. The elastic lamina was visualized following incubation with 0.2 μmol/L Alexa Fluor 633 hydrazide. Arteries were excited at 488 and 633 nm, the fluorescence emitted at 515 and 660 nm from cells through the bottom wall of the artery were acquired through a water immersion objective (40x, NA 0.9, WD 0.15 mm, Olympus, Japan, 1024 x 1024 pixels) using the same laser, pinhole, and photomultiplier tube settings in all experiments. z-Stacks through the wall of the artery were obtained in 0.5 μm increments (FluoView software Version 5.0, Olympus) and reconstructed in 3-D using Imaris software (Version 5.5, Bitplane). For an estimate of staining within the holes through the IEL, the fluorescent spots corresponding to holes through the IEL were counted, and calculated as a percentage of the total number of holes.

Primary antibodies: 1:100 Anti-SK3 ($K_{Ca2.3}$, SK$_{Ca3}$, KCNN3), raised in rabbit against aas 2-21 of human $K_{Ca}$ 2.3 (100% homology to rat sequence), Alomone labs (APC-025); 1:100 Anti-SK4 ($K_{Ca3.1}$, IK$_{Ca1}$, KCNN4), raised in rabbit against aas 350-363 of rat $K_{Ca3.1}$, Alomone labs (APC-064); 1:50 Anti-NaK-ATPase-alpha2, raised in rabbit against aas 432-445 of human sequence (100% homology to rat), Upstate (07-674); 1:100 Anti-NaK-ATPase-alpha3, α3-isoform-specific monoclonal XVIF9-G10 raised in mouse against canine cardiac microsomes (detects rat sequence), Affinity BioReagents (MA3-915); and 1:100 Anti-$K_{ir2.1}$, raised in rabbit against aas 392-410 of human sequence (89% homology to rat), Chemicon (AB5374). Secondary antibodies: 1:100 Alexa Fluor 488 goat anti-mouse and goat anti-rabbit, Molecular Probes (A-11029 and A-11034).

Drugs and dyes

Drugs were all from Sigma except for apamin (Latoxan); 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34, selective IK$_{Ca}$-channel blocker, a gift from Dr H
Wulff, UC Davis); and M372049 (NPR-C antagonist, a gift from Dr AJ Hobbs). Stock solutions of nifedpine, ouabain, and TRAM-34 were prepared in dimethylsulfoxide (DMSO); all other stock solutions were prepared in distilled water. Dyes were all obtained from Molecular Probes: Oregon Green 488 BAPTA-1 AM (O6807); and Pluronic F-127 (P3000MP).

Analysis of data

Values for changes in membrane potential and % relaxation evoked by each agonist were taken as the maximum (peak) responses. Values for tension stimulated by of PE were taken as the plateau (rather than peak) values. All data are expressed as mean ± s.e.mean from n experiments, and were analysed non-parametrically with the Mann Whitney test. A P value of <0.05 was considered significant.

References


Cumulative addition of Ca\(^{2+}\) (1.0 – 3.0 mmol/L) stimulated concentration-dependent relaxation of phenylephrine-contracted arteries. This relaxation was insensitive to apamin, but equally sensitive to TRAM-34 and to endothelial cell damage (Figure 1A), suggesting that the endothelium-dependent relaxation was due to stimulation of K\(_{Ca3.1}\)-channels. Changing the bath [Ca\(^{2+}\)]\(_o\) from 1.0 mmol/L to either 2.0 or 3.0 mmol/L increased the sensitivity to ACh (log EC\(_{50}\): -7.2 ± 0.02, -8.0 ± 0.05, -8.1 ± 0.04, respectively), and in the presence of TRAM-34, the responses to ACh were effectively identical, regardless of [Ca\(^{2+}\)]\(_o\).

Legend for Online Figure 1. Effects of [Ca\(^{2+}\)]\(_o\) alone and on responses to ACh in rat mesenteric arteries mounted in a wire myograph. A, Summarized data showing the average relaxation to increases in [Ca\(^{2+}\)]\(_o\), from 1.0 to 3.0 mmol/L under control conditions (n=14) or
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in the presence of TRAM-34 (1 µM) either alone (n=6) or together with apamin (50 nmol/L, n=5) or following endothelial cell damage (with a hair, n=3). B, Summarized data showing the effect of the bath [Ca^{2+}]_o on the relaxation to ACh either under control conditions (n=14-21) or in the presence of TRAM-34 (1 µmol/L, n=12-14).

**Online Supplementary Figure 2**

Cumulative addition of the putative EDHF, CNP (1 nmol/L – 1 µmol/L) did not stimulate any hyperpolarization in quiescent mesenteric arteries (1 µmol/L: 0.4 ± 0.7 mV, n=4), even though 1 µmol/L ACh subsequently increased potential by 35.8 ± 1.2 mV (n=4). EDHF-hyperpolarization to ACh was not modified by the selective natriuretic peptide receptor-C (NPR-C) antagonist, M372049 (100 nmol/L) or with 30 µmol/L Ba^{2+}, but was significantly reduced with apamin (50 nmol/L, n=4, online Figure 2A). During PE contraction, CNP stimulated a slow relaxation (to a maximum of 65.8 ± 8.3%, n=6), associated with inconsistent, and at best modest hyperpolarization (1 µmol/L: 2.5 ± 3.9 mV, n=6; hyperpolarization recorded in only 2 of 6 experiments, maximum 11 mV). M372049 blocked relaxation to CNP (online Figure 2B), but did not modify EDHF-hyperpolarization and relaxation to ACh, online Figure 2C. Ouabain suppressed both EDHF-hyperpolarization and relaxation, but this inhibition was not increased by the additional presence of M372049 (online Figure 2C).
Legend for Online Figure 2. Lack of contribution by CNP to EDHF responses in rat mesenteric arteries mounted in a wire myograph. A, Summarized data showing the average changes in membrane potential (Δ Em) to cumulative increases in [ACh] in quiescent arteries in the absence (n=5-6) or presence of either barium (30 µmol/L, n=4), M372049 (100 nmol/L, n=4), or apamin (50 nmol/L, n=4). M372049 had no effect on ACh-mediated hyperpolarization, despite fully blocking the relaxation to CNP (B, n=4-6). C, Summarized data showing average changes in membrane potential and relaxation (%) to ACh in arteries stimulated by PE (Control, n=8) or in the additional presence of either M372049 (100 µmol/L, n=4), ouabain (100 µmol/L, n=4-7), or the combination of both agents (n=4-6). Asterisk indicates significant difference relative to control (P<0.05).