Involvement of Myoendothelial Gap Junctions in the Actions of Endothelium-Derived Hyperpolarizing Factor

Shaun L. Sandow,* Marianne Tare,* Harold A. Coleman, Caryl E. Hill, Helena C. Parkington

Abstract—The nature of the vasodilator endothelium-derived hyperpolarizing factor (EDHF) is controversial, putatively involving diffusible factors and/or electrotonic spread of hyperpolarization generated in the endothelium via myoendothelial gap junctions (MEGJs). In this study, we investigated the relationship between the existence of MEGJs, endothelial cell (EC) hyperpolarization, and EDHF-attributed smooth muscle cell (SMC) hyperpolarization in two different arteries: the rat mesenteric artery, where EDHF-mediated vasodilation is prominent, and the femoral artery, where there is no EDHF-dependent relaxation. In the rat mesenteric artery, stimulation of the endothelium with acetylcholine (ACh) evoked hyperpolarization of both ECs and SMCs, and characteristic pentalaminar MEGJs were found connecting the two cell layers. In contrast, in the femoral artery, ACh evoked hyperpolarization in only ECs but not in SMCs, and no MEGJs were present. Selective hyperpolarization of ECs or SMCs evoked hyperpolarization in the other cell type in the mesenteric artery but not in the femoral artery. Disruption of gap junctional coupling using the peptide Gap 27 markedly reduced the ACh-induced hyperpolarization in SMCs, but not in ECs, of the mesenteric artery. These results show that transfer of EC hyperpolarization or of a small molecule to SMCs through MEGJs is essential and sufficient to explain EDHF. (Circ Res. 2002;90:1108-1113.)

Key Words: endothelium-derived hyperpolarizing factor • myoendothelial gap junctions • endothelium • smooth muscle • electrical coupling

The endothelium plays a central role in the regulation of vascular tone.¹ The endothelium is capable of exerting a profound relaxing influence on the underlying smooth muscle, mediated by at least three different factors, depending on the vascular bed. These include nitric oxide (NO) and prostacyclin, both diffusible factors.²–⁶ In addition, after blockade of NO and prostacyclin synthesis, stimulation of the endothelium is capable of evoking vascular smooth muscle relaxation that has been attributed to a third factor, endothelium-derived hyperpolarizing factor (EDHF).⁷–¹⁵

The hallmark of the vasorelaxation attributed to EDHF is that it is accompanied by membrane hyperpolarization. Consensus regarding the nature of EDHF is lacking, with evidence suggesting the involvement of a diffusible factor(s) released from the endothelium in some vascular beds.¹¹,¹²,¹⁶,¹⁷ Others propose that the hyperpolarization generated in endothelial cells (ECs) is capable of spreading electrotonically to the underlying smooth muscle cells (SMCs),¹⁸,¹⁹,²¹,²³,²⁵ most likely via myoendothelial gap junctions (MEGJs).²⁰,¹⁸,¹⁹,²¹,²³–²⁶ Evidence to support the transfer of a small molecule (eg, cAMP) via MEGJs has also been presented.²⁰,²² Support for the MEGJ hypothesis has come from studies in which gap junctions have been pharmacologically blocked, either with peptide mimetics of connexin 43 (Cx43)²⁷–³⁰ or with glycyrrhetinic acid derivatives.¹³,²⁶,³¹–³³ Although the former have been demonstrated to indeed reduce dye coupling between Cx-transfected cells,³⁴ the latter seem to have some nonspecific actions.³¹–³³,³⁵

An alternative and complementary approach would be to study an artery that lacked an EDHF-dependent relaxation and to test for the existence of agonist-induced hyperpolarization in the ECs and for the presence of MEGJs. The femoral artery represents such a tissue because EDHF does not contribute to endothelium-dependent SMC relaxation in this vessel.³⁶,³⁷ In the present study, this artery was compared with the mesenteric artery, where vasodilation to EDHF is prominent. We tested the hypothesis that the ECs of both beds could generate hyperpolarization, but that the lack of EDHF in the SMCs of the femoral artery was due to the absence of MEGJs.

Materials and Methods

The experiments were carried out under the guidelines of the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes.

Electrophysiological Experiments

Sixteen-week-old male Wistar rats (Monash University Central Animal Services, Victoria, Australia) were anesthetized with chlo-
roform and killed by exsanguination. Femoral (first order) and mesenteric (first and second order) arteries were isolated. A segment of artery was then cut longitudinally and pinned to the base of a recording chamber with the endothelium uppermost. In some experiments, arterial segments were left as intact tubes. Preparations were continuously superfused with physiological saline (mmol/L: NaCl 120, KCl 5, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1, NaHCO₃ 25, and glucose 11) at 35°C and bubbled with O₂ 95% and CO₂ 5%. The NO synthase inhibitor N⁵-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) and the cyclooxygenase inhibitor indomethacin (1 μmol/L) were present in all experiments to inhibit NO and prostanoid production, respectively. Membrane potentials of ECs and SMCs were recorded using intracellular glass microelectrodes with resistances of 100 to 150 MΩ and bubbled with O₂ 95% and CO₂ 5%. The NO synthase inhibitor N⁵-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) and the cyclooxygenase inhibitor indomethacin (1 μmol/L) were present in all experiments to inhibit NO and prostanoid production, respectively. Membrane potentials of ECs and SMCs were recorded using intracellular glass microelectrodes with resistances of 100 to 150 MΩ. The tips of the electrodes were filled with 2% Lucifer Yellow CH and backfilled with 1 mol/L KCl.33–35 During the experiments, Lucifer Yellow readily diffused into the impaled cell, thus allowing unequivocal identification of every cell impaled.

The ECs were stimulated for 1 minute by the addition of acetylcholine (ACh) to the superfusate to evoke the response attributed to EDHF. Selective stimulation of ECs with 1-ethyl-2-benzimidazolinone (1-EBIO) and of SMCs with levocromakalim was also for 1 minute by addition of the channel opener to the superfusate.

Cell-to-cell coupling was disrupted by the connexin mimetic peptide Gap 2727 (sequence SRPTKETFII; Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University; purity >97%). Gap 27 was dissolved directly into the physiological saline and the solution was oxygenated, warmed, and recirculated over the preparations for 1 to 3 hours. In a pilot study, we confirmed that this recirculation procedure had no effect on the resting membrane potential or on the hyperpolarization evoked by 10 μmol/L ACh in either ECs or SMCs. Responses to ACh were tested at 0.5- and/or 1-hour intervals.

### Electron Microscopy

#### Animals and Tissue Preparation

Sixteen-week-old male Wistar rats were anesthetized with an intraperitoneal injection of ketamine and xylazine 2% (44 and 8 mg/kg, respectively) and perfused (~60 mm Hg) via the left ventricle with physiological saline (0.9% NaCl) containing 0.1% NaNO₃, 0.1% BSA, and 5 U/mL aprotinin. Animals were then cut from both the femoral and mesenteric arteries of each animal. Tissues were subsequently postfixed in 2% osmium tetroxide (pH 7.35) at 25°C for 2 hours. Care was taken to obtain the same region of vessel from each animal. Tissues were subsequently postfixed in 2% osmium tetroxide in the same buffer for 2 hours, stained with saturated aqueous uranyl acetate for 2 hours, and embedded in Araldite 502 according to conventional procedures.

#### Serial-Section Electron Microscopy

Methods for serial-section analysis were similar to those previously described by Sandow and Hill. Briefly, 50 transverse serial sections (one segment totaling ~5 μm of vessel, each from a different animal) were cut from both the femoral and mesenteric arteries of three animals. Low-magnification micrographs (~2500) of the central section of each series were taken on plate film on a Hitachi 7100 transmission electron microscope, and the vessel circumference was estimated as the length of the internal elastic lamina (IEL). The number of medial SMC layers was determined by averaging the number of individual SMC profiles, ~5 μm in length, from the outer edge of the IEL to the inner edge of the external elastic lamina along each of four linear plots 90° apart, from each of 3 vessels each from a different animal.

To detect MEGJs, the IEL in each serial section was examined at ×10,000. All MEGJs through serial sections were photographed on plate film at high magnification (~20,000 to ~40,000), and the numbers of MEGJs with characteristic pentalaminar membrane structure were noted. Reconstruction was performed using the MCID Imaging system (Imaging Research). Photographed MEGJ profiles and their surrounding EC and SMC regions were digitized from contact prints using a flatbed scanner (HP Scanjet 4c), at a resolution of 300 dots per inch. Selected gap junctions between adjacent SMCs and adjacent ECs were photographed at ~20,000 to ~40,000.

### Morphology of ECs and Determination of the Number of MEGJs per EC

To determine EC dimensions, whole-mount regions of femoral and mesenteric arteries (as above) were processed for CX40 immunohistochemistry (1:100), because this staining highlights the cell periphery. Antibodies were raised in sheep against a peptide corresponding to amino acids 254 to 270 of rat CX40, as used previously. Staining was detected after incubation in Cy3-conjugated donkey anti-goat immunoglobulins (1:100, Jackson Immuno-Research) for 1 hour at 25°C. Preparations were mounted in buffered glycerol, and 1-μm optical sections were taken through the EC layer using a Leica TCS 4D-confocal microscope, with images being recombined to produce a single image of the labeled ECs.

The number of MEGJs per EC was estimated from the vessel circumference at the level of the IEL, the number of ECs in this region, and MEGJs present in the 5-μm region.

#### Statistical Analysis

Data are expressed as mean±SEM; n indicates the number of animals tested. Student’s t test, paired or unpaired, as appropriate, was used to test significance using the software package InStat 3 (GraphPad Corp). Concentration-hyperpolarization curves were fitted using the least-squares method (GraphPad), which were used to determine the pD₂ (−log EC₅₀). For the histological data, statistical significance was tested using a one-way ANOVA followed by pairwise t tests with Bonferroni correction for multiple group comparisons and with paired or unpaired t tests for groups of two. Values of P<0.05 were considered statistically significant.

### Results

#### EDHF-Attributed Responses in Identified ECs and SMCs

Resting membrane potentials (RMPs) in dye-identified femoral artery ECs were only ~26±1 mV (n=5) compared with ~58±2 mV in the SMCs of the same tissues (Figure 1). In contrast, the RMPs in mesenteric ECs were not significantly different from those of mesenteric SMCs (Figures 1A through 1C; Table). RMPs of ECs in femoral and mesenteric arteries were not significantly different (Figure 1C; Table). Stimulation of the endothelium of the femoral artery with ACh evoked 45±2 mV (n=5) hyperpolarization in ECs but failed to initiate any hyperpolarization in the SMCs (Figures 1B and 1C), even in SMCs that were immediately beneath the IEL. Conversely, in the mesenteric artery, ACh evoked comparable concentration-dependent hyperpolarizations in both ECs and SMCs (Figures 1B and 1C; Table). The maximum amplitude of EC hyperpolarization in the mesenteric artery was only half of that in femoral ECs, but the level of membrane potential reached during the maximum hyperpolarization was not different in the two arteries (Figure 1C).

Intermediate- and small-conductance Ca²⁺-activated K⁺ channels have been implicated in EDHF in many arterioles. Mesenteric EC and SMC hyperpolarizations in response to ACh (10 μmol/L) were abolished by charybdotoxin (ChTx; 30 to 50 nmol/L) plus apamin (0.1 to 0.5 μmol/L).
μmol/L; n=4), which are blockers of these channels. Removal of the endothelium abolished EDHF-mediated SMC hyperpolarization in the mesenteric artery. In the femoral artery, ChTx plus apamin all but abolished (95±2%, n=4) EC hyperpolarization. That the hyperpolarization attributed to EDHF was recorded in the ECs, but not in SMCs, of the femoral artery is consistent with findings that the ChTx- and apamin-sensitive K⁺ channels underpinning this response are located on the ECs.⁴⁰,⁴¹

### Identification of MEGJs

MEGJs did not occur in the femoral artery: there were no MEGJs in the 5-μm region examined (n=3), equivalent to an

### Properties of ECs and SMCs in Mesenteric and Femoral Arteries

<table>
<thead>
<tr>
<th></th>
<th>Mesenteric Artery</th>
<th>Femoral Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel circumference, μm</td>
<td>648±57 (3)</td>
<td>963±38 (3)</td>
</tr>
<tr>
<td>No. of SMC layers</td>
<td>4.8±0.27 (3)</td>
<td>9.8±0.62 (3)</td>
</tr>
<tr>
<td>SMCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMP, mV</td>
<td>-57±1 (5)</td>
<td>-58±2 (5)</td>
</tr>
<tr>
<td>Maximum EDHF-mediated hyperpolarization, mV</td>
<td>18±2 (5)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>pD₂</td>
<td>6.78±0.13 (5)</td>
<td>...</td>
</tr>
<tr>
<td>ECs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMP, mV</td>
<td>-54±1 (5)</td>
<td>-26±3 (5)</td>
</tr>
<tr>
<td>Maximum hyperpolarization, mV</td>
<td>22±2 (5)</td>
<td>45±2 (5)</td>
</tr>
<tr>
<td>pD₂</td>
<td>7.32±0.12 (5)</td>
<td>6.3±0.15 (5)</td>
</tr>
<tr>
<td>No. of MEGJs in 5-μm vessel length</td>
<td>7.3±1.5 (3)</td>
<td>0 (3)</td>
</tr>
<tr>
<td>EC dimensions, μm²</td>
<td>351±5</td>
<td>363±6</td>
</tr>
</tbody>
</table>

Results are mean±SEM, with No. of animals in parentheses. First- and second-order mesenteric arteries and first-order femoral arteries were used.
Area of the endothelium occupied by 13.3 ECs (Figure 2A). In contrast, in the mesenteric artery, MEGJs occurred with a frequency of 0.79 per EC, ie, 7.3/1006/1.5 MEGJs in the 5/m9262/m region examined (n=3), equivalent to an area of the endothelium occupied by 9.2 ECs (Figures 2C and 2D). All MEGJs in the mesenteric arteries occurred on projections arising from ECs. Pentalaminar gap junctions were identified between adjacent SMCs and between adjacent ECs in both vessels (eg, Figure 2B). Vessel circumference and the number of SMC layers were significantly less, whereas EC dimensions were not significantly different in the mesenteric compared with the femoral artery (Table).

Separate Generation of Hyperpolarization in ECs and SMCs

We further tested the existence or otherwise of electrical crosstalk between the two cell types in each artery by generating hyperpolarizations in either SMCs or ECs. In rat and rabbit mesenteric arteries, the ATP-sensitive K+ channel opener levcromakalim generates hyperpolarization in SMCs.42,43 Levcromakalim evoked SMC hyperpolarization in both femoral and mesenteric arteries, but hyperpolarization was recorded in the ECs in only the mesenteric artery (Figure 3A). Intermediate-conductance Ca2+-sensitive K+ channels, present on native ECs, can be activated using 1-EBIO.44,45 Concentrations =100 μmol/L selectively evoked EC hyperpolarization in both arteries (Figure 3B). SMC hyperpolarization also occurred in the mesenteric, but not in femoral, SMCs (Figure 3B). In both arteries, EC hyperpolarization evoked by 1-EBIO was blocked by ChTx plus apamin. 1-EBIO (100 μmol/L) failed to evoke SMC hyperpolarization in mesenteric arteries denuded of endothelium. Taken together, these results demonstrate that there is electrical coupling between the ECs and SMCs in the mesenteric, but not in the femoral, artery.

Influence of the Gap Junction Inhibitor Gap 27 on EDHF in the Mesenteric Artery

EC and SMC hyperpolarizations were recorded in response to 1-minute stimulation with 10 μmol/L ACh. Incubation with 300 μmol/L Gap 27 (for 1 to 3 hours) had no effect on the RMP of the SMCs, but the amplitude of EDHF-attributed hyperpolarization in SMCs was significantly attenuated (progressive reduction to 33±11% over 3 hours, n=5; Figure 4). In time controls run for 2 to 3 hours in the absence of Gap 27, EDHF-attributed hyperpolarization in SMCs was not significantly different to their initial response (97±5% of the initial response, n=7). In contrast to the effect of Gap 27 on SMCs, EDHF-attributed hyperpolarization in ECs after incubation in Gap 27 for 1 to 3 hours was not significantly different to their initial response (110±9% of the initial response, n=5, Figure 4).

Discussion

The present study demonstrates that a lack of MEGJs in the femoral artery accounts for the absence of electrical coupling between ECs and SMCs and a lack of EDHF in this vessel. The large difference in RMPs of ECs and SMCs in the femoral artery is consistent with this absence of functional...
electrical connectivity. Endothelium-dependent relaxation in the femoral artery thus relies entirely on the release of a diffusible factor, NO. Conversely, MEGJs allow the transmission of electrical responses between ECs and SMCs, and they function as a pathway for the spread of EC hyperpolarization to SMCs in the mesenteric artery. This also accounts for the similar RMPs in ECs and SMCs in this vessel. The role of MEGJs in conducting EDHF-attributed hyperpolarization, either electrically or via a small intermediate molecule, is further supported by the effectiveness of Gap 27 in reducing the hyperpolarization in only the SMCs, while leaving intact the response in the ECs, similar to previous observations in this vascular bed.

The prominence of EDHF generally increases as vessel size decreases. A strong syncytial relationship between ECs and SMCs exists in hamster retractor muscle feed arteries and mesenteric arterioles, where EDHF is prominent. The role of EDHF in vessels with larger numbers of medial SMCs may be diminished, despite the existence of MEGJs, because of the dissipation of the hyperpolarizing current into the “electrical sink” of the increased number of layers of SMCs. These large arteries thus rely on the release of a diffusible factor. In the present study, however, the absence of an EDHF response in the femoral artery seems due to the absence of MEGJs and the absence of an EDHF-mediated hyperpolarization in the SMCs.

At least two EDHFs can be recognized on the basis of their susceptibility to ion channel/transport blockade. In some arteries, the hyperpolarization is abolished by blockers of large-conductance Ca$^{2+}$-activated K$^+$ channels (BK$_{Ca}$ channels) such as iberiotoxin. Products of the cytochrome P-450 pathway, epoxycosatrienoic acids (EETs) produced by ECs, are likely candidates because they activate BK$_{Ca}$ channels on SMCs. In other vessels, especially small arteries and arterioles, however, the EDHF hyperpolarization is resistant to iberiotoxin but is blocked by the combination of ChTx plus apamin. In some of these vessels, the responses are also blocked by Ba$^{2+}$ and ouabain and these have been attributed to the release of K$^+$ from ECs acting on inwardly rectifying K$^+$ channels and the Na$^+$/K$^+$/H$^+$-ATPase on the SMCs. In many other vessels, however, the EDHF hyperpolarization is resistant to iberiotoxin or Ba$^{2+}$ and ouabain. The hyperpolarization generated in the ECs in both the femoral and mesenteric arteries in the present study is blocked by ChTx plus apamin.

Because the ChTx- and apamin-sensitive K$^+$ channels underpinning the EDHF response are located on the ECs, a recurring hypothesis has been that the SMC hyperpolarization attributed to this particular EDHF relies on the flow of current generated in ECs via MEGJs. Our study has tested this hypothesis from a different perspective by using the femoral artery, which lacks EDHF-attributed SMC relaxation. The responses in this artery were compared with those occurring in the mesenteric artery, which exhibits EDHF-attributed SMC relaxation.

Figure 4. Effects of Gap 27 on EDHF-attributed hyperpolarization in ECs and SMCs of mesenteric arteries. EC (n=5) and SMC (n=5) hyperpolarization recorded before and after incubation with Gap 27. Only SMC hyperpolarization was significantly reduced after exposure to Gap 27. *Significantly different (P<0.05).

Acknowledgments

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