Endothelial Cell Pathway for Conduction of Hyperpolarization and Vasodilation Along Hamster Feed Artery

Geoffrey G. Emerson, Steven S. Segal

Abstract—Acetylcholine (ACh) evokes the conduction of vasodilation along resistance microvessels. However, it is not known which cell layer (endothelium or smooth muscle) serves as the conduction pathway. In isolated, cannulated feed arteries (≈70 μm in diameter at 75 mm Hg; length ≈4 mm) of the hamster retractor muscle, we tested the hypothesis that endothelial cells provide the pathway for conduction. Microiontophoresis of ACh (500 ms, 500 nA) onto the distal end of a feed artery evoked hyperpolarization (−13±2 mV) of both cell layers with vasodilation (15±1 μm) along the entire vessel. To selectively damage endothelial cells (confirmed by loss of vasodilation to ACh and labeling of disrupted cells with propidium iodide), an air bubble was perfused through a portion of the vessel lumen, or a 70-kDa fluorescein-conjugated dextran (FCD) was illuminated within a segment (300 μm) of the lumen. After endothelial cell damage, hyperpolarization and vasodilation conducted up to, but not through, the treated segment. To selectively damage smooth muscle cells (confirmed by loss of vasoconstriction to phenylephrine and labeling with propidium iodide), FCD was perfused around the vessel and illuminated. Vasodilation and hyperpolarization conducted past the disrupted smooth muscle cells without attenuation. We conclude that endothelial cells provide the pathway for conducting hyperpolarization and vasodilation along feed arteries in response to ACh. (Circ Res. 2000;86:94-100.)

Key Words: feed artery • conduction • endothelium • hyperpolarization • vasodilation

Conducted vasodilation contributes to the coordination of blood flow control within microvascular resistance networks. Vasodilation arising within skeletal muscle can “ascend” into the arteries supplying the muscle. Feed arteries, which lie external to a muscle, are a key site of resistance for controlling blood flow into the muscle. Thus, the conduction of vasodilation from intramuscular arterioles into their feed arteries substantially augments the hyperemic response. However, little is known about the mechanism of conduction in feed arteries, which (in rodents) appear similar to arterioles in wall morphology.

In arterioles of the hamster cheek pouch, the conduction of vasodilation evoked by acetylcholine (ACh) reflects the spread of hyperpolarization into endothelial cells and smooth muscle cells along the vessel wall. Gap junctions are expressed in both endothelium and smooth muscle of arterioles, indicating that hyperpolarization could spread along either (or both) of these cell monolayers. Gap junctional plaque surface area and intercellular dye coupling suggest that endothelial cells are better suited than smooth muscle cells for conducting electrical signals along the microvessel wall. However, there have been no direct, functional tests of this relationship.

The goal of the present study was to investigate the mechanism by which vasodilation is conducted along feed arteries of the hamster retractor muscle. Specifically, we tested (1) whether the spread of hyperpolarization underlies the conduction of vasodilation along these vessels and (2) whether the endothelium or smooth muscle layer provides the cellular pathway for conduction. Experiments were performed in isolated, cannulated microvessels in which endothelial cells or smooth muscle cells were selectively disrupted to evaluate the role of each cell layer in the conduction of vasodilation evoked by ACh.

Materials and Methods

Procedures were approved by the Institutional Animal Care and Use Committee. Feed arteries to the retractor muscle of anesthetized (pentobarbital; 60 mg/kg IP) male Golden hamsters (~100 g; Charles River Breeding Laboratories, Kingston, NY) were excised, cannulated in a vessel chamber, superfused with physiological salt solution (PSS), and observed using video microscopy on a vibration-isolated tabletop.

After equilibration, vessels were stimulated using microiontophoresis of ACh (1 mol/L; 500 ms, 500 nA) to activate endothelial cells or phenylephrine (PE; 0.5 mol/L; 500 ms, 500 nA) to activate smooth muscle cells. In preliminary experiments, we confirmed that vasodilation (to ACh) conducted readily along isolated feed arteries whereas vasoconstriction (to PE) was confined to the site of stimulation. Protocols (see below) were then designed to test which cell layer (endothelium or smooth muscle) conducted the signal for vasodilation in response to ACh.
electrical responses were sampled at 100 Hz; effective resolutions were 1
m and 100 ms, respectively.

Experiment 1: Conducted Vasodilation and Hyperpolarization
To determine the electrophysiological basis of conducted vasodilation, a cell was impaled with a microelectrode near the upstream end of the vessel (Figure 1). The microelectrode was aligned with the vessel axis and cells were penetrated at 60° to the vessel wall. ACh was delivered at 0, 500, 1000, or 2000 µm downstream from the recording site (Figure 1). With 2 minutes of rest in between, this stimulus was repeated at each distance in random order. The impaled cell was identified by characteristic dye labeling (Figure 2).

Experiment 2: Air Treatment
To selectively damage endothelial cells, an air bubble was delivered into the upstream end of the vessel; it exited through a side branch located midway along the vessel (Figure 3A), which was then resealed with suture. ACh was delivered at the downstream end (untreated region); responses were evaluated along the entire vessel before and after treatment.

Experiment 3: Luminal Light-Dye Treatment
To selectively damage endothelial cells along a defined segment, a 70-kDa fluorescein-conjugated dextran (FCD, 0.5%) was added to the luminal perfusate. From preliminary experiments that defined the region of damage required to interrupt conduction, a 300-µm segment near the middle of the vessel was illuminated for 3 to 5 periods of 30 seconds (each separated by 5 minutes of recovery) using a mercury lamp and fluorescein filter (Figure 4A). Before and after treatment, vasodilation was triggered at the downstream end of the vessel, and vasomotor and electrophysiological responses were recorded from each side of the illuminated segment.

Figure 1. Vasomotor and electrophysiological responses during conducted vasodilation. Feed artery segments (n=7; resting diameter was 56±2 µm, maximal diameter 89±4 µm, and length ∼4 mm) were impaled with a microelectrode. After 1 minute of a stable membrane potential (Em, −31±2 mV), ACh was microiontophoresed onto the vessel at defined distances downstream (A). Vasomotor and electrical responses evoked from each distance were recorded at the site of impalement. After recording, the impaled cell was identified by dye labeling (Figure 2); all recordings in this analysis were from endothelial cells. B, Representative tracings of diameter and Em taken with ACh stimulus (arrows) delivered at defined distances downstream by repositioning the stimulus micropipette relative to the impaled cell. C, Summary data showing the magnitude of the vasomotor response (ΔDiameter−peak diameter−resting diameter) to ACh stimuli delivered at respective distances downstream. D, Summary data showing the magnitude of the change in Em (ΔEm=peak Em−resting Em) at corresponding distances. Vasodilation and hyperpolarization at distance 0 were greater than respective conducted responses (P<0.05, repeated-measures one-way ANOVA with post hoc Tukey’s comparisons); however, there was no significant difference among conducted responses evoked at 500, 1000, or 2000 µm from the electrode. Flow indicates direction of superfusion with PSS (also in Figures 3 through 5).

Figure 2. Fluorescent dye labeling during intracellular recording. A, Smooth muscle cell after impalement with a microelectrode containing Lucifer yellow. B, Endothelial cell after impalement with a microelectrode containing a 3-kDa FCD. Dye was injected (−5 nA, 200-ms pulses, 1 Hz) for 10 seconds. C, Feed artery segment after 3 separate impalments (2 smooth muscle cells and 1 endothelial cell) with a microelectrode containing Lucifer yellow. Note that smooth muscle cell staining is confined to a single cell, whereas Lucifer yellow diffuses through the endothelium after recording from an endothelial cell. White lines depict vessel wall. Bar=25 µm in panels A through C.
Experiment 4: Abluminal Light-Dye Treatment
To selectively damage smooth muscle cells, a 70-kDa FCD was perifused around a feed artery segment (Figure 5A) via a pressurized (10 psi) micropipette. The protocol otherwise followed that of experiment 3.

After experiments 2 through 4, the effectiveness and selectivity of cell damage were assessed with microiontophoresis of ACh (to test endothelial cell function) and PE (to test smooth muscle function). Cellular damage was further evaluated (Figure 6) by labeling with luminal and abluminal propidium iodide (PI, 1 μmol/L) along with luminal Hoechst 33342 (HOE33342, 1 μmol/L). Whereas PI permeates only dead cells, HOE33342 permeates all cells to which it has access.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
Upon pressurization to 75 mm Hg (in vivo pressure), vessels (n=43) initially dilated to their maximal (passive) diameter (99±3 μm) and then constricted spontaneously to a stable resting diameter (71±2 μm) during equilibration. All vessels in the present study responded to ACh and PE, indicating that both endothelial cell and smooth muscle cell monolayers were functional. Vessels were typically studied for 2 to 3 hours and remained stable for up to 6 hours.

Experiment 1: Conducted Vasodilation and Hyperpolarization
ACh evoked vasodilation and endothelial cell hyperpolarization at the site of stimulation (magnitude 19±2 μm and −19±2 mV, respectively) and both responses traveled along the vessel...
Dye microinjection during intracellular recording clearly distinguished between respective cell types (Figure 2). Conducted responses were sustained with distance (15 ± 6 mm and 211 ± 6 mV at 2 mm, respectively). The onset of the ACh stimulus preceded the onset of hyperpolarization by 0.8 ± 0.1 second and the onset of vasodilation by 3.0 ± 0.3 seconds at each site along the vessel. Thus, hyperpolarization preceded vasodilation by 2 seconds at all distances from the stimulus, with an estimated velocity of conduction 20 mm/s.

Smooth muscle cells also hyperpolarized (see below) in response to ACh. However, we were unable to keep an electrode lodged in a smooth muscle cell for 4 successive responses (ie, to stimuli at all 4 distances). Therefore, smooth muscle cells were not included in this analysis.

Experiment 2: Air Treatment
After perfusion of air through the upstream half of vessels, ACh delivered at the downstream (untreated) end evoked vasodilation that conducted up to the treated region but no further (Figure 3B). Moreover, the direct response to ACh was inhibited in the treated region (Figure 3C). Nevertheless, responses to PE were unchanged (Figure 3D), and the addition of sodium nitroprusside (SNP; 10⁻⁵ mol/L) to the PSS caused maximal dilation (to 108 ± 7 mm; resting diameter 78 ± 2 mm) in the treated segment as well as the untreated segment, indicating smooth muscle cell integrity throughout.

Along the treated segment, endothelial cells were labeled with PI (indicating membrane disruption) whereas smooth muscle cells were not (Figure 6A). HOE33342 labeled smooth muscle as well as endothelial cells (Figure 6A'), indicating disruption of the integrity of the endothelial cell layer and access of luminal dye to smooth muscle cells. In the
Effect of Light-Dye Treatments on Vasomotor Responses

<table>
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<th>Stimulus</th>
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<th>Abluminal (n=9)</th>
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<tr>
<td>ACh</td>
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<td>16 ± 1</td>
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<td>PE</td>
<td>−20 ± 2</td>
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A 300-μm segment near the center of feed arteries (resting diameter 68 ± 5 μm) was illuminated after luminal or abluminal application of 70-kDa FCD to damage the inside or outside cell layer of the vessel, respectively. ACh or PE was microiontophoresed onto the illuminated segment to evaluate endothelial and smooth muscle integrity, respectively. The PE response (vasoconstriction) was monitored in the treated region; the ACh response (vasodilation) was monitored in the treated region. The ACh response (vasodilation) was measured 500 μm upstream because of loss of tone in the treated segment (see Results). Vasomotor responses in the Table (μm) were recorded before and after treatment and calculated by subtracting the resting diameter from the diameter at peak response. Responses before and after treatment were compared using paired Student’s t tests (*P < 0.05).

untreated half of these vessels, neither endothelial cells nor smooth muscle cells were labeled with either dye (not shown).

Resting diameter remained the same after air bubble treatment (78 ± 2 versus 82 ± 6 μm) in 5 of 7 vessels. The remaining 2 vessels lost tone after air bubble treatment, making vasodilatory responses unmeasurable. When treated with PE (10⁻⁷ mol/L) to generate tone,12 these vessels responded to ACh, PE, and SNP in the manner observed for the other 5 vessels and were therefore included in this analysis. For control vessels (n=5) perfused with PSS instead of air, direct and conducted responses to ACh remained intact along the entire vessel (data not shown).

Experiment 3: Luminal Light-Dye Treatment

Illumination of dye in the vessel lumen caused endothelial (but not smooth muscle) cell damage, as shown by selective loss of the response to ACh (Table) and by labeling with vital dyes (Figure 6B and 6B'). Resting diameter increased (from 74 ± 7 to 89 ± 10 μm; P<0.05, Student’s t test) in the illuminated segment, which was less than maximal diameter (102 ± 10 μm; P<0.05, Student’s t test). ACh applied downstream of the illuminated segment evoked vasodilation (Figure 4B) and hyperpolarization (Figure 4C) that conducted up to, but not through, the segment. Beyond the illuminated segment, resting diameter was unchanged, and both endothelial (n=4) and smooth muscle cells (n=3) hyperpolarized by 15 to 20 mV (with vasodilation) when stimulated directly with ACh.

Because luminal light-dye treatment dilated the illuminated segment, controls were performed to test the effect of such dilation on conduction. Thus, SNP (10 μmol/L) was applied from a micropipette (tip: 5 μm, 10 psi) to produce a fusiform dilation (to 97 ± 6 μm) ~500 μm long in the center of vessels (n=5; resting diameter 80 ± 7 μm, maximal diameter 108 ± 8 μm). Responses to ACh initiated 750 μm downstream from the dilated segment conducted through the segment with no attenuation (18 ± 3 versus 17 ± 3 μm at 2000 μm upstream from the ACh stimulus). Furthermore, FCD did not affect resting diameter or responses to ACh or PE unless illuminated (n=4), nor did the illumination protocol have an effect on vessels in the absence of FCD (n=4).

Experiment 4: Abluminal Light-Dye Treatment

Abluminal light-dye treatment caused smooth muscle (but not endothelial) cell damage, as assessed by PE and ACh (Table) and by labeling with vital dyes (Figure 6C and 6C'). Although direct responses to ACh and PE were abolished in the treated segment, conducted responses to ACh initiated from the treated segment and measured 500 μm upstream were preserved. Doubling the duration of the PE stimulus nearly doubled the magnitude of vasoconstriction in untreated regions of vessels, yet remained without effect in abluminally treated segments (n=4; data not shown). ACh applied downstream of the illuminated segment evoked vasodilation (Figure 5B) and hyperpolarization (Figure 5C) that conducted through the segment without impairment, indicating that smooth muscle damage was without effect on conducted responses. During abluminal light-dye treatment, the illuminated segment initially constricted (by ~20 μm); on completion of the illumination protocol, segments dilated (to 97 ± 6 μm) beyond the original resting diameter (80 ± 7 μm).

Discussion

The present study is the first to demonstrate the essential role of endothelial cells in providing the pathway for conducting hyperpolarization, and thereby vasodilation, along resistance microvessels supplying skeletal muscle. Using ACh to stimulate feed arteries of the hamster retractor muscle, our findings show that selective damage to endothelial cells (with smooth muscle cell integrity) within a defined vessel segment interrupts the conduction of hyperpolarization and vasodilation through the site of injury. In striking contrast, selective damage to smooth muscle cells (with endothelial cell integrity) had no effect on the conduction of hyperpolarization or vasodilation past the injured region. We conclude that the conduction of hyperpolarization along the endothelium coordinates smooth muscle cell relaxation along these resistance microvessels in a rapid and highly effective manner.

Electrophysiological responses of smooth muscle cells in feed arteries were indistinguishable from those of endothelial cells (Figures 4 and 5). This behavior is consistent with the hypothesis that smooth muscle and endothelial cells are electrically coupled by myoendothelial gap junctions.7,15 In contrast, electrical responses of smooth muscle cells have been dissociated from those of endothelial cells in cheek pouch arterioles.9 Whereas myoendothelial coupling has been implicated in these arterioles,20 an alternative mechanism for relaxing arteriolar smooth muscle cells involves the release of endothelial-derived hyperpolarizing factor.21 We have excluded a crucial role for nitric oxide in the conduction of vasodilation in feed arteries11; however, additional experiments are required to resolve the specific nature of coupling between smooth muscle and endothelium in these vessels.

The present data represent the first sustained recordings of endothelial cell membrane potential from isolated, pressurized microvessels. Previous studies of endothelial cell cou-
pling have come from preparations of cultured cells\textsuperscript{22} or conduit arteries.\textsuperscript{23,24} However, neither arterial nor cultured endothelium can be presumed to reflect the functional properties of endothelial cells that govern microvascular resistance. We show that, under extremely stable experimental conditions, endothelial cells in isolated, pressurized microvessels can be penetrated for extended periods, thereby enabling electrophysiological and vasomotor responses to be recorded throughout repeated vasoactive stimuli. In contrast, microelectrodes frequently dislodged during the first or second vasomotor response when recording from smooth muscle cells. This difference between cell types may be explained by the electrode being more deeply embedded in the vessel wall when endothelial cells are impaled than when smooth muscle cells are impaled from the abluminal surface.

Neither electrical nor vasomotor responses to ACh decayed significantly over distances exceeding 2 mm. This effective distance for conduction along feed arteries is considerably greater than reported for arterioles of the hamster cheek pouch\textsuperscript{2,4} and skeletal muscle\textsuperscript{11,25} or for guinea pig submucosa,\textsuperscript{16} where conducted responses decayed by >50\% within 1 to 2 mm. Given that the wall morphology of the feed arteries in the present study (Figures 2 and 6) appears similar to that of proximal arterioles,\textsuperscript{6,8} the greater effective distance for conduction along feed arteries may be explained instead by their lack of branching (and signal dissipation) when compared with arteriolar networks.\textsuperscript{11,16}

Either Lucifer yellow\textsuperscript{6,9} or a 3-kDa FCD\textsuperscript{15} was used in the microelectrode to identify the cell impaled during intracellular recording (Figure 2). Whereas Lucifer yellow has been our dye of choice because of its strong fluorescence,\textsuperscript{28} it has been suggested that the dual sulfate groups on this molecule can selectively block myoendothelial gap junctions whereas fluorescein does not.\textsuperscript{10} Nevertheless, our electrophysiological responses to ACh were indistinguishable in smooth muscle and in endothelium whether microelectrodes contained Lucifer yellow or a 3-kDa FCD. We therefore conclude that our measurements of membrane potential are independent of the dye used to label the cell from which we recorded.

Our modified version of the air bubble technique\textsuperscript{27} relied on a side branch to allow air to exit midway along the vessel, thereby maintaining endothelial cell integrity in the unexposed half of the vessel. The loss of vasomotor responses to ACh (but not to PE or SNP) in the treated region indicated selective endothelial cell damage and was confirmed with vital dye staining. Our second method for selectively disrupting endothelial cells was illumination of fluorescein within a segment of the vessel lumen that was defined by the diameter of the illumination beam. Fluorescein was selected for its superior phototoxicity,\textsuperscript{28} whereas conjugation of a 70-kDa dextran prevented its leakage from within (or into) the luminal compartment. As with air bubble treatment, functional indicators along with vital dye labeling confirmed selective damage to the exposed endothelial cells while preserving the integrity of surrounding smooth muscle cells as well as adjacent endothelial cells. Moreover, light-dye treatment along a segment of endothelium prevented hyperpolarization as well as vasodilation from conducting through the treated segment. Collectively, these findings indicate that endothelial cell integrity throughout the vessel is necessary for the conduction of vasodilation to effectively reduce the resistance to blood flow along the entire feed artery.

The finding that smooth muscle cell hyperpolarization could not spread past the region of endothelial cell damage (Figure 4) suggests relatively poor coupling between smooth muscle cells\textsuperscript{11} when compared with those in cheek pouch arterioles\textsuperscript{6} or coronary arteries.\textsuperscript{29} When smooth muscle cells were selectively damaged with abluminal light-dye treatment, vasomotor responses were abolished within the damaged segment. Nevertheless, an ACh stimulus delivered in the region of damage evoked conducted vasodilation that spread along the rest of the vessel, confirming the integrity of the endothelial cell signaling pathway. Moreover, when triggered beyond the region of smooth muscle damage, the integrity of the endothelial cell pathway enabled the spread of hyperpolarization past the damaged smooth muscle cells and into viable cells, thereby effecting a vasodilatory response. This behavior highlights the effectiveness of endothelial cell conduction as the basis for coordinating smooth muscle cell relaxation along feed arteries of the hamster retractor muscle in response to ACh.

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References


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