Endothelial and smooth muscle cell conduction in arterioles controlling blood flow

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Welsh, Donald G., and Steven S. Segal. Endothelial and smooth muscle cell conduction in arterioles controlling blood flow. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H178–H186, 1998.—We performed intracellular recording with Lucifer yellow dye microinjection to investigate the cellular pathway(s) by which constriction and dilation are conducted along the wall of arterioles (diameter 47 ± 1 µm, n = 63) supplying blood flow to the cheek pouch of anesthetized hamsters. At rest, membrane potential ($E_m$) of endothelial (−36 ± 1 mV) and smooth muscle (−35 ± 1 mV) cells was not different. Micropipette delivery of norepinephrine (NE) or phenylephrine (PE) produced smooth muscle cell depolarization (5–41 µm) and vasoconstriction (7–49 µm) at the site of release and along the arteriole with no effect on $E_m$ of endothelial cells. KCl produced conduction of depolarization and vasoconstriction with similar electrical kinetics in endothelial and smooth muscle cells. Acetylcholine triggered conduction of vasodilation (2–25 µm) and hyperpolarization (3–33 µm) along both cell layers; in smooth muscle, this change in $E_m$ was prolonged and followed by a transient depolarization. These cell-specific electrophysiological recordings uniquely illustrate that depolarization and constriction are initiated and conducted along smooth muscle, independent of the endothelium. Furthermore, conduction of vasodilation is explained by the spread of hyperpolarization along homologously coupled endothelial and smooth muscle cells, with distinctive responses between cell layers. The discontinuity between endothelium and smooth muscle indicates that these respective pathways are not electrically coupled during blood flow control.

Conduction of vasomotor responses enables the coordination of blood flow magnitude and distribution within resistance networks (27, 36). The conduction of vasodilation and vasoconstriction have been taken to reflect the electrotonic spread of electrical signals through gap junctions between cells of the arteriole wall (18, 27, 29, 38). Early studies of electrical conduction in arterioles assumed that the coupling between smooth muscle cells provided the signaling pathway (13). Subsequently, both electrical and dye coupling between endothelial cells were found to be prevalent (16, 17, 26). Whereas such homologous coupling within these respective cell types is now established, the nature of heterologous coupling between smooth muscle and endothelial cells has remained controversial (1, 2, 19, 26). In part, such controversy may reflect differences between measurements performed using cultured cells (16, 17), excised arteries (1, 2), and microvesSEL preparations (13, 19, 26, 29, 38).

To date, electrophysiological studies of conduction in arterioles have utilized isolated preparations studied in vitro (13, 29, 37, 38), in which transmural pressure and luminal flow had been eliminated. A variety of second messengers, which are able to regulate the conductance of gap junctions (e.g., cyclic nucleotides, Ca$^{2+}$, and nitric oxide (20, 30)), are produced in response to both pressure and flow. Therefore, in addition to the physical trauma induced by dissection, the elimination of tangential wall stress and luminal shear stress may alter gap junctional coupling relative to in vivo conditions.

The goal of this study was to define the cellular pathway(s) by which electrical responses are initiated and conducted along arterioles in vivo. We tested the hypothesis that each cell layer (endothelial, smooth muscle) could provide a pathway for conducting vasmotor responses along the arteriolar wall. In the hamster cheek pouch (27) $\alpha$-adrenoceptor and muscarinic agonists were applied via micropipettes onto arterioles to initiate responses on smooth muscle and endothelial cells, respectively (14, 37); membrane potential ($E_m$) and diameter were monitored at the site of application or at a remote site along the vessel. Our data from cells identified by dye labeling indicate that homologous gap junctional coupling enables current to spread between endothelial cells or between smooth muscle cells; however, heterologous electrical coupling was not prevalent between the two cell types. Thus smooth muscle and endothelium each provide a signaling pathway, with the nature of the stimulus determining which cell layer conducts the signal along the vessel wall.

METHODS

Hamster cheek pouch preparation. All procedures were approved by the Animal Care and Use Committee of The J ohn B. Pierce Laboratory and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (revised 1996). Male Golden hamsters (80–110 g, n = 35; Charles River Breeding Laboratories) were anesthetized with pentobarbital sodium (60 mg/kg ip) and tracheotomized (PE-190 tubing) to ensure airway patency. A cannula (PE-50) secured in the left femoral vein enabled continuous replacement of fluids and maintenance of anesthesia throughout experiments (10 mg pentobarbital sodium/ml isotonic saline, infused at 0.41 ml/h). Esophageal temperature was maintained at 37–38°C with conductive heating. With the use of a stereomicroscope, the cheek pouch was exteriorized onto a Plexiglas board and superficial connective tissue was removed for observation of microvessels (27). The preparation was superfused continuously with a bicarbonate-buffered Ringer solution at 37°C (pH 7.2–7.4) of the following composition (in mM): 137 NaCl, 4.7 KCl, 1.2 MgSO$_4$, 2.0 CaCl$_2$, and 18 NaHCO$_3$ and equilibrated with 5% O$_2$–5% CO$_2$–95% N$_2$. These reagents were obtained from J. T. Baker or Sigma; others were obtained from Sigma unless noted.
The preparation was placed on the stage of an intravitral microscope (model 20T, Zeiss) and transilluminated with Köhler illumination using a Leitz UM 32 objective (NA = 0.20), which provided a 15-mm working distance to facilitate microelectrode penetration. The optical image was coupled to a video camera (model NC-70X, Dage-MTI); total magnification on the monitor face (model PV M 133, Sony) was ×470.

Membrane potential, intracellular dye injection, and diameter recordings. Recording microelectrodes were pulled (model P-87, Sutter Instruments) from borosilicate glass tubes (no. GC120F-10, Warner Instruments) to produce a flexible tip that could move with the vessel wall during vasomotor activity. Tips were filled with Lucifer yellow dye (4% solution in deionized water); the remainder of the electrode (resistance 300–400 MΩ) was backfilled with 150 mM LiCl2. The reference electrode (Ag/AgCl pellet) was positioned in the effluent solution, and Em was monitored using an electrometer (model 773, World Precision Instruments).

The criteria for a successful cell penetration were 1) sharp negative deflection of potential on entry, 2) stable E, for at least 1 min after entry, and 3) stable positive deflection on exit. In some experiments, on completion of E, recordings, negative current (5 nA) was passed through the recording electrode to label the cell with Lucifer yellow dye. In most cases, cells were readily identified without current injection (e.g., with loss of impalement) due to diffusion of the dye from the microelectrode during the 10- to 20-min recording period.

Cell type was identified with the use of an immersion lens (×40/0.75, Zeiss) with epifluorescence (Zeiss filter set 48 77 05); a 75-W xenon lamp provided epi-illumination. Internal vessel diameter was recorded from the monitor face using a video caliper (model 321, Colorado Video); spatial resolution was ~2 µm. Simultaneous outputs from the electrometer and the caliper were acquired at 40 Hz using a MacLab system (model 8s, AD Instruments).

Microiontophoresis and pressure microejection of vasoactive stimuli. Norepinephrine (NE; 1 M), phenylephrine (PE; 0.5 M), and acetylcholine (ACCh; 1 M) were prepared daily in deionized H2O and micropipettes using microiontophoresis (model 260, World Precision Instruments). Ejection current was held at 500 nA, and stimulus duration varied from 150 to 2,000 ms; neurotransmitter currents were ~200 nA. KCl (0.5 M) was ejected from micropipettes using pressure (20–40 psi, 250–2,000 ms; model PL1-100, Medical Systems). Stimulus pipettes (tip inner diameter: 1 µm for microiontophoresis and 4–5 µm for pressure microejection) were fabricated with the same capillary tubes and puller used for the recording electrodes.

Experimental protocol. Suitable sites for study were identified during the period of equilibration (~60 min) of the preparation on the intravitral microscope. Hydraulic micromanipulators were used to minimize mechanical disturbance. A stimulus pipette was secured in one manipulator (model MO-102, Narishige) and positioned with its tip adjacent to a second- or third-order arteriole. With reference to a calibrated eyepiece reticle, the recording microelectrode was positioned parallel to the vessel axis with its tip located at a distance of 530 µm upstream from the stimulus pipette; this distance nearly spanned the field of view. The tip of the microelectrode was lowered carefully (model MX510, SOMA) onto the edge of the arteriole, and a cell was penetrated. After a stable E, and vessel diameter were attained (~1 min), a stimulus was applied; 1–2 min of recovery were allowed between successive stimuli. At the upstream site, changes in E, and vessel diameter were recorded as "conducted" responses. Once a range of conducted responses were obtained (3−4 stimuli), the stimulus pipette was repositioned with its tip adjacent to the recording site. Identical stimuli were applied, and the changes in E, and vessel diameter were recorded as "local" responses. In such a manner, both conducted and local responses were obtained from the same cell within a given experiment. If a particular stimulus elicited vasomotor responses without a corresponding change in E, (see results), KCl was added to the 50-ml reservoir of superfusion solution to produce an external K+ concentration of 55 mM; depolarization confirmed successful impalement and intracellular recording.

Data analysis. Recordings were selected to illustrate the characteristic effects of each stimulus on endothelial cells and smooth muscle cells. Because each experiment involved recording from a single cell, endothelial cell and smooth muscle cell recordings within each of Figs. 2–5 are from separate experiments and are shown together for illustrative purposes. To help quantify the discordant electrical responses between endothelial cells and smooth muscle cells to ACh (see results), the total duration of an electrical response was expressed as a percentage of the total duration of the vasomotor response; each was defined as the time between the response onset and complete recovery. Summary data from all recordings are presented in Fig. 6.

RESULTS

Identification of recording sites. The cell type from which a recording was obtained was ascertained by examining the labeling pattern following dye microinjection (Fig. 1). Individually labeled smooth muscle cells appeared as a narrow band that wrapped circumferentially around the arteriole; there was no evidence of dye spread into adjacent smooth muscle cells or underlying endothelial cells. In contrast, labeled endothelial cells appeared as a band parallel to the vessel axis. Typically, dye spread from the injected cell into many endothelial cells along the vessel axis, with little circumferential labeling; there was no evidence of dye coupling to surrounding smooth muscle cells.

Resting conditions. The diameter of arterioles at rest was 47 ± 1 µm (n = 63); all vessels studied exhibited brisk and reversible dilation (typical magnitude, 20–30 µm) to the addition of sodium nitroprusside (10 µM) to the superfusate. Vessel diameters were unaltered with successful impalement with microelectrodes. The E, of endothelial cells was −36 ± 1 mV (n = 40) and that of smooth muscle was −35 ± 1 mV (n = 23); these values are not different from those recorded in preliminary experiments using microelectrodes filled with 1 M KCl (data not shown). With constant arteriole diameter at rest, E, often fluctuated slightly (by 2–3 mV), particularly when recording from smooth muscle cells.

Local and conducted vasoconstriction. Microiontophoresis of NE or PE elicited vasoconstriction that was conducted along the arteriole wall (Figs. 2 and 3). A rapid depolarization of smooth muscle cells preceded the vasomotor response by ~2 s; repolarization was typically slower and corresponded with the return to resting diameter. In contrast to these consistent effects on smooth muscle cells, neither NE nor PE had any effect on the E, of endothelial cells (Figs. 2 and 3). Nevertheless, these same endothelial cells promptly depolarized (to −13 ± 2 mV; n = 18) when superfusate KCl was raised to 55 mM. Such dichotomous electrical...
Fig. 1. Identification of endothelial cells and smooth muscle cells in hamster cheek pouch arterioles in vivo. Third-order arterioles were viewed (×40/0.75; Zeiss) with bright-field and epifluorescent illumination following Lucifer yellow dye microinjection. Endothelial cells (A) run parallel to vessel axis and show strong dye coupling. Smooth muscle cells (B) wrap circumferentially around arteriole. Dye is constrained to injected cell; 2 separate smooth muscle cell injections are present in this photomicrograph. Scale bar = 20 µm.
responses to NE release were also found in preliminary recordings using KCl microelectrodes (data not shown). When KCl was released from a stimulus pipette, similar depolarizations were initiated in both endothelial and smooth muscle cells, and these conducted readily along both cell layers (Fig. 4).

Local and conducted vasodilation. ACh release triggered the conduction of vasodilation along the arteriole. Vasomotor responses to ACh were preceded (2–3 s) by hyperpolarization in endothelial cells and in smooth muscle cells (Fig. 5). Whereas the onset of hyperpolarization was rapid in both cell types, the shape and duration of electrical responses varied markedly and characteristically between cell types. Typically, endothelial cell hyperpolarization was followed by an equally rapid repolarization that occurred at or before the peak of the dilation. In contrast, smooth muscle cell repolarization was prolonged and typically (in 6 of 8 experiments) followed by an “afterdepolarization” that increased (magnitude 2–19 mV) with stimulus intensity. As a consequence, the duration of the entire electrical response (see Fig. 5) in smooth muscle cells was approxi-
mately twice that of endothelial cells (P < 0.05; t-tests) when expressed as a percentage of the duration of vasomotor response for respective cell types (local: 79 ± 8 vs. 37 ± 4%; conducted: 82 ± 5 vs. 44 ± 4%).

Summary data. Diameter and E_m responses to NE, PE, KCl, and ACh are summarized in Fig. 6. Note that electrical responses were monitored over a full range of vasomotor responses by varying the pulse duration of respective stimuli. The magnitude of the vasomotor response in an endothelial cell or a smooth muscle cell typically increased with the magnitude of the electrical response, indicating a role for electromechanical coupling in response to each stimulus tested. The notable exception was the lack of endothelial cell response to NE and PE, in which no change in electrical activity was recorded throughout a wide range of vasoconstriction.
DISCUSSION

The conduction of vasomotor responses reflects the electrotonic spread of current along the arteriolar wall. This study has distinguished the cellular pathways by which electrical responses are conducted in vivo. Using the hamster cheek pouch preparation, we show that \( \alpha \)-adrenoceptor agonists initiated a depolarization on and conducted between smooth muscle cells, independent of endothelial cells. In contrast, ACh elicited hyperpolarization that spread along both the endothelial cell layer and the smooth muscle cell layer. However, the shape and the duration of hyperpolarization varied substantively and characteristically between the respective cell types. Our findings indicate that current can readily pass between adjacent endothelial cells or adjacent smooth muscle cells; thus homologous electrical coupling is present in vivo. In contrast, electrical coupling between endothelial and smooth muscle cells was not prevalent during blood flow control. In light of these findings, we propose that the endothelial cell layer and the smooth muscle cell layer can each function as a distinct electrical pathway. In vivo, the initial stimulus may thereby determine which electrical pathway(s) may be used in signal transmission along the arteriolar wall.

**Cell labeling.** The lack of Lucifer yellow dye coupling between smooth muscle and endothelial cells in cheek pouch arterioles (Fig. 1) confirms earlier reports (19, 26). In the present study, this property has enabled us to rigorously define the cell type from which electrical recordings were obtained. Whereas the spread of dye between endothelial cells indicates gap junctional coupling (26), the absence of dye coupling in smooth muscle cells (Fig. 1) does not reflect the electrical coupling clearly present within this cell layer (Figs. 2 and 3). Such discrepancy between electrical coupling and dye coupling (i.e., conductance vs. permeability of gap junctions) can be explained by the permeability of respective gap junctions to ions versus dye molecules (6, 19, 32). Lucifer yellow has been shown to be a nontoxic, intensely fluorescent dye that is readily visualized in living cells (31). Although this agent has recently been reported (19) to impair dye coupling in arteriolar smooth muscle cells, it has no effect on electrophysiological responses or on cell-to-cell electrical coupling (31). Moreover, this dye remains widely used to identify the electrophysiological properties of specifically labeled cells. In the present experiments, if Lucifer yellow dye could effectively block electrical coupling, then a labeled smooth muscle cell should have been electrically isolated from other smooth muscle cells. It is evident from findings in Figs. 2, 3, and 4 that electrical responses were readily and consistently conducted into and beyond smooth muscle cells that were labeled with this dye.

**Em at rest.** At rest, the \( E_m \) of endothelial and smooth muscle cells averaged \(-286\) and \(-235\) mV, respectively. These resting values are near the equilibrium potential for chloride [approximately \(-20\) to \(-30\) mV (12, 24)], suggesting that endothelial and smooth muscle cells may have considerable chloride permeability under physiological conditions. However, if endothelial and smooth muscle cells are "clamped" at or near chloride's equilibrium potential, then fluctuations in \( \text{K}^+ \) and \( \text{Na}^+ \) permeability may have little effect on \( E_m \), and one would predict stable recordings at rest. In light of the
rapidity of electrical responses when stimulated (see Results) and the fluctuations in $E_m$ observed at rest, we suggest that $E_m$ largely reflects (changes in) the relative permeabilities to cations (7, 9, 28, 33).

It should be recognized that our values for $E_m$ in arterioles in vivo are 30–40 mV more positive than those reported for arterioles isolated from either hamster cheek pouch (37, 38) or guinea pig submucosa (13, 29) for in vitro studies. These differences can be accounted for as follows. First, arterioles in vivo remain exposed to physiological levels of transmural pressure, which depolarizes smooth muscle cells to elicit constriction (21). Second, the superfusate used in this study was equilibrated with 5% $O_2$, which can further depolarize arterioles that had been traumatized during surgery. Consistent with this view, we found that arterioles in vivo were equilibrated with 5% $O_2$, which can further depolarize arterioles that had been traumatized during surgery. Consistent with this view, we found that arterioles that had been traumatized during surgery were both dilated and had more negative $E_m$ at rest (greater than $-50$ mV) compared with arterioles that had not been damaged (unpublished observations). Thus, loss of pressure and physical trauma may increase K$^+$ conductance in both endothelial and smooth muscle cells, producing resting potentials that are closer to the equilibrium potential for K$^+$ than exist in arterioles actively controlling blood flow. With hyperpolarization and loss of vasomotor tone, the ability to study the conduction of vasodilatation is precluded. Hence, electro-physiological studies of conduction in isolated arterioles have focused on depolarization and vasoconstriction (29, 37, 38).

Local and conducted vasoconstriction. Focal activation of adrenoceptors initiated a characteristic depolarization of smooth muscle cells that, within $-2$ s, gave rise to vasoconstriction (Figs. 2 and 3). At both local and conducted sites, these electrical events were rapid in onset and were followed by a slower phase of repolarization that coincided with the return to resting diameter. The similarity between the effects of NE and PE observed (Figs. 2 and 3), together with the selectivity of PE for the $\alpha_1$-adrenoceptors, indicates that NE acted primarily through the $\alpha_2$-subtype. These depolarizing responses were observed through a corresponding range of vasoconstriction (Fig. 6) and may be explained by the activation of a cation channel permeable to both Na$^+$ and Ca$^{2+}$ (33), along with a reduction in K$^+$ conductance (22). Nevertheless, the ionic bases of these responses remain to be defined.

Whereas the $E_m$ of smooth muscle cells typically decreased by 10–20 mV on adrenoceptor activation, the $E_m$ of endothelial cells was unaffected by either NE or PE. Superfusion with NE or PE (1 $\mu$M) also failed to depolarize endothelial cells (data not shown), despite maximal constriction of arterioles and elimination of blood flow. This lack of endothelial responsiveness indicates that $\alpha_1$-adrenoceptors (or their signaling pathway for depolarization) were not present on endothelial cells and that smooth muscle cells were not electrically coupled to endothelial cells. It is important to recognize that these findings cannot be explained by the inability of endothelial cells to depolarize in vivo, because superfusing the cheek pouch with 55 mM KCl depolarized these same cells by an average of 23 mV.

The inability of PE to influence the $E_m$ of endothelial cells is in contrast to a recent in vitro study (38) in which this agonist was found to elicit depolarization in endothelial as well as smooth muscle cells. On the basis of the similarity of electrical responses between cell types and the assumption that PE activated $\alpha_1$-adrenoceptors only on smooth muscle cells, it was concluded that myoendothelial gap junctions (i.e., heterologous cell couplings) were present in cheek pouch arterioles and that this enabled the rapid spread of current from smooth muscle to endothelium (38). As an explanation of such a discrepancy with the present findings, it is possible that myoendothelial junctions are indeed present but are effectively closed under physiological conditions. As a result, current would not pass between the two cell types, as observed in the present study (Figs. 2 and 3). We hypothesize that myoendothelial gap junctional conductance could be reduced by cyclic nucleotides, Ca$^{2+}$, or nitric oxide (20, 30); the production of these second messengers is sensitive to both pressure and flow. Alternatively, the input resistance of endothelial cells in vivo may be considerably less than that of smooth muscle cells; this could selectively limit the effect of current spread from smooth muscle cells on endothelial cell $E_m$ (6). Finally, high local concentrations of PE may well activate an endothelial cell adrenoceptor (11). If such receptors were coupled to chloride channels, then cells with a resting $E_m$ near $-65$ mV will rapidly depolarize, whereas those in the present study, which rest closer to chloride's equilibrium potential, may experience little or no change in $E_m$. Because this interpretation remains speculative, it is clear that additional work is necessary to resolve the differences between in vivo and in vitro findings.

The release of KCl from a micropipette initiates the conduction of vasoconstriction and depolarization in arterioles (28, 29, 37, 38). Figure 4 uniquely illustrates that in vivo electrical responses to KCl were quite similar between smooth muscle and endothelial cells. Moreover, the onset of the electrical response preceded the vasomotor response by $-2$ s, as seen with the adrenoceptor agonists. Unlike NE and PE, which must first interact with receptors to elicit a cellular response, the elevation of external K$^+$ produces depolarization through a Nernst effect via changes in the K$^+$ equilibrium potential. Although KCl has been useful in defining the contractile behavior of vascular smooth muscle (23) and the cable properties of arterioles (29), its lack of specificity on the $E_m$ of excitable cells disqualifies it as a probe for myoendothelial coupling. Nevertheless, the consistency of the delay between the onset of depolarization and vasoconstriction across these stimuli indicates that a common final pathway is used in the process of electromechanical coupling (37).

Local and conducted vasodilatation. Whereas ACh triggered hyperpolarization in both endothelial and smooth muscle cells, there was a concomitant and
The afterdepolarization of smooth muscle cells may provide insight into this dichotomy. Thus, once the action of an EDHF wanes at the site of ACh release, the afterdepolarization may reflect the direct actions of any residual ACh on smooth muscle cells (4). Because the direct effect of ACh is present only where the agonist is released (15, 27, 28), the presence of afterdepolarization along the vessel implies that that electrical events in smooth muscle originated locally and were conducted along the smooth muscle layer. In contrast, if EDHF was released along the vessel as hyperpolarization spread along the endothelium, the afterdepolarization should not have been present at conducted sites. Although six of eight experiments were consistent with this explanation, additional studies are required to resolve this matter.

In summary, the present data represent the first cell-specific electrophysiological records of conduction obtained in arterioles controlling blood flow. α-Adrenoceptor and muscarinic agonists elicited local changes in E_{m} that were readily conducted along the vessel wall. Specifically, NE and PE initiated a depolarization that spread along smooth muscle cells independent of endothelial cells. In contrast, ACh elicited hyperpolarizing responses that were distinct between cell types and were conducted along the respective cell layers. Our findings indicate that current flow along the wall of arterioles occurs through homologous coupling between endothelial cells and between smooth muscle cells; heterologous coupling between endothelial and smooth muscle cells was not prevalent. We propose that the endothelium and the smooth muscle monolayers form parallel electrical pathways along the arteriole wall. Whereas hyperpolarization and depolarization can rapidly spread along both cellular pathways, it appears that the particular stimulus will determine which cell layer is used for signaling. Differences between the behavior of similar vessels studied in vitro may be explained by the loss of physiological regulation of gap junctional coupling.

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