Endothelial cell signaling during conducted vasomotor responses

Kim A. Dora, Jun Xia, and Brian R. Duling
Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, Virginia 22906-0011

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Dora, Kim A., Jun Xia, and Brian R. Duling. Endothelial cell signaling during conducted vasomotor responses. Am J Physiol Heart Circ Physiol 285: H119–H126, 2003; 10.1152/ajpheart.00643.2002.—ACh and KCl stimulate vasomotor responses that spread rapidly and bidirectionally along arteriole walls, most likely via spread of electric current or Ca2+ through gap junctions. We examined these possibilities with isolated, cannulated, and perfused hamster cheek pouch arterioles (50- to 80-μm resting diameter). After intraluminal loading of 2 μM fluo 3 to measure Ca2+ or 1 μM di-8-ANEPPS to measure membrane potential, photometric techniques were used to selectively measure changes in intracellular Ca2+ concentration ([Ca2+]i) or membrane potential in endothelial cells. Activation of the endothelium by micropipette application of ACh (10-4 M, 1.0-s pulse) to a short segment of arteriole (100–200 μm) increased endothelial cell [Ca2+]i, and caused hyperpolarization at the site of stimulation. This response was followed rapidly by vasodilatation of the entire arteriole (~2-mm length). Change in membrane potential always preceded dilation, both at the site of stimulation and at distant sites along the arteriole. In contrast, an increase in endothelial cell [Ca2+]i was observed only at the application site. Micropipette application of KCl, which can depolarize both smooth muscle and endothelial cells (250 mM, 2.5-s pulse), also caused a rapid, spreading response consisting of depolarization followed by vasconstriction. With KCl stimulation, in addition to changes in membrane potential, increases in endothelial cell [Ca2+]i were observed at distant sites not directly exposed to KCl. The rapid longitudinal spread of both hyperpolarizing and depolarizing responses support electrical coupling as the mode of signal transmission along the arteriolar length. In addition, the relatively short distance between heterologous cell types enables the superimposed radial Ca2+ signaling between smooth muscle and endothelial cells to modulate vasomotor responses.

GAP JUNCTIONS LINKING smooth muscle and endothelial cells in arterioles provide a pathway for intercellular communication. We showed (9, 28) that agents known to act via signal transduction pathways that are found specifically in smooth muscle cells can cause parallel changes in endothelial cell signaling, suggesting the two cell types are functionally coupled. Cells in the arteriolar wall often behave as an electrical syncytium (15, 28), and thus changes in membrane potential in one cell type can alter the function of voltage-gated Ca2+ channels in the other cell type (28). Furthermore, the radial Ca2+ signaling from smooth muscle to the endothelium may stimulate the synthesis of endothelium-derived vasodilators, which then relax the vascular smooth muscle (9, 29). The smooth muscle-endothelial cell pair seems to function in a linked fashion, and therefore the endothelial cell Ca2+ response to stimulation with agents such as KCl in an arteriole cannot be directly extrapolated from isolated cells where depolarization decreases endothelial cell intracellular Ca2+ concentration ([Ca2+]i) (4, 19, 21, 23).

The complex relationship between membrane potential and Ca2+ in the two cell types of the arteriolar wall led us to investigate the relative contributions to arteriolar responses made by either 1) membrane potential change or 2) Ca2+ flux. Changes in endothelial cell [Ca2+]i and membrane potential were measured in isolated, perfused arterioles with photometric and electrophysiological techniques. We made measurements at a site of stimulation and at distant sites along the arteriolar length. We examined the temporal patterns of changes in [Ca2+]i, membrane potential, and diameter in response to brief pulsatile stimulation with either a receptor-mediated agonist (ACh) or a depolarizing agent (KCl).

METHODS

All procedures and protocols used in this study were conducted on cannulated, perfused cheek pouch arterioles isolated from male golden hamsters with protocols approved by the University of Virginia Animal Care and Use Committee. Hamsters (128 ± 3 g, n = 28; Charles River) were anesthetized with pentobarbital sodium (50 mg/kg ip), and the cheek pouch was excised and spread out in a refrigerated (4°C) dissection chamber filled with MOPS-buffered saline solution (in mM: 145 NaCl, 4.70 KCl, 2.0 CaCl2, 1.17 MgSO4, 1.2 NaH2PO4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.75 NaOH, and 2.0 MOPS) containing 1% low-endotoxin BSA (MOPS-BSA; U.S. Biochemical). Arterioles (resting diameter 65.1 ± 1.7 μm, maximum diameter 88.0 ± 2.2 μm; n = 28) were dissected and cannulated according to methods described previously (9, 11, 13). A segment of a second-order arteriole ~3 mm in length without visible branches was dissected and transferred to a temperature-controlled experimental chamber mounted on an inverted microscope (Olympus IMT-2).

Address for reprint requests and other correspondence: B. R. Duling, Dept. of Molecular Physiology and Biological Physics, Univ. of Virginia Health Sciences Center, P.O. Box 10011, Charlottesville, VA 22906-0011 (E-mail: brd@virginia.edu).

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Arterioles were cannulated at both ends with glass pipettes and attached to MOPS-BSA-filled reservoirs (13). The heights of the upstream and downstream reservoirs were initially set 40 mmHg above the vessel with a pressure gradient of 2.6 mmHg between the two ends, thereby establishing flow within the range found in vivo (11). A 30- to 60-min equilibration period followed, during which temperature in the tissue chamber was gradually raised to 37°C. All vessels developed spontaneous tone by constricting to 20% of maximum diameter. Throughout the experiment, the tissue bath was continuously flushed with MOPS-buffered saline solution at a rate of 2 ml/min, equivalent to a bath turnover rate of 1.5 times/min. At the end of each experiment, maximal diameter was determined by exposure of the vessel to nominally Ca²⁺-free MOPS-buffered saline solution with 0.1 mM adenosine at room temperature.

Stimulating pipettes (tip diameter 5 μm) were positioned near the vessel (~20 μm distant) with the aid of a motor-driven micromanipulator (STM/III), and agonists were pressure ejected onto the vessel with a brief pressure pulse by using a Picospritzer (General Valve), thereby allowing rapid and precise control of delivery. Vascular responses were observed at the site of the stimulating pipette (designated the local site) or at sites 500 and 1,000 μm upstream from the stimulating pipette. Both the superfusion and perfusion flows were directed along the length of the vessel toward the stimulating pipette (Fig. 1).

Arterioles were visualized with either bright-field (Olympus halogen illuminator) or fluorescence (LEP Hg lamp with 1.75% neutral density filter in light path) microscopy at a magnification of ×900 (Olympus ×40, 0.7 NA objective). In fluorescence studies, a slit placed at an object plane limited the field of illumination to a 100-μm vessel segment, thereby reducing photo damage, a critical issue in studies of this type. The image of an arteriolar segment produced by an intensified charge-coupled device camera (Dage-MTI, Gen II or Gen III with extended resolution and blue response, Stanford Photonics) was displayed on a video monitor (Dage-MTI) and recorded (model BV-1000, Mitsubishi) for subsequent image analysis (Image 1, Universal Imaging). The video signal was also used for diameter measurement (video caliper, Microcirculation Research Institute). An image splitter (Nikon, with appropriate filters) was mounted on the microscope side port to direct fluorescent light to a pair of photomultiplier tubes (PMTs; Hamamatsu) for measurement of fluorescence from the potential-sensitive or Ca²⁺-sensitive dyes. Videocaliper and PMT voltages were sampled by an analog-to-digital board, and the data were processed and stored with data acquisition software (Workbench PC, Strawberry Tree). This system allowed simultaneous measurement of both vessel diameter and changes in fluorescence intensity. The video and PMT measurements were synchronized by a signal from the pipette pressure-pulse trigger, allowing the establishment of precise temporal correlation between changes in fluorescence intensity and diameter.

**Measurement of endothelial cell [Ca²⁺].** Fluo 3 was selectively loaded into endothelial cells by perfusing arterioles with MOPS-BSA containing 2.2 μM fluo 3-AM (in 0.1% DMSO; Molecular Probes) for 40–60 min, followed by a 20-min wash. The fluo 3 was excited at 488 nm, and the resultant emissions sampled at 530 nm were five- to sixfold above the vessel autofluorescence observed before dye loading. Changes in endothelial cell fluorescence intensity in response to stimuli were assessed with software allowing high dynamic range (Workbench PC; measured at 5 Hz). Ca²⁺ calibrations were performed on completion of experiments. Agents were added either to the superfusion solution or to the luminal perfusion solutions. Minimum and maximum fluorescence (F₀₉₅ and F₉₉₅) were determined with concentrations of 10 μM ionomycin and solutions containing either 0 mM Ca²⁺ and 5 mM EGTA or 2 mM Ca²⁺. Adenosine (0.1 mM) was used to maximally dilate the vessels to eliminate motion artifact. After F₉₉₅ was obtained, 0 Ca²⁺ and 0.05% saponin were added to permeabilize the membranes to release free cytosolic Ca²⁺ and determine the extent of membrane-bound dye. This treatment was found to consistently return the fluorescence intensity to F₀₉₅.

**Measurement of endothelial cell membrane potential.** The emission spectrum of the voltage-sensitive dye di-8-ANEPPS has a broad peak centered in the range of 590 nm and shifts toward longer wavelengths in response to increases in membrane potential. Comparison of the ratio of the emission intensities at 560 and 620 nm (F₆₂₀/F₅₆₀) with simultaneous microelectrode recordings has shown that the ratio changes linearly with potential over the range of −56 to −6 mV and that a 1% decrease in the ratio corresponds roughly to a 10-mV depolarization (2).

Di-8-ANEPPS was selectively loaded into endothelial cell membranes by perfusing arterioles with MOPS-BSA containing 6 μM Pluronic (Molecular Probes) and 1 μM di-8-ANEPPS-AM (in 0.02% DMSO; Molecular Probes) for 20 min, followed by a 20-min wash. Resultant levels of fluorescence

![Fig. 1. Schematic diagram of the experimental setup to measure endothelial cell intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) and membrane potential in isolated, perfused arterioles. Brief pulses of each agent were applied via a stimulating pipette. The direction of perfusate and superfusate flow oppose the diffusion of agents to observation sites located 500 and 1,000 μm from the site of stimulation. Both the microscope objective and the stimulation pipette could be positioned variably along the arteriole length. In the fluorescence experiments, the stimulation pipette remained at the local site and the objective was positioned along the arteriole length. In the microelectrode experiments, the objective and microelectrode were fixed and the stimulation pipette was moved along the arteriole.](http://ajpheart.physiology.org/)

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(excitation 488 nm, emission 560 and 620 nm) were at least 10-fold above the vessel autofluorescence observed before dye loading. Fluorescence ratios were calculated on-line (Workbench PC software) at a rate of 10 Hz. In these experiments it was not possible to obtain absolute value calibrations of the membrane potentials measured with di-8-ANEPPS. To ensure that the changes reflected changes in membrane potential as accurately as possible, we performed numerous controls during each experiment. The excitation light intensity was changed by placement of neutral-density filters in the light path, and it was established that the absolute light level had no effect on $F_{560}/F_{620}$.

In a few experiments, intracellular microelectrodes were used to confirm the findings obtained with di-8-ANEPPS. Arterioles were isolated as described above and were transferred to a dual-vessel chamber (model CH/2/A; Living System Instrumentation) for cannulation. One end of the arteriole was cannulated and tied with a thread onto the cannulating pipette mounted on one side of the chamber under a Zeiss dissection microscope. The chamber was then transferred to the stage of an Olympus BH2-RFCA microscope for cannulation of the free end of the arterioles with a modified V-track, double-pipette cannulating system (as above). The microelectrode was passed through the cannulating pipette to impale cells from the luminal surface of the vessel (Fig. 1).

Membrane potential was recorded with glass microelectrodes pulled from borosilicate glass tubes with an inner filament (1.5-mm OD, 0.86-mm ID; GC150F-15, Clark Electromechanical) on a Brown-Flaming micropipette puller (model P-77, Sutter). Microelectrodes filled with 2 M KCl had a tip resistance of 80–120 MΩ and were connected to an Axoclamp-2 amplifier. A reference electrode (a Ag/AgCl pellet) was connected to the bath via a flexible salt bridge tube (WPI) filled with 3 M KCl. The output of the Axoclamp-2 was displayed on an oscilloscope (V-650F, Hitachi) and stored to disk. To minimize the effects of capacitance in the recording system, a minimal fluid level superfusion solution in the tissue bath was maintained. Cells in the arteriolar wall were impaled by lowering the micropipette with a Leitz manipulator at 70–80° to horizontal. Successful penetration of the cell was marked by a sharp deflection of the potential to a stable level and a depolarization response to pulse application of KCl.

This approach allowed successful cell penetration, but the capacitance between perfusion solution and recording electrode was very high because of the immersion of the outer surface of the recording pipette in MOPS-buffered saline solution. Capacitance could not be fully compensated for by using the capacitance neutralization function of the Axoclamp 2B amplifier, and as a result, the electrical recordings were somewhat damped in both time and amplitude.

The arteriole was viewed with a ×10 eyepiece and a Leitz ×32 UM objective. The image was projected to a television camera (model SIT 66, Dage-MTI) and displayed on a video monitor (model PMM-935, Lenco). The diameter of the arteriole was continuously measured with Diamtrak software (Monteck).

RESULTS

All the arterioles displayed myogenic tone (resting diameter 65.1 ± 1.7 μm, maximum diameter 88.0 ± 2.2 μm; $n = 28$) and maintained a relatively constant diameter during the experiment unless stimulated to dilate or contract. In general, resting membrane potential remained constant, but in a few experiments, the potential oscillated with upstrokes from the baseline with amplitudes of −8 mV and at −1.2 Hz. The resting membrane potential was taken at the most hyperpolarized level and had a mean value of $−37.6 ± 1.1$ mV ($n = 14$).

As previously shown (9, 10, 29), luminal perfusion of the fluorescent dyes confined loading to the endothelial cells and changes in arteriolar diameter did not affect the fluorescence intensity except as a function of a change in $[Ca^{2+}]_i$ or membrane potential. Cell selectivity of dye loading was assessed by examining the orientation of stained cells (10). Di-8-ANEPPS appeared to localize to the cell membranes, whereas fluo-3 was largely intracellular as indicated by the strong fluorescence signal from the nuclear region. Under no circumstances were experiments performed when circumferential outlines (smooth muscle cells) were visible.

Local responses. Application of ACh caused an increase in endothelial cell $Ca^{2+}$ and an increase in the fluorescence ratio ($F_{560}/F_{620}$, i.e., hyperpolarization) followed by vasodilation (Fig. 2). Changes in endothelial cell $[Ca^{2+}]_i$ occurred well in advance of changes in arteriolar diameter (Figs. 3 and 4), supporting the conclusion that the fluorescence change originated from the endothelium and that the change in endothelial cell $Ca^{2+}$ initiated the vasomotor response. Hyperpolarization followed the rise in $[Ca^{2+}]_i$ and clearly preceded changes in diameter (Figs. 2 and 3). In contrast, the endothelium-independent vasodilator sodium nitroprusside (SNP) caused only a slight decrease in endothelial cell $[Ca^{2+}]_i$ and a small hyperpolarization despite stimulating vasodilation of the same magnitude as observed with ACh (Figs. 2 and 4). This was confirmed with microelectrode experiments, in which SNP (0.5-s pulse) also stimulated an increase in diameter ($8.2 ± 0.6$ μm) and only a negligible change in membrane potential ($0.3 ± 0.4$ mV; $n = 3$).

To establish a link between these results and those published previously (2, 9), KCl was also used to alter membrane potential. As shown in Fig. 5, pipette application of KCl caused vasoconstriction, a decrease in the fluorescence ratio (i.e., a depolarization), and a sharp transient increase in endothelial cell $[Ca^{2+}]_i$. Because KCl would likely alter the membrane potential in both smooth muscle and endothelium, we cannot be certain which cell type was depolarized during KCl application. In any case, blockade of L-type, voltage-gated $Ca^{2+}$ channels with nifedipine sharply reduced the magnitude of the KCl-induced rises in $[Ca^{2+}]_i$ and the associated contractile responses. The membrane potential changes were unaltered (Fig. 6; Ref. 27).

The sensitivity of these vessels to $K^+$ was assessed in experiments in which the concentration of KCl in the bath was modified. Elevations >30 mM evoked depolarization and vasoconstriction, whereas small rises in extracellular [KCl] (to 10 or 15 mM) caused hyperpolarization and relaxation of arterioles (Fig. 7).

To determine whether a change in diameter per se influenced fluorescence intensity, indo-1, which acts by sensitizing the contractile proteins to $Ca^{2+}$ (17), was used to cause vasoconstriction without a change in $Ca^{2+}$ or membrane potential (Fig. 6). The observations
indicate that the two fluorescent dyes were responding to changes in Ca^{2+} and membrane potential directly rather than to movement of the arteriole.

Conducted responses. The vasodilation induced by ACh spread along the length of the arteriole and was always preceded by hyperpolarization (Figs. 2 and 3). Endothelial cell [Ca^{2+}]_{i}, in contrast, increased at the local site but not at sites 500 and 1,000 μm upstream, clearly dissociating changes in endothelial cell [Ca^{2+}]_{i} from the vasodilation (Fig. 2). The sensitivity of the endothelial cells to muscarinic agonists at these upstream sites was tested by applying ACh directly. In all cases, application of ACh to a vessel segment induced an increase in endothelial cell [Ca^{2+}]_{i} at that site (data not shown), the expected outcome if endothelial cell [Ca^{2+}]_{i} rather than smooth muscle cell [Ca^{2+}]_{i} was being measured (9).

The pattern seen with KCl was quite different, with the changes in Ca^{2+} and membrane potential in response to KCl being qualitatively similar along the arteriolar length. KCl caused a depolarization and a constriction that roughly mirrored the change in membrane potential. Both membrane potential and diameter change spread rapidly along the vessel length.
Shortly after the onset of the depolarization, there was a transient elevation in endothelial cell \([\text{Ca}^{2+}]_i\) (Fig. 5).

**DISCUSSION**

Short-bolus micropipette applications of ACh and KCl were used to examine the temporal patterns of changes in membrane potential, diameter, and endothelial cell \([\text{Ca}^{2+}]_i\) in the arteriolar wall after stimulation. By using \([\text{Ca}^{2+}]_i\)- and membrane potential-sensitive fluorescent dyes in combination with microelectrode recording, we have demonstrated a clear distinction between patterns of changes in \([\text{Ca}^{2+}]_i\), membrane potential, and diameter, depending on the agent used for stimulation and the site of measurement. The rapid spread of vasomotor responses suggests that electrical conduction is the most likely mechanism of the longitudinal spread of signal. It is possible that over the time frame investigated, intercellular \([\text{Ca}^{2+}]_i\) signaling (e.g., \([\text{Ca}^{2+}]_i\) waves) may well have occurred between small groups of adjacent cells. Importantly, because smooth muscle to endothelial cell signaling falls into this latter category, heterocellular \([\text{Ca}^{2+}]_i\) signaling across the wall of the arteriole can play an important physiological role in the modulation of contraction.

The use of a potential-sensitive dye instead of microelectrodes has experimental advantages and disadvantages. An advantage is that the fluorescence measurement is relatively easy, whereas it is technically very difficult to penetrate (and hold for long periods of time) either smooth muscle or endothelial cell membrane potential.
cells with microelectrodes. A major disadvantage of the fluorescence ratio technique is that absolute calibration is difficult or impossible, although our data do seem quite reasonable, showing a 2–3% increase in the fluorescence ratio in response to ACh that would be roughly equivalent to a hyperpolarization of 20–30 mV (2). This is quite consistent with the membrane potential changes observed in the cannulated arteriole (Fig. 3).

The finding that ACh elevates endothelial cell [Ca\textsuperscript{2+}] (Fig. 2), despite the rapid spread of hyperpolarization and vasodilation along the arteriolar length, has two important implications. First, it supports the suggestion that the vasodilation at distant sites is due to a conducted response intrinsic to the arteriolar wall and not to diffusion of the agonist. We previously demonstrated (18) the presence of gap junctions between the longitudinally orientated endothelial cells, which could serve as a path for cell-cell communication of these signals. Second, the absence of a change in Ca\textsuperscript{2+} at the distant sites suggests that neither Ca\textsuperscript{2+} nor inositol 1,4,5-trisphosphate [Ins(1,4,5)P\textsubscript{3}] is the signal responsible for the cell-cell communication that leads to relaxation of the smooth muscle. Indeed, the rapid spread of conduction of the mechanical responses along the vessel axis makes the diffusional transfer of a signaling molecule such as Ca\textsuperscript{2+} or Ins(1,4,5)P\textsubscript{3} unlikely as the underlying mode of transmission. Root mean squared diffusion times for Ca\textsuperscript{2+} and/or Ins(1,4,5)P\textsubscript{3} over a 1-mm distance would likely be on the order of minutes, not the fractions of a second observed here (3, 8). In this study, we were unable to determine the distance of interendothelial cell Ca\textsuperscript{2+} diffusion. However, if we assume that in a 500-μm segment of vessel there could be as few as 3 endothelial cell lengths (16), the lack of endothelial cell response at this distance from the site of application indicates that the short-pulse stimulation with ACh does not stimulate rapid intercellular Ca\textsuperscript{2+} spread. Overall, the data presented here strongly support the proposal that electrical current spread was the longitudinally communicated signal.

The presence of myoendothelial gap junctions in these arterioles (12, 18) may provide the pathway responsible for the movement of current from the endothelial cells to the smooth muscle cells at the distant sites. The receptor-independent hyperpolarization of the endothelium at the distant sites would be expected to cause a small rise in endothelial cell [Ca\textsuperscript{2+}]\textsubscript{i} (4, 19, 21, 23). However, the fall in smooth muscle Ca\textsuperscript{2+} associated with dilation at the distant site would contribute to a lowering of the endothelial cell Ca\textsuperscript{2+}. The fact that SNP-induced dilation was associated with a small decrease in endothelial cell [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 4; Ref. 9) is consistent with the idea that a decrease in smooth muscle cell [Ca\textsuperscript{2+}]\textsubscript{i} is readily transferred to the endothelium. A decrease in smooth muscle cell [Ca\textsuperscript{2+}]\textsubscript{i} is also associated with the hyperpolarization evoked by ACh (9). Thus, at the upstream sites in the absence of endothelial cell muscarinic receptor stimulation, this decrease in smooth muscle [Ca\textsuperscript{2+}]\textsubscript{i} may transfer to the underlying endothelial cells and negate any drive from membrane potential to increase intracellular Ca\textsuperscript{2+} levels.

An endothelium-derived hyperpolarizing factor (EDHF) might be involved in ACh-induced hyperpolarization of the smooth muscle at both the local and distant sites, but because the release of EDHF is thought to be dependent on an increase in endothelial cell [Ca\textsuperscript{2+}]\textsubscript{i} (5), and endothelial cell [Ca\textsuperscript{2+}]\textsubscript{i} decreased

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**Fig. 6.** Effect of pressure-pulse ejected vasoconstrictors on vessel diameter, endothelial cell [Ca\textsuperscript{2+}]\textsubscript{i}, and membrane potential at the site of stimulation. Responses were obtained in arterioles loaded with fluo 3 or di-8-ANEPPS to measure changes in [Ca\textsuperscript{2+}]\textsubscript{i} and membrane potential, respectively. KCl: 250 mM, 2.2-s pulse (fluo 3) or 10-s pulse (di-8-ANEPPS) in the presence and absence of nifedipine (Nif, 1 μM); indolactam (Indo): 10⁻⁶ M, 1.2 s pulse. Values are means ± SE of peak responses from n = 8 and 7 (KCl ± Nif), n = 5 and 4 (KCl ± Nif), and n = 4 and 4 (Indo) arterioles loaded with fluo 3 and di-8-ANEPPS, respectively; diameter values were pooled.

**Fig. 7.** Relationship between diameter (○) and membrane potential (●) in response to cumulative concentrations of KCl. Extracellular [KCl] was raised from 4.7 mM (resting membrane potential −38.0 ± 7.1 mV; resting diameter 74.3 ± 2.5 μm) by addition of KCl to the bath. Values are means ± SD of steady-state responses from 2 arterioles.
at the distant sites (Fig. 4), this seems unlikely. Further evidence to support our data that endothelial cell [Ca^{2+}]_{i} did not rise at the distant sites is the observation that L-arginine analogs do not block the conducted vasodilation mediated by short pulses of ACh (11). A rise in endothelial cell [Ca^{2+}]_{i} at the distant sites would be expected to evoke a release of nitric oxide and thus dilation. A similar observation was made in hamster cheek pouch arterioles in situ (26), a preparation in which it was proposed that the release of an EDHF at the upstream sites contributes to a conducted dilation (1), as it appeared myoendothelial junctions were not open (1, 25, 26). If the myoendothelial junctions are not open at the distant sites, it is possible that there is a small rise in endothelial cell [Ca^{2+}]_{i} associated with hyperpolarization. Whether this occurs and whether it is sufficient to stimulate the release of an EDHF remain to be established.

An additional possibility to explain the distant dilation in response to ACh is the release of K+ from endothelial cells during ACh-induced hyperpolarization. When released into the restricted space between the smooth muscle and endothelial cells, the K+ might cause smooth muscle hyperpolarization and thus relaxation (14). Only small rises in extracellular [KCl] are necessary to evoke both hyperpolarization and relaxation (Fig. 7). The mechanism responsible for the release of K+ at the distant sites has not been defined, but opening of inwardly rectifying K channels may contribute (20).

The time course of the changes in endothelial cell [Ca^{2+}]_{i}, in response to depolarization concentrations of KCl (Fig. 5) is an important observation and is difficult to explain. As mentioned above, the rapidity of the rise in endothelial cell [Ca^{2+}]_{i} along the length of the arterioles argues against longitudinal intercellular spread of Ca^{2+} and/or Ins(1,4,5)P_{3} as the mechanism. Furthermore, the onset of the spreading contraction to KCl is extremely rapid, ruling out a major contribution by smooth muscle-smooth muscle Ca^{2+} signaling, as again this would be too slow (7). An alternative explanation is that the K+ induces a rapid spread of depolarizing current through gap junctions to the neighboring smooth muscle cells and activates voltage-gated Ca^{2+} channels, causing a rise in smooth muscle [Ca^{2+}]_{i}, and that this drives radial Ca^{2+} movement from smooth muscle cells to endothelial cells through the myoendothelial cell junctions. The radial Ca^{2+} signaling might involve simple diffusion of Ca^{2+}, be secondary to elevation of Ins(1,4,5)P_{3} (9, 22, 29), or be the result of the extracellular diffusion of an as yet unidentified factor (9).

In view of the fact that the recovery of endothelial cell Ca^{2+} is so rapid compared with the arteriolar contraction and depolarization (Fig. 5), additional factors must be involved in determining the time course of the endothelial cell Ca^{2+} transient. Application of nifedipine produced almost complete blockade of the contractile response (and presumably the changes in smooth muscle Ca^{2+}) but left a small portion of the endothelial cell Ca^{2+} response intact (Fig. 6). Thus the endothelial cell Ca^{2+} may not be simply tracking the pattern set by the rise in smooth muscle cell calcium after activation of L-type Ca^{2+} channels. One possible explanation for the abrupt termination of the Ca^{2+} response to KCl is that the endothelial cell membrane Ca^{2+} pumps, working with the endoplasmic reticulum, reduce the cytoplasmic Ca^{2+} very rapidly after the initial Ca^{2+} transient in the smooth muscle (30). This could result in substantial dissociation between the time course of recovery of the smooth muscle cytoplasmic Ca^{2+} and that of the endothelium.

A second possible contributor to the time course of the KCl-induced Ca^{2+} transient shown in Fig. 5 is movement of a conducted depolarization along the arteriolar length through endothelial cells, with associated activation of voltage-sensitive Ca^{2+} channels in either or both cells. Although it is often assumed that voltage-sensitive Ca^{2+} channels are not present in the endothelium (6), T-type Ca^{2+} channels have now been reported (see, e.g., Ref. 24) and these could explain the brief calcium transient shown in Fig. 6. This is certainly an important avenue for future investigation. Regardless of the mechanism, as current flows longitudinally down the arteriole through smooth muscle and/or endothelium, there is an elevation in endothelial cell [Ca^{2+}]_{i} that can in turn, modulate arteriolar contraction (9, 29).

An effect of a change in endothelial cell [Ca^{2+}]_{i} is often masked by the Ca^{2+} signaling from activated smooth muscle (9, 29). Indeed, in the case of depolarization mediated by phenylephrine, the local rise in endothelial cell [Ca^{2+}]_{i} attenuates the local contraction and manifests itself as conducted vasodilation (29). Simply by either blocking the rise in endothelial cell [Ca^{2+}]_{i} or blocking the endothelial cell Ca^{2+}-activated K+ channels, which are crucially important for endothelial cell hyperpolarization, the response to phenylephrine reverts to one of conducted constriction (29). Together, these findings underline the importance of the endothelium in modulating vasoconstriction, even under circumstances that are not usually thought to be “endothelial cell-dependent mechanisms.”

In summary, these results reveal the complex interactions that occur between cells in the arteriole wall before the onset of vasomotor responses. The current coupling and Ca^{2+} signaling between smooth muscle cells and endothelial cells, when considered both separately and in unison, afford signaling processes that can augment or attenuate the magnitude of responses, depending on the signaling processes activated. The observation that changes in membrane potential precede Ca^{2+} responses at the distant sites supports the view that the rapid spread of current signals a change in smooth muscle Ca^{2+} and hence the onset of vasomotor responses. Secondary to this activation, smooth muscle cells can influence the endothelium to modulate vasomotor responses.

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Present address of K. A. Dora: Dept. of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK.

REFERENCES


