Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 6529–6534, June 1997
Physiology

Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles

(gap junctions/intercellular signaling/vasoconstriction/modulation)

K. A. DORA, M. P. DOYLE*, AND B. R. DULING†

Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, P.O. Box 10011, Charlottesville, VA 22906-0011

Communicated by Robert M. Berne, University of Virginia, Charlottesville, VA, March 31, 997 (received for review January 8, 1997)

ABSTRACT **It is well known that vascular smooth muscle tone can be modulated by signals arising in the endothelium (e.g., endothelium-derived relaxing factor, endothelium-derived hyperpolarizing factor, and prostaglandins). Here we show that during vasoconstriction a signal can originate in smooth muscle cells and act on the endothelium to cause synthesis of endothelium-derived relaxing factor. We studied responses to two vasoconstrictors (phenylephrine and KCl) that act by initiating a rise in smooth muscle cell intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and exert little or no direct effect on the endothelium. Fluo-3 was used as a Ca^{2+} indicator in either smooth muscle or endothelial cells of arterioles from the hamster cheek pouch. Phenylephrine and KCl caused the expected rise in smooth muscle cell $[Ca^{2+}]_i$ that was accompanied by an elevation in endothelial cell $[Ca^{2+}]_i$. The rise in endothelial cell $[Ca^{2+}]_i$ was followed by increased synthesis of NO, as evidenced by an enhancement of the vasoconstriction induced by both agents after blockade of NO synthesis. The molecule involved in signal transmission from smooth muscle to endothelium is as yet unknown. However, given that myoendothelial cell junctions are frequent in these vessels, we hypothesize that the rise in smooth muscle cell Ca^{2+} generates a diffusion gradient that drives Ca^{2+} through myoendothelial cell junctions and into the endothelial cells, thereby initiating the synthesis of NO.**

Smooth muscle and endothelial cell functions in the arteriolar wall are unified by complex intercellular signaling processes. In arterioles comprised of one layer of smooth muscle cells surrounding the endothelium, the close apposition of the two cell types enables a signal derived from one cell to rapidly diffuse to neighboring cells. For example, agents that stimulate a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of endothelial cells can cause the generation of vasodilators such as endothelium-derived hyperpolarizing factor and endothelium-derived relaxing factor (1, 2). Endothelial cell products can also modulate the magnitude of a response to a vasoconstrictor, as evinced by the greater constriction after endothelium removal or NO synthesis blockade (3–5). Interestingly, such effects have been reported during stimulation with agents such as phenylephrine (PE) and KCl that are thought to have little or no direct effect on endothelial cells.

In prior studies we have demonstrated that smooth muscle depolarization induced by PE is conducted to adjacent endothelial cells (6), and that the two cell types are dye coupled (7), suggesting the presence of myoendothelial gap junctions in these arterioles (8, 9). Based on the observation that Ca^{2+} and inositol triphosphate (IP_3) can also pass through gap junctions (10, 11) we hypothesized that there might be a previously unrecognized coupling process linking smooth muscle cell activation to endothelial cell activation. In this study we have used the Ca^{2+}-sensitive dye fluo-3 to measure changes in smooth muscle and endothelial cell $[Ca^{2+}]_i$ in response to smooth muscle stimulation in perfused arterioles isolated from the hamster cheek pouch.

MATERIALS AND METHODS

Isolated Vessel Preparation. Procedures and protocols used in this study were approved by the University of Virginia Animal Care and Use Committee. In all experiments, male Golden hamsters (122 ± 2 g, $n = 40$; Charles River Breeding Laboratories) were anesthetized with pentobarbital sodium (50 mg/10 g, i.p.) and the cheek pouch was excised and spread out in a refrigerated (4°C) dissection chamber containing 3-(*N*-morpholino)propane-sulfonic acid (Mops)-buffered saline (145 mM NaCl/4.70 mM KCl/2.0 mM $CaCl_2$/1.17 mM $MgSO_4$/1.2 mM NaH_2PO_4/5.0 mM glucose/2.0 mM pyruvate/0.02 mM EDTA/2.75 mM NaOH/2.0 mM Mops) and 1% low endotoxin BSA (United States Biochemical; Mops/BSA). Arterioles (58.8 ± 1.6 μm, $n = 40$, resting diameter) were isolated, cannulated at both ends, and perfused according to methods described previously (12, 13). In brief, a segment of a second order arteriole ≈3 mm in length without visible branches was dissected and transferred to a temperature-controlled tissue chamber mounted on an inverted microscope (Olympus IMT-2). Arterioles were cannulated at both ends with glass pipettes and attached to Mops/BSA-filled reservoirs (12). The heights of the upstream and downstream reservoirs were initially set 40 mmHg (1 mmHg = 133 Pa) above the vessel with a gradient of 2.6 mmHg between the two ends, thereby establishing flow within the range found *in vivo* (13). A 30- to 60-min equilibration period followed, during which temperature in the tissue chamber was gradually raised to 37°C. Throughout the experiment, the tissue bath was continuously flushed with Mops-buffered saline at a rate of 2 ml/min, equivalent to a bath turnover rate of 1.5 times/min. For all agents the appropriate vehicle controls were carried out and found to have no effect on vessel diameter or fluorescence responses.

To initiate agonist responses, a stimulating pipette (tip diameter 5 μm) was positioned near the vessel (within 20 μm) with the aid of a motor-driven micromanipulator. All agonists were pressure ejected onto the abluminal surface of the vessel using a Picospritzer (General Valve, Fairfield, NJ), thereby allowing rapid and precise control of delivery. Each value is the average of two sequential responses at the same arteriolar location. In all experiments (except those presented in Fig. 3) measurements were made while focusing at the vessel midplane.

Fluorescence Imaging. Optical techniques were used to measure intracellular Ca^{2+} using the fluorescent probe fluo-3

Abbreviations: PE, phenylephrine; ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; L-NAME, N^{ω}-nitro-L-arginine methyl ester; IP_3, inositol triphosphate.

*Present address: Somatogen, Inc., 2545 Central Avenue, Suite FD1, Boulder, CO 80301-2857.
†To whom reprint requests should be addressed. e-mail: kbrd@virginia.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

and simultaneous arteriolar diameter responses were measured with video calipers. Arterioles were visualized by illumination with either transmitted light or epifluorescence [LEP Ltd. (Hawthorne, NY) Hg lamp with 1.75% neutral density filter in light path] at a magnification of ×900 (Olympus ×40, 0.7 NA objective). In fluorescence studies, slits between the light source and the vessel limited the illumination field to a 100 μm segment, thereby reducing damage to the vessel.

Fluo-3 was selectively loaded into endothelial cells by perfusing arterioles with Mops/BSA containing 2.2 μM fluo-3 AM (Molecular Probes) for 40–60 min, followed by a 20-min wash. Resultant levels of fluorescence (excitation 488 nm, emission 530 nm) were 5- to 6-fold above vessel autofluorescence. In experiments where smooth muscle cells were loaded, the same dye concentration was added to the bath for 120–200 min. Use of a single emission wavelength Ca^{2+}-sensitive dye in combination with an image splitter (Nikon) permitted simultaneous use of a photomultiplier tube (Hamamatsu) and intensified CCD camera [GenII, Dage–MTI (Michigan City, IN) or GenIII extended resolution and blue response, Stanford Photonics (Stanford, CA)] for fluorescence measurement. Images were obtained for measurement of luminal diameter (video caliper, Microcirculation Research Institute, Texas A & M University), and subsequent image analysis (Image 1, Universal Imaging, Media, PA). Video caliper and photomultiplier tube voltages were sampled at 5 Hz by an analog-digital board, and the data were processed and stored with WORKBENCH PC data acquisition software (Strawberry Tree, Sunnyvale, CA) that allowed simultaneous, on line measurement of both vessel diameter and changes in fluorescence intensity. The video and photomultiplier tube measurements were synchronized by a signal from the pressure-pulse trigger, allowing the establishment of precise temporal relations between drug application time and changes in fluorescence intensity and diameter.

Ca^{2+} calibrations were performed upon completion of experiments. $[Ca^{2+}]_i$ was manipulated by adding agents to both the superfusion and luminal perfusion solutions. Ionomycin (10^{-5} M, calcium salt, Sigma) was used to determine the maximum range of dye sensitivity; and saponin (0.05%, Sigma) was subsequently added to determine the extent of membrane-bound dye. The single wavelength calibration equation was according to the equation of Grynkiewicz *et al.* (14): $[Ca^{2+}] = K_d[(F - F_{min})/(F_{max} - F)]$, where K_d = 400 nM; F_{min} = intensity in the presence of 0 Ca^{2+} Mops plus 5 mM EGTA (0 Ca^{2+}), 0.1 mM adenosine, and 10 mM ionomycin; and F_{max} = intensity in the presence of 2 mM Ca^{2+} Mops, 0.1 mM adenosine, and 10 mM ionomycin. After obtaining F_{max}, addition of 0 Ca^{2+} and 0.05% saponin returned the fluorescence intensity to F_{min}, a level indiscernible from the vessel autofluorescence.

RESULTS AND DISCUSSION

Cell-Specific Fluo-3 Dye Load. Several facts support the idea that cell-specific dye loading could be achieved, depending on the side of application. First, luminal loading of fluo-3 yielded increases in fluorescence upon addition of acetylcholine (ACh, Fig. 1 *A* and *C*), as would be expected if the fluorescence changes reflected mainly an increase in endothelial cell $[Ca]_i$, which then acted as the stimulus for vasodilation (1, 15). Second, comparable dilations induced by sodium nitroprusside, a dilator known to act directly on smooth muscle, caused a slight decrease in fluorescence of luminally loaded fluo-3 rather than the increase seen with ACh (Fig. 1 *A* and *C*). Third, after loading fluo-3 from the abluminal side of vessels, fluorescence decreased during application of ACh (Fig. 2), consistent with the expected smooth muscle $[Ca^{2+}]_i$ response during vasodilation (15). These findings strongly support the conclusion that an endothelial cell Ca^{2+} signal predominates during the response observed after luminal loading of fluo-3.

Endothelial Cell Ca^{2+} Response to Vasoconstrictors. To test the hypothesis that changes in Ca^{2+} in smooth muscle are mirrored by changes in endothelial cell Ca^{2+}, the vasoconstrictors PE, KCl, and indolactam were used as stimuli. Use of these agents was based on several lines of logic. First, we have shown that PE, a selective α_1-adrenoreceptor agonist, does not stimulate endothelial cells directly in this preparation (16–18). When applied selectively to the arteriolar lumen (i.e., to endothelial cells) PE produces either no effect, or a vasoconstriction at very high doses as the drug penetrates the endothelium and gains access to the smooth muscle cells. Under no circumstances does PE produce a vasodilatory response (17, 18). Second, the cells of these arterioles are electrically coupled, and PE and KCl depolarize both smooth muscle and endothelial cells (6). In smooth muscle cells, depolarization initiates opening of L-type Ca^{2+} channels and permits Ca^{2+} influx. However, since endothelial cells lack L-type Ca^{2+} channels, depolarization causes a modest reduction in Ca^{2+} influx into these cells due to the diminution of the electrochemical gradient (19, 20). Finally, indolactam was selected since it is reported to activate protein kinase C directly, and thereby cause vasoconstriction without large changes in smooth muscle $[Ca^{2+}]_i$ (21, 22).

Application of either PE or KCl to an arteriole whose endothelial cells were loaded with fluo-3 caused a rapid transient increase in endothelial cell fluorescence that peaked prior to the onset of the vasoconstriction (Fig. 1 *B* and *C*). Remarkably, the magnitude of the peak in endothelial cell $[Ca^{2+}]_i$ induced by these vasoconstrictors was similar to the endothelial response induced by ACh application at the same sites (compare Fig. 1 *A* and *B*). Indolactam (LC Laboratories, Woburn, MA), in contrast to the other two agents, had no discernible effect on endothelial cell $[Ca^{2+}]_i$ (Fig. 1 *B* and *C*), despite producing a constriction of similar magnitude. These results tend to exclude changes in diameter, flow or shear stress *per se* as causes of the changes in fluo-3 fluorescence. They suggest strongly that PE and KCl caused increases in endothelial cell $[Ca^{2+}]_i$ by some other mechanism.

More details on the changes in endothelial cell $[Ca^{2+}]_i$ in response to vasoconstrictors were obtained by focusing the microscope at the plane of the endothelial cells lining the lower surface of the arteriole. This allowed visualization of a substantial portion of the endothelial cells, rather than only their cross-section as was the case when focusing in the vessel mid plane. This procedure did, however, exclude the possibility for simultaneous measurement of diameter and fluo-3 fluorescence. The absolute values of nuclear fluorescence were higher than cytosolic, and both nuclear and cytosolic fluorescence intensity increased in response to ACh, PE, and KCl (Fig. 3), with the magnitude of the changes being proportional to the resting fluorescence intensity in both compartments (Fig. 3*A*). The relative contribution by each compartment to the overall Ca^{2+} signal could not be established in this study, but since the ratio of fluorescence in the two compartments was essentially constant, we were confident that ACh, KCl, and PE each increased endothelial cell $[Ca^{2+}]_i$.

Endothelial Cell Damage Blocks the Ca^{2+} Response. Further support for the idea that fluorescence signals from luminally loaded vessels originated from the endothelium was obtained by passing air bubbles through the vessel lumen, to permeabilize endothelial cells, thereby allowing efflux of fluo-3. After obtaining control diameter and Ca^{2+} responses to ACh and PE, the endothelial cell membranes were disrupted, and responses to ACh and PE were repeated. The mechanical responses to ACh were eliminated as would be expected if the endothelium were destroyed, and no change in the fluorescence signal could be detected. PE-mediated vasoconstrictions were unaffected by air perfusion, but the fluorescence response was markedly reduced or eliminated (Fig. 4*A*). To establish that air perfusion had not adversely affected the integrity of smooth muscle membranes, fluo-3 was subsequently loaded into smooth muscle cells by abluminal application of the dye. Peak smooth muscle Ca^{2+}

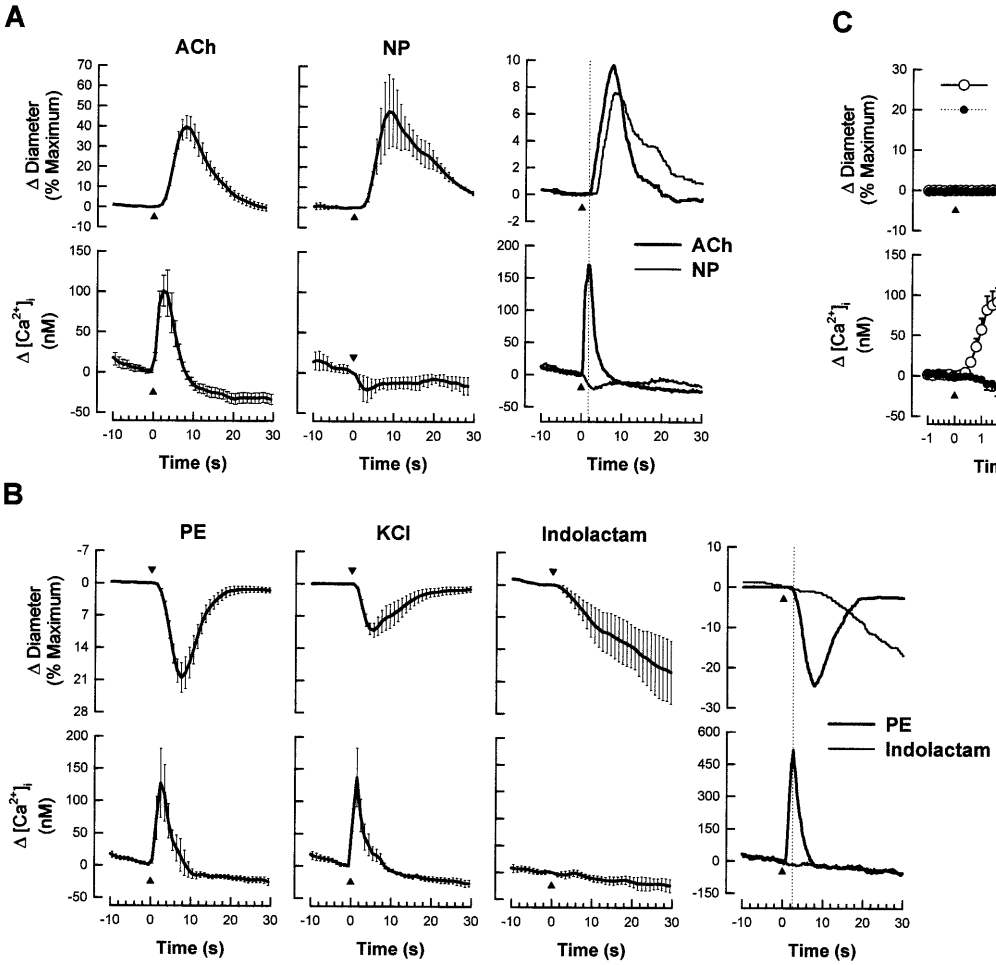

FIG. 1. Time course of changes in vessel diameter and endothelial cell $[Ca^{2+}]$ in response to abluminal pressure-pulse application of agonists to isolated perfused resistance arterioles. (*A*) ACh (10^{-4} M, 1.2-sec pulse, $n = 15$) and sodium nitroprusside (NP 5×10^{-5} M, 1.2-sec pulse, $n = 3$) both stimulated vasodilation, but had opposite effects on $[Ca^{2+}]_i$. (*Right*) The rapid rise in $[Ca^{2+}]_i$ in response to ACh, but not sodium nitroprusside, at the same arteriolar location suggests changes in fluorescence intensity relate to changes in endothelial cell rather than smooth muscle $[Ca^{2+}]_i$ (typical trace). (*B*) PE (10^{-5} M, 1.2-sec pulse, $n = 11$) and KCl (250 mM, 2.2-sec pulse, $n = 8$) both stimulated vasoconstriction and rises in endothelial cell $[Ca^{2+}]_i$. In contrast, indolactam (10^{-5} M, 1.2-sec pulse, $n = 4$) caused vasoconstriction without a change in $[Ca^{2+}]_i$. (*Right*) No increase in $[Ca^{2+}]_i$ was observed in association with indolactam-stimulated vasoconstriction at the same arteriolar location shown to be responsive to PE (typical trace). (*C*) For each agonist, changes in endothelial cell $[Ca^{2+}]_i$ occurred well in advance of changes in arteriolar diameter. Values are means ± SE; ▲ denotes time at which agonist application commenced.

responses to PE after disruption of the endothelial cells were 57% (calculated as percent change in fluorescence intensity, $n = 3$) of the smooth muscle Ca^{2+} responses to PE presented in Fig. 2 (unpaired Student's *t* test, $n = 6$). Support that endothelial cells were selectively disrupted after air perfusion was obtained by addition of propridium iodide (10^{-5} M) to the bath, which stained predominantly endothelial cell nuclei (data not shown).

Do the Vasoconstrictors Directly Stimulate Endothelial Cells? Further evidence that neither KCl nor PE exerted a direct effect on endothelial cells was obtained using nifedipine. Prior studies showed that the vasomotor response to depolarization of these vessels is dependent on opening voltage gated Ca^{2+} channels that are sensitive to nifedipine (23). Therefore the net effect of KCl application should be the aggregate of

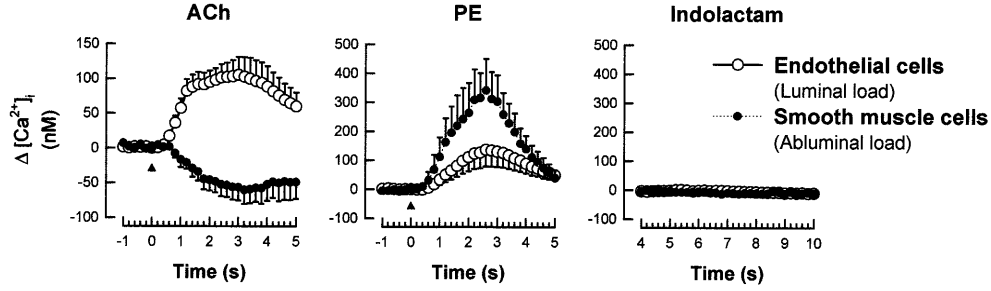

FIG. 2. Comparison between smooth muscle and endothelial cell $[Ca^{2+}]_i$ responses to abluminal pressure-pulse application of agonists. Data from Fig. 1 are compared with responses obtained after loading smooth muscle cells with fluo-3 by adding the dye to the superfusion medium (abluminal load) rather than perfusion medium (luminal load). ACh ($n = 5$), PE ($n = 6$), and indolactam ($n = 4$) were applied at the same concentration and for the same duration as described in Fig. 1. Peak diameter responses were the same between groups. Values are means ± SE; ▲ denotes time at which agonist application commenced.

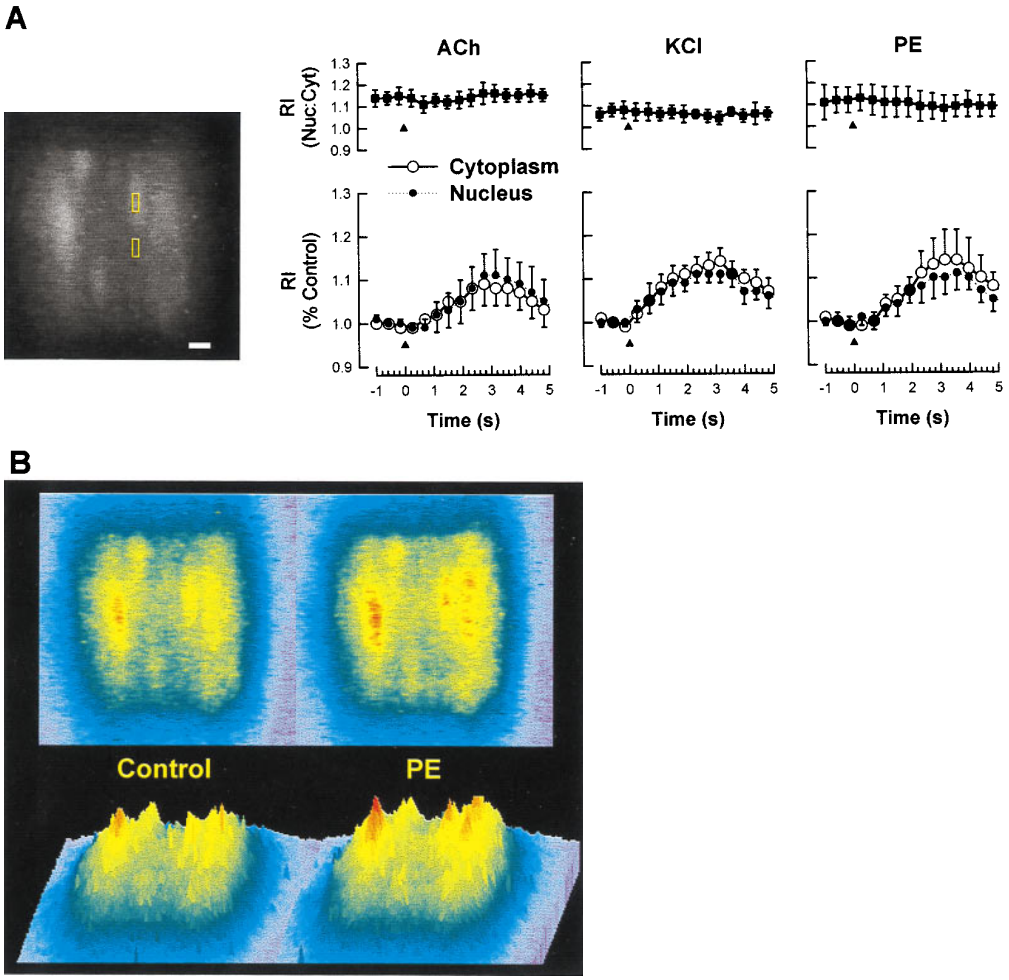

FIG. 3. Image analysis of arteriolar endothelial cell $[Ca^{2+}]_i$ responses to agonists. In these experiments the plane of focus was lowered to the layer of endothelial cells lining the bottom surface of the arteriole (hence no diameter responses were obtained). (*A*) Analysis of recorded video images allowed determination of nuclear and cytosolic changes in fluorescence intensity. (*Left*) Endothelial cells are orientated longitudinally along the arteriole (top to bottom) and are readily distinguished from smooth muscle cells (not visible). Small boxes were positioned in the two compartments (three cells per image) and relative intensity (RI) over time was recorded. Note: In all experiments only a short segment of arteriole was exposed to epi-illumination. (Bar = 10 μm.) (*Right*) Each agonist increased both nuclear (Nuc) and cytosolic (Cyt) fluorescence intensity in equal proportions. ACh (n = 6), PE (n = 4), and KCl (n = 4) were applied at the same concentration and for the same duration as described in Fig. 1. After a response to ACh was obtained, PE and/or KCl was applied at the same arteriolar location. Values are means ± SE; ▲ denotes time at which agonist application commenced. (*B*) Pseudocolor images of arteriolar endothelial cells before (Control) and 1 sec after (PE) stimulation with 10^{-4} M PE. Bottom panels are profiles of the corresponding top panel images.

actions on L-type channels in smooth muscle (increased Ca^{2+} influx) and the reduced inward electrochemical gradient for Ca^{2+} due to the change in membrane potential. Nifedipine virtually abolished the vasoconstrictor-mediated change in endothelial cell fluorescence (Fig. 4*B*) and we therefore conclude that the Ca^{2+} influx into the endothelial cells in response to PE or KCl originated largely from Ca^{2+} flux through smooth muscle L-type Ca^{2+} channels. Taken together with the ability of ACh to stimulate rises in $[Ca^{2+}]_i$ in the presence of nifedipine (Fig. 4*B*), the data are fully consistent with the idea that neither KCl nor PE acted via a primary effect on the endothelial cell, but rather, support the novel idea that the increase in endothelial cell $[Ca^{2+}]_i$ was dependent on a primary activation of smooth muscle cells and an elevation of its $[Ca^{2+}]_i$.

Functional Role for Increases in Endothelial Cell $[Ca^{2+}]_i$. The time courses of vasoconstrictor-mediated changes in $[Ca^{2+}]_i$ were strikingly similar in smooth muscle and endothelial cells, and our recording system (sample rate = 5 Hz) could detect no latency between the two (Fig. 2). Based on the conclusion that neither PE nor KCl directly increased endothelial cell $[Ca^{2+}]_i$, these results suggest that the intercellular signaling process coupling smooth muscle Ca^{2+} to endothelial cell Ca^{2+} is very rapid. In the absence of voltage gated channels in the endothelium, it is reasonable to propose that diffusional transfer of some solute might couple the changes in Ca^{2+} in the two cell types. Such a signal might be either Ca^{2+} itself, IP_3, or a previously unrecognized smooth muscle-derived relaxing factor. Since we have presented evidence that the smooth muscle cells are both electrically and dye coupled to the adjacent endothelial cells in the arterioles we study (6, 7), the parsimonious hypothesis is that the signal is the diffusion of Ca^{2+} itself or perhaps IP_3 through myoendothelial gap junctions. Others have provided evidence for intercellular diffusion of both of these molecules (10, 11). We favor the proposal that Ca^{2+} penetrates the junction since in dye transfer studies we have shown a strong bias against the diffusion of anionic solutes with molecular weights similar to IP_3 from smooth muscle to the endothelium through myoendothelial junctions (7). Though we cannot exclude the participation of some form of smooth muscle-derived relaxing factor in the endothelial response, in light of present evidence, we have no reason to evoke an additional signaling process beyond simple diffusion of Ca^{2+} down its concentration gradient from activated smooth muscle to endothelium.

Though cell–cell movement of Ca^{2+} and IP_3 are relatively slow in confluent populations of cells in culture (10, 11), the

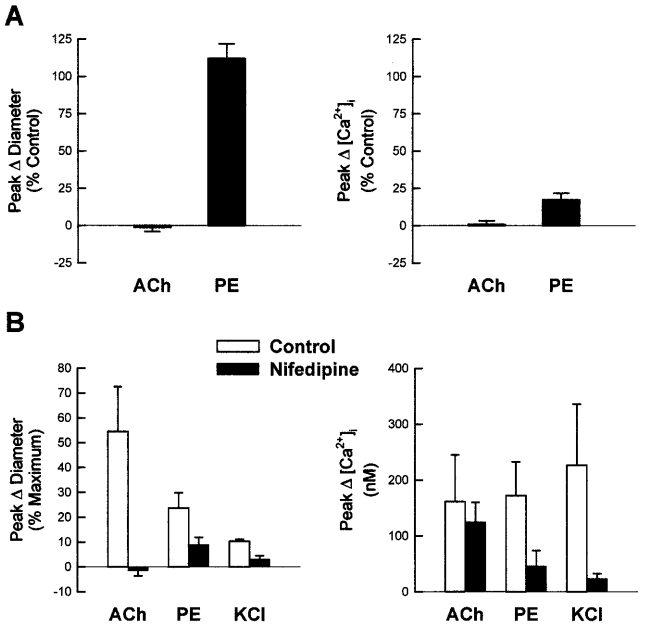

FIG. 4. (*A*) Effect of endothelium-specific damage on the diameter and fluorescence responses to agonists. Perfusion of air bubbles through the lumen of arterioles changed resting diameter from 64.9 ± 4.9 to 60.5 ± 10.5 μm (n = 4). The responses to ACh were abolished (n = 4), whereas PE-mediated vasoconstriction was unaffected, yet the increase in fluorescence was markedly attenuated (n = 4). (*B*) Effect of nifedipine on changes in vessel diameter and endothelial cell $[Ca^{2+}]$ in response to agonists. Addition of nifedipine (10^{-6} M) to the superfusion solution resulted in near maximal dilation of the arteriole (Δ diameter 64 ± 4 to 84 ± 5 μm, n = 8). In the presence of nifedipine, the rise in $[Ca^{2+}]_i$ in response to ACh was not affected (106.3 ± 27.6% of control) despite an absence of vasodilation (−8.1 ± 8.1% of control, n = 5). In contrast, both the rise in $[Ca^{2+}]_i$ and vasoconstriction in response to PE were reduced (30.9 ± 14.8%, 37.1 ± 9.6% of control, respectively, n = 4) and were markedly reduced in response to KCl (23.4 ± 9.2%, 25.7 ± 10.4% of control, respectively, n = 5). For all agents, values are means ± SE of paired responses before and after each treatment. The stimulation pipette was positioned at the same arteriolar location to obtain each pair of responses.

unique geometry of the arterioles would favor a very rapid diffusional coupling of a change in smooth muscle solute concentration to a change in endothelial cell solute concentration for two reasons. First, the volume of endothelial cells is estimated to be <10% of smooth muscle cells, and the maximum number of smooth muscle cells each endothelial cell could be coupled to is estimated to be 20 (24). The relatively small endothelial cell volume would reduce the dilution incurred by a solute diffusing from smooth muscle to endothelium and facilitate the heterocellular radial transfer of ions. Second, based on a 2 μm diffusion distance (i.e., twice the thickness of an endothelial cell) and on the diffusion coefficient for Ca^{2+} in cytosolic extracts (25), an estimate for the diffusion time from a smooth muscle cell to an adjacent endothelial cell is <100 ms, a period within our present detection limit. It must be noted that the reported diffusion coefficient for IP_3 is even greater (25), but since we were unable to determine either the presence of Ca^{2+} waves or the buffering capacity for Ca^{2+} within the cells, we could not accurately estimate the diffusion time for Ca^{2+} relative to IP_3.

If such transfer of Ca^{2+} from smooth muscle to endothelium via myoendothelial gap junctions does occur, it would be expected to cause the generation of various endothelium-derived vasoactive materials including a hyperpolarizing factor and NO (1, 2, 26). To test the latter possibility, NO synthase activity during vasoconstriction was assessed pharmacologically using N^{ω}-nitro-L-arginine methyl ester (L-NAME) blockade. Exposure to L-NAME did not affect the resting level of spontaneous tone in these isolated arterioles (at 40 mmHg), a finding consistent with an earlier report (13). Stimulation of arterioles with ACh, KCl, and PE each increased NO synthase activity as shown by a partial blockade of the ACh-mediated vasodilation, and an augmentation of KCl and PE-mediated vasoconstrictions by L-NAME (Fig. 5*A*).

Previous work has shown that there is an L-NAME-sensitive and an L-NAME-insensitive component to the ACh response (13). Therefore, since L-arginine analogs are reported to be without effect on ACh-mediated endothelial cell hyperpolarization (27), it was predictable that L-NAME would attenuate, but not fully block, the vasodilatory response to ACh (13). Inhibitors of NO synthase have been previously reported to augment KCl- and norepinephrine-induced vasoconstriction both *in vivo* and *in vitro* (3, 4, 28–30). The observation with KCl had remained unexplained until now, whereas the response to norepinephrine was attributed to the presence of α_2-adrenoreceptors on endothelial cells (3). In the present study, the use of the relatively selective α_1-adrenoreceptor agonist PE limited the stimulation to smooth muscle cells, and thereby unmasked what we believe to be Ca^{2+} signaling between smooth muscle and endothelial cells. The possibility that stimulation with PE and KCl caused a rapid formation of endothelium-derived hyperpolarizing factor was not assessed in this study. It is possible that the endothelium-derived hyperpolarizing factors released after stimulation with ACh (31, 32) are also released after addition of the vasoconstrictors and may further modulate vascular tone.

It is also important to note that the magnitude of the effect of L-NAME on vasoconstriction mediated by KCl and PE was time-dependent. There was no discernible effect of L-NAME during the first minute of constriction, consistent with a previous report using N^{ω}-nitro-L-arginine against PE in the same preparation (17). The delayed appearance of the L-NAME inhibitable component is largely predicable from the known behavior of Ca^{2+} and NO synthase. ACh-mediated stimulation of NO production appears to be small during the early period of stimulation (Fig. 5*A*; ref. 13), despite a rapid increase in endothelial cell $[Ca^{2+}]_i$ (Fig. 1). The delayed development of L-NAME sensitivity may also reflect the pattern of changes in smooth muscle $[Ca^{2+}]_i$ in response to stimulation. Agonist stimulation typically causes a transient peak in $[Ca^{2+}]_i$ followed by a smaller, sustained plateau phase (21), and since the vasodilatory action of NO involves a decrease in smooth muscle $[Ca^{2+}]_i$ (33) an effect of NO would more likely be manifest after the early, transient elevation of smooth muscle $[Ca^{2+}]_i$. In attempting to synthesize this information, it is important to recognize that the response of NO synthase to a change in Ca^{2+} is also complex. ACh stimulation is known to cause a biphasic $[Ca^{2+}]_i$ response in endothelial cells despite steady vasodilation during the period of agonist application (15). The biphasic Ca^{2+} response to ACh is also reported in cultured endothelial cells (34), and may involve Ca^{2+} release from intracellular stores, which triggers tyrosine kinase-dependent hyperpolarization and a maintained influx of Ca^{2+} (35). Thus, it can be inferred that other mechanisms maintain the signal during the later phases, perhaps a Ca^{2+}-sensitization of NO synthase involving protein kinase C, or as reported by Fleming *et al.* (26), stimulation of NO synthase by a Ca^{2+}-independent mechanism.

In summary, we find that changes in smooth muscle $[Ca^{2+}]_i$ can influence the level of Ca^{2+} in adjacent endothelial cells and secondarily stimulate the synthesis of vasodilators. We hypothesize that the cell–cell signaling involves diffusion of solute from smooth muscle to the endothelium through myoendothelial gap junctions, and though there are other possibilities, the most likely candidate appears to be radial movement of Ca^{2+} (Fig. 5*B*).

However, in the absence of a specific blocker of gap junctional transfer (6) we cannot test the hypothesis directly until new experimental approaches are developed. We note that our findings have profound implications with regard to vasomotor responses in arterioles, the portion of the vascula-

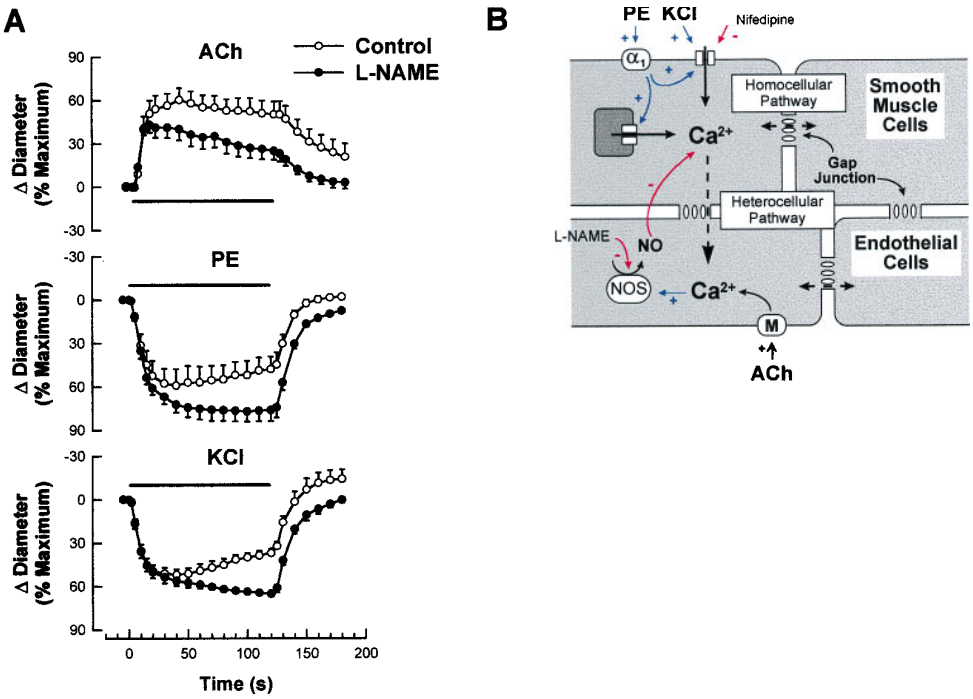

FIG. 5. (*A*) Effect of L-NAME on the time course of changes in vessel diameter in response to agonists. ACh (10^{-4} M, $n = 5$), PE (10^{-5} M, $n = 5$), and KCl (250 mM, $n = 5$) were pressure-pulse ejected adjacent to a segment of the arteriole for a period of 2 min (■). L-NAME (10^{-5} M) was added to the superfusion solution and allowed to equilibrate for 20 min, and had no effect on vessel diameter (Δ Diameter from 64.5 ± 3.4 to 66.8 ± 3.4 μm, $n = 15$). Without changing the stimulation pipette position, the time course for each agonist was repeated in the presence of L-NAME. Values are means ± SE of paired responses before and after L-NAME. (*B*) Schematic of the proposed heterocellular Ca^{2+} diffusion pathway. Increases in smooth muscle $[Ca^{2+}]_i$ stimulated by either PE or KCl diffuse radially to underlying endothelial cells through myoendothelial gap junctions. The secondary increase in endothelial cell $[Ca^{2+}]_i$ stimulates NO synthase to generate NO and modulate smooth muscle $[Ca^{2+}]_i$ and hence contraction. The extent of homocellular Ca^{2+} diffusion is limited by cell volume and distance.

ture where myoendothelial junctions are especially prevalent and control of flow predominates. Any vasoconstrictor that increases smooth muscle $[Ca^{2+}]_i$ has the potential to induce the counterregulatory response in endothelial cells. Signaling originating in smooth muscle and acting on endothelium must be considered when attempting to understand the complex mechanisms involved in the regulation of vessel diameter, blood pressure, and flow distribution within the microcirculation.

We are grateful to D. N. Damon, J. M. Beach, and J. Xia for technical assistance and valuable discussions. The work here was supported by National Institutes of Health Grant HL 53318, and by a C. J. Martin Fellowship from the National Health and Medical Research Council (Australia).

1. Busse, R., Pohl, U. & Luckhoff, A. (1989) *Z. Kardiol.* **78,** Suppl. 6, 64–69.
2. von der Weid, P. Y. & Beny, J. L. (1992) *Am. J. Physiol.* **262,** H1823–H1831.
3. Angus, J. A., Cocks, T. M. & Satoh, K. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45,** 2355–2359.
4. Amerini, S., Mantelli, L. & Ledda, F. (1995) *Pharmacol. Res.* **31,** 175–181.
5. Ayajiki, K., Kindermann, M., Hecker, M., Fleming, I. & Busse, R. (1996) *Circ. Res.* **78,** 750–758.
6. Xia, J., Little, T. L. & Duling, B. R. (1995) *Am. J. Physiol.* **269,** H2031–H2038.
7. Little, T. L., Xia, J. & Duling, B. R. (1995) *Circ. Res.* **76,** 498–504.
8. Duling, B. & Sleek, G. (1988) in *Mechanical Aspects of Endothelial Cell-Smooth Muscle Cell Interactions in Resistance Vessels*, eds. Bevan, J., Majewski, H. & Maxwell, R. (IRL, Washington, DC), Vol. 1, pp. 23–30.
9. Little, T. L., Beyer, E. C. & Duling, B. R. (1995) *Am. J. Physiol.* **268,** H729–H739.
10. Rooney, T. A. & Thomas, A. P. (1993) *Cell Calcium* **14,** 674–690.
11. Sanderson, M. J., Charles, A. C., Boitano, S. & Dirksen, E. R. (1994) *Mol. Cell. Endocrinol.* **98,** 173–187.
12. Duling, B. R. & Rivers, R. J. (1986) in *Isolation, Cannulation, and Perfusion of Microvessels*, eds. Baker, C. H. & Nastuk, W. G. (Academic, Orlando FL), pp. 265–280.
13. Doyle, M. P. & Duling, B. R. (1997) *Am. J. Physiol.* **272,** H1364–H1371.
14. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
15. Falcone, J. C., Kuo, L. & Meininger, G. A. (1993) *Am. J. Physiol.* **264,** H653–H659.
16. Lew, M. J. & Duling, B. R. (1992) *J. Vasc. Res.* **29,** 341–346.
17. Matsuki, T., Hynes, M. R. & Duling, B. R. (1993) *Am. J. Physiol.* **264,** H1251–H1258.
18. Lew, M., Rivers, R. & Duling, B. (1989) *Am. J. Physiol.* **257,** H10–H16.
19. Luckhoff, A. & Busse, R. (1990) *Naunyn-Schmiedebergs Arch. Pharmacol.* **342,** 94–99.
20. Schilling, W. P. (1989) *Am. J. Physiol.* **257,** H778–H784.
21. Meininger, G. A., Zawieja, D. C., Falcone, J. C., Hill, M. A. & Davey, J. P. (1991) *Am. J. Physiol.* **261,** H950–H959.
22. Hill, M. A., Davis, M. J., Song, J. B. & Zou, H. (1996) *J. Pharmacol. Exp. Ther.* **276,** 867–874.
23. Xia, J. & Duling, B. R. (1995) *Am. J. Physiol.* **269,** H2022–H2030.
24. Haas, T. L. & Duling, B. R. (1997) *Microvasc. Res.* **53,** 113–120.
25. Allbritton, N. L., Meyer, T. & Stryer, L. (1992) *Science* **258,** 1812–1815.
26. Fleming, I., Hecker, M. & Busse, R. (1994) *Circ. Res.* **74,** 1220–1226.
27. Chen, G. F. & Cheung, D. W. (1992) *Circ. Res.* **70,** 257–263.
28. Jones, C. J., DeFily, D. V., Patterson, J. L. & Chilian, W. M. (1993) *Circulation* **87,** 1264–1274.
29. Kaley, G., Koller, A., Rodenburg, J. M., Messina, E. J. & Wolin, M. S. (1992) *Am. J. Physiol.* **262,** H987–H992.
30. Pohl, U. & de Wit, C. (1996) *Pflügers Arch.* **432,** R107–R110.
31. Campbell, W. B., Gebremedhin, D., Pratt, P. F. & Harder, D. R. (1996) *Circ. Res.* **78,** 415–423.
32. Chen, G. F. & Cheung, D. W. (1996) *Circ. Res.* **79,** 827–833.
33. Blatter, L. A. & Wier, W. G. (1994) *Cell Calcium* **15,** 122–131.
34. Busse, R., Fichtner, H., Luckhoff, A. & Kohlhardt, M. (1988) *Am. J. Physiol.* **255,** H965–H969.
35. Sharma, N. R. & Davis, M. J. (1996) *Am. J. Physiol.* **270,** H267–H274.