Arteriolar smooth muscle Ca\(^{2+}\) dynamics during blood flow control in hamster cheek pouch

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Arteriolar smooth muscle Ca\(^{2+}\) dynamics during blood flow control in hamster cheek pouch. J Appl Physiol 101: 307–315, 2006. First published February 2, 2006; doi:10.1152/japplphysiol.01634.2005.—Intracellular calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) governs the contractile status of arteriolar smooth muscle cells (SMC). Although studied in vitro, little is known of SMC [Ca\(^{2+}\)]\(_{i}\) dynamics during the local control of blood flow. We tested the hypothesis that the rise and fall of SMC [Ca\(^{2+}\)]\(_{i}\), underlies arteriolar constriction and dilation in vivo. Aperenchymal segments of second-order arterioles (diameter 35 ± 2 μm) were prepared in the superfused cheek pouch of anesthetized hamsters (n = 18) and perfused with the ratiometric dye fura PE-3 (AM) to load SMC [Ca\(^{2+}\)]\(_{i}\); was 406 ± 37 nM. Elevation of superfusate O\(_{2}\) from 0 to 21% produced constriction (11 ± 2 μm) that was unaffected by dye loading; [Ca\(^{2+}\)]\(_{i}\) increased by 108 ± 53 nM (n = 6, P < 0.05). Cycling of [Ca\(^{2+}\)]\(_{i}\), during vasomotion (amplitude, 150 ± 53 nM; n = 4) preceded corresponding diameter changes (7 ± 1 μm) by ~2 s. Microiontophoresis (1 μm pipette tip; 1 μA, 1 s) of phenylephrine (PE) transiently increased [Ca\(^{2+}\)]\(_{i}\), by 479 ± 64 nM (n = 8, P < 0.05) with constriction (26 ± 3 μm). Flushing blood from the lumen with saline increased fluorescence by 510 nm by ~45% during excitation at both 340 and 380 nm with no difference in resting [Ca\(^{2+}\)]\(_{i}\), diameter or respective responses to PE (n = 7). Acetylcholine microiontophoresis (1 μA, 1 s) transiently reduced resting SMC [Ca\(^{2+}\)]\(_{i}\), by 131 ± 21 nM (n = 6, P < 0.05) with vasodilation (17 ± 1 μm). Superoxide of sodium nitro-prusside (10 μM) transiently reduced SMC [Ca\(^{2+}\)]\(_{i}\), by 124 ± 18 nM (n = 6, P < 0.05), whereas dilation (23 ± 5 μm) was sustained. Resolution of arteriolar SMC [Ca\(^{2+}\)]\(_{i}\) in vivo discriminates key signaling events that govern the local control of tissue blood flow.

The control of peripheral vascular resistance is integral to the regulation of tissue blood flow and the maintenance of arterial perfusion pressure. Dynamic changes in peripheral resistance are mediated by the dilation and constriction of arterioles. In turn, the intracellular calcium ion concentration ([Ca\(^{2+}\)]\(_{i}\)) of arteriolar smooth muscle cells (SMC) is a key determinant of arteriolar diameter. Since the development of techniques for the isolation and cannulation of microvessel segments (15), the study of SMC [Ca\(^{2+}\)]\(_{i}\) dynamics in arterioles has relied on in vitro experimentation. These preparations afford rigorous control of transmural pressure, luminal flow, temperature, and the chemical environment of luminal and abluminal fluid compartments. Great insight has been gained with respect to functional relationships between vasomotor and [Ca\(^{2+}\)]\(_{i}\), responses of arteriolar SMC to pressure (11, 27), of endothelial cells to flow (20), and of respective cell layers to a variety of agonists in vitro. Nevertheless, the isolation and preparation of segments for in vitro study remove arterioles from the networks they comprise while eliminating blood flow and subjecting vessels to temperature transitions exceeding 30°C. Moreover, key signaling pathways (e.g., oxygen sensitivity) are typically lost once arterioles are removed from their native environment (22, 24). Whereas recent in vivo studies have indicated a key role for [Ca\(^{2+}\)]\(_{i}\), in arteriolar endothelium during functional dilation in skeletal muscle (16), little is known of how arteriolar smooth muscle [Ca\(^{2+}\)]\(_{i}\), responds either to direct or indirect (i.e., endothelium-dependent) stimuli in vivo. Thus there is a need to bridge in vitro studies of vasomotor control with signaling events underlying the local control of tissue blood flow.

The goal of this study was to evaluate [Ca\(^{2+}\)]\(_{i}\), dynamics in arteriolar SMC in vivo. For this purpose, we developed methods based on fluorescence microscopy and photometry while preserving the integrity of blood flow control. A ratiometric calcium-sensitive dye (fura PE-3) was utilized to minimize the effect of movement artifacts and changes in vessel wall thickness associated with dilation and constriction (35). To reduce the potential for nonspecific signals while preserving the structural and functional integrity of arteriolar networks, aperenchymal segments were prepared in the epithelial region of the hamster cheek pouch for selective loading of SMC using abluminal perfusion of dye from a micropipette. To evaluate arteriolar SMC reactivity in light of [Ca\(^{2+}\)]\(_{i}\), dynamics through a spectrum of stimuli, measurements were performed during changes in ambient O\(_{2}\), during spontaneous vasomotion, and in response to agonists that acted either on SMC directly or via endothelium-dependent signaling. To establish the validity of our methods, complementary experiments were performed to evaluate the effects of blood flow within the vessel lumen on fluorescent signals. We tested the hypothesis that the rise and fall of SMC [Ca\(^{2+}\)]\(_{i}\), underlies arteriolar constriction and dilation in vivo. Although such relationships are implied from in vitro studies, it is essential that they be verified and extended in vivo. Our findings support this hypothesis and demonstrate that the resolution of arteriolar SMC [Ca\(^{2+}\)]\(_{i}\), in vivo discriminates key signaling events governing the local control of tissue blood flow.

METHODS

Animal Care

All procedures were approved by the Institutional Animal Care and Use Committee of The John B. Pierce Laboratory and were performed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male golden hamsters (90 ± 2 g; n = 18; Charles River Breeding Laboratories, Kingston, NY) were maintained at 24°C on a 14:10-h light-dark cycle and provided rodent chow and water ad libitum. Surgical procedures typically required ~3 h and were performed using a stereo microscope. Experimental procedures typically lasted 4–5 h. At the end of each day’s experiments, the hamster was euthanized with an overdose of pentobarbital sodium delivered intravenously.

### Surgical Procedures

A hamster was anesthetized with pentobarbital sodium (65 mg/kg, intraperitoneal injection) and tracheotomized to ensure airway patency. The right femoral vein was cannulated for continuous replacement of fluid and maintenance of anesthesia (10 mg pentobarbital/ml in sterile saline infused at 0.41 ml/h). Depth of anesthesia was maintained according to the spontaneous rate of ventilation and lack of withdrawal to toe pinch. Body temperature was maintained at 37°C by using conducted heat.

The hamster was positioned on a custom-fabricated (Technical Services, Pierce Laboratory, New Haven, CT) Plexiglas platform. The left cheek pouch was everted and opened longitudinally, and the epithelial region was pinned flat over an UV-fused silica glass window (25 mm diameter × 2 mm thick; Edmund Scientific, Barrington, NJ) mounted on a pedestal that was integral to the platform. Superficial connective tissue was removed using microdissection. A defined region along second-order arterioles was selected for study on the basis of 1) being located at least 1 cm away from the edge of the preparation, 2) lack of branching for a distance of ~500 μm, and 3) absence of adjacent venules. The epithelial tissue was carefully removed to obtain aparenchymal segments (22, 24).

The exposed cheek pouch was superfused continuously (~5 ml/min) with bicarbonate-buffered physiological saline solution (PSS, in mmol/l: 137.0 NaCl, 4.7 KCl, 1.2 MgSO4, 2.0 CaCl2, 18.0 NaHCO3; pH 7.4, 37°C) prepared with deionized water (dH2O) and equilibrated in sterile saline infused at 0.41 ml/h). The superfusate was equilibrated with 10 or 21% O2 (5% CO2-95% N2). The effluent solution was aspirated with a dye concentration of 1 μM (with 0.1% pluronic, 0.1% DMSO). Aperifusion micropipettes (tip ID 100 μm) from borosilicate glass capillaries were pulled 30 min before experimental procedures were initiated. One micropipettes (described below). Each preparation equilibrated for at least 30 min before experimental procedures were initiated. One micropipettes (described below). Each preparation averaged 1 mm in diameter (45 μm; Polysciences, Warrington, PA) were added to maintain a constant optical path length between slide and coverslip when positioned on the microscope stage. For each experiment, fluorescence ratios were determined at 0 [Ca2+]i (Rmin) and at 39 μM [Ca2+]i (Rmax), respectively; values were recorded three times and averaged. The constant β was calculated from the fluorescence during excitation at 380 nm with 0 μM [Ca2+]i divided by that with 39 μM [Ca2+]i. As illustrated in Fig. 1, plotting these data as log β(R − Rmin)/(Rmax − R)] vs. log [Ca2+]i enabled log Ks to be determined as the y-intercept. These experiments (n = 5) yielded the following values for our intravital microscopy system: Rmin = 0.5 ± 0.02, Rmax = 4.1 ± 0.01, β = 4.4 ± 0.01, and Ks = 303 ± 8 nM. [Ca2+]i, for arteriolar SMC was calculated by using the equation [Ca2+]i = Km × β × (R − Rmin)/(Rmax − R) (21).

### In Vivo Experiments

**Source of [Ca2+]i, signal.** To minimize the possibility of signals arising from cells other than SMC, epithelial tissue was dissected...
away to obtain aparenchymal arteriolar segments (22, 24). In preliminary experiments, ionomycin (10 μM) was added to the superfusion solution while the [Ca\(^{2+}\)] signal was recorded to confirm successful loading of fura PE-3 into the aparenchymal segment. Mast cells near the adventitia of cheek pouch arterioles (14) could incorporate dye and thereby affect the [Ca\(^{2+}\)] signal as well as arteriolar reactivity. To test for the presence of mast cells on aparenchymal segments, preparations were stained with toluidine blue (0.1% solution) at the end of experiments.

Vasomotor tone, oxygen sensitivity, and vasomotion. Key functional indexes of the physiological integrity of arterioles include the level of spontaneous vasomotor tone and sensitivity to changes in superfusate O2 content (22, 24). To test the O2 sensitivity of aparenchymal arteriolar segments in vivo, superfusate oxygen was increased from 0 to 21% for ~5 min before and after dye loading to evaluate [Ca\(^{2+}\)] responses during vasoconstriction. The level of spontaneous vasomotor tone was evaluated by adding sodium nitroprusside (SNP, 1 mM) to the superfusate. When vasomotion was present, [Ca\(^{2+}\)] was monitored while cyclic changes in arteriolar diameter were recorded, and no other interventions were performed in these preparations.

Endothelium-dependent vasodilation. Robust vasodilation in response to the endothelium-dependent vasodilator ACh provides another critical index of the functional integrity of arterioles in vivo (2, 4). With EC [Ca\(^{2+}\)], shown to increase upon activation of muscarinic receptors (3, 7, 9, 13, 36), a fall in the [Ca\(^{2+}\)] signal indicates SMC-specific responses. Furthermore, the ability to monitor changes in SMC [Ca\(^{2+}\)], during relaxation (dilation) as well as during contraction (constriction; below) is critical to understanding a full range of [Ca\(^{2+}\)] dynamics during blood flow control.

For these experiments, microiontophoresis was used to deliver ACh (1.0 M in dH2O; 1 μA, 1 pulse from a 1-μm micropipette tip) locally onto the dye-loaded segment (2, 38), while internal diameter and SMC [Ca\(^{2+}\)], were recorded simultaneously at the site of stimulation. Vehicle controls were performed to determine whether the loading solution affected vasomotor responses. Vessels were stimulated with ACh (as above) before and after the dye-loading protocol with the exception that the solution did not contain fura PE-3.

Optical effects of blood flow. The presence of red blood cells flowing in the vessel lumen can influence the optical properties of arterioles by scattering and absorbing light. To investigate these effects on recordings of SMC [Ca\(^{2+}\)] dynamics, aparenchymal arteriolar segments were monitored under control conditions and while blood was flushed from the lumen with PSS. Perfusion micropipettes were sharpened (OD 15 μm) by triple beveling using a rotating disk with an abrasive surface of 0.3 μm. The micropipette was filled with PSS containing heparin (10 U/ml; Elkins-Sinn; Cherry Hill, NC), secured in a micromanipulator, and inserted into a side branch located upstream from the aparenchymal segment. A hydrostatic water column connected to the micropipette was elevated until the effluent PSS completely flushed blood from the lumen of the observed arteriolar segment. The height of the column corresponded to pressures (45–50 cm H2O) that approximated values recorded in vivo (12). The effluent PSS was directed into the dye-loaded segment by occluding side branches between the perfusion micropipette and the recording site. Local responses to SMC activation via α1-adrenoceptors with phenylephrine (PE; 0.5 M in dH2O) microiontophoresis (1 μA, 1 s) were obtained under control conditions and during PSS perfusion. For the purpose of comparing responses with and without blood flow, a brief transient stimulus enabled the time course of responses to be observed as well as peak values.

Data Analyses

All data were acquired at 10 Hz. Diameter changes (μm) were calculated as the difference between the peak response and the preceding baseline diameter. Values for SMC [Ca\(^{2+}\)], were calculated on the basis of Rmin, Rmax, and Kd as determined in vitro, and the fluorescence intensities were recorded in vivo. Changes in SMC [Ca\(^{2+}\)], are reported as the difference between the peak response and the preceding baseline. Data were analyzed (SigmaStat v. 3.1, SPSS; Chicago, IL) by Student’s paired t-test or the Mann-Whitney rank sum test. Differences were considered statistically significant with P < 0.05. Summary data are presented as means ± SE.

RESULTS

Across experiments, resting SMC [Ca\(^{2+}\)], averaged 406 ± 37 nM (n = 18). Because these resting Ca\(^{2+}\) values were higher than previously reported for arterioles that were isolated from rat (27) and hamster (42) cremaster muscles for in vitro experiments using fura 2, we also measured resting SMC [Ca\(^{2+}\)], in cheek pouch arterioles in vivo using segments similar to those studied in vivo. Thus arterioles were isolated, cannulated, and loaded with fura 2-AM (Molecular Probes) at transmural pressure = 70 cm H2O. Smooth muscle [Ca\(^{2+}\)], and internal diameter were measured as described (6). In the presence of spontaneous (myogenic) tone (diameters: rest = 69 ± 5 μm; maximum = 93 ± 8 μm; n = 6), SMC [Ca\(^{2+}\)], was 284 ± 34 nM. On the basis of the overlap of respective 95% confidence intervals, this value recorded by a different system and alternate dye is not significantly different (P > 0.05) from that determined in vivo. For these in vitro experiments, Kd (259 μM) was determined by using the K+ salt form of fura 2 as described (see METHODS), and Rmin (0.54 ± 0.03) and Rmax (6.6 ± 0.1) were determined from arterioles incubated with 3 μM ionomycin and either 0 Ca\(^{2+}\) + 10 mM EGTA or 10 mM Ca\(^{2+}\) in the bath, respectively, with β = 6.7 ± 0.7.

As assessed with toluidine blue, arterioles of similar branch order within intact epithelial regions of the cheek pouch showed numerous perivascular mast cells. In contrast, mast cells were not readily apparent along aparenchymal segments (n = 3).

Vasomotor Tone, Oxygen Sensitivity, and Vasomotion

In preparations evaluated for vasomotor tone, resting diameter was 33 ± 2 μm. Elevating superfusate O2 from 0 to 21% produced constriction (11 ± 2 μm) that was unaffected by dye loading. In these preparations (n = 6), baseline [Ca\(^{2+}\)], was 511 ± 120 nM and increased by 108 ± 53 nM (P < 0.05), recorded 5 min after raising O2 to 21% during a sustained constriction. Superfusion of SNP reduced SMC [Ca\(^{2+}\)], by 124 ± 18 nM (n = 6, P < 0.05) during the first minute as dilation (23 ± 5 μm) ensued. As arterioles reached maximal diameter (48 ± 5 μm), SMC [Ca\(^{2+}\)], recovered to within 57 ± 21 nM of baseline (P > 0.05). Spontaneous vasomotion (3–4 cycles/min) was observed in four experiments. Representative traces of SMC [Ca\(^{2+}\)], and diameter changes during vasomotion in vivo are shown in Fig. 2. Across these experiments (n = 4), SMC [Ca\(^{2+}\)], changes (150 ± 53 nM) preceded diameter changes (7 ± 1 μm) by ~2 s.

Endothelium-Dependent Vasodilation

Acetylcholine dilated arterioles transiently by 17 ± 1 μm and was associated with a transient fall in SMC [Ca\(^{2+}\)], of 131 ± 21 nM (Fig. 3; n = 6, P < 0.05). The amplitude of vasodilation to ACh before dye loading (20 ± 2 μm) was not significantly different. Independent vehicle controls confirmed...
that there was no effect of the loading solution on vasodilation to ACh ($n = 4$; data not shown).

**Effect of Blood Flow**

The resting diameter of aparenchymal arteriolar segments containing blood flow ($40 \pm 3 \, \mu m, n = 8$) was not different from that when flushed with PSS ($42 \pm 3 \, \mu m, n = 7$). Flushing blood from the arteriolar lumen increased fluorescence (at 510 nm) by $\sim 45\%$ during excitation at both 340 and 380 nm (Fig. 4A). Nevertheless, the 340/380 nm ratio from fura PE-3 indicated that baseline SMC $[Ca^{2+}]_{i}$ during blood flow ($371 \pm 24 \, nM$) was not significantly different from that when the lumen contained PSS ($366 \pm 29 \, nM, n = 12$; Fig. 4B).

In response to PE (1 $\mu A$, 1 s), $[Ca^{2+}]_{i}$ increased transiently by $479 \pm 64 \, nM (P < 0.05)$ with a constriction of $26 \pm 3 \, \mu m$ (Fig. 5A) that eliminated blood flow as the lumen closed. With PSS perfusing the vessel lumen, neither the rise in SMC $[Ca^{2+}]_{i}$, ($498 \pm 78 \, nM$) nor the magnitude of vasoconstriction ($25 \pm 4 \, \mu m$) was significantly different from control (Fig. 5B). Reducing stimulus duration from 1,000 to 500 ms significantly ($P < 0.05$) attenuated the amplitude of both vasoconstriction and the underlying $Ca^{2+}$ transient to the same extent under both conditions (data not shown).

To resolve the effect of blood on fluorescence originating from arteriolar SMC in vivo, responses to PE recorded under control conditions were compared with those obtained when the lumen was flushed with PSS. During blood flow, the numerator increased rapidly at first and then more slowly while the denominator initially decreased and then increased above baseline near the end of the transient rise in SMC $[Ca^{2+}]_{i}$ (Fig. 6A). These events coincided with peak vasoconstriction (and flow cessation, above). In contrast, during the PE response with PSS in the lumen, the divergence between numerator and denominator was maintained during the $[Ca^{2+}]_{i}$ transient through recovery to respective baselines (Fig. 6B). Despite the difference in behavior of respective 340 and 380 signals between conditions, $[Ca^{2+}]_{i}$ transients based on the fura PE-3 ratio were nearly indistinguishable (Figs. 5 and 6). The lack of difference in time course for the onset and peak responses between conditions (Table 1) further confirms the reproducibility of $[Ca^{2+}]_{i}$ responses, irrespective of blood in the vessel lumen.

**DISCUSSION**

Our goal was to investigate SMC $[Ca^{2+}]_{i}$ dynamics concomitant with vasomotor responses in arterioles controlling tissue blood flow. For this purpose, the ratiometric $Ca^{2+}$-sensitive dye fura PE-3 was selectively loaded into arteriolar SMC without compromising functional integrity of smooth muscle or endothelium. Whereas the presence of blood attenuated fluorescent signals by scattering and absorbing light, $[Ca^{2+}]_{i}$ dynamics of SMC were not different from those recorded when blood was flushed from the lumen. The isolation and cannulation of arterioles (15) has enabled in vitro studies of the regulation of $[Ca^{2+}]_{i}$ dynamics in microvascular smooth muscle and endothelium (13, 27, 42). However, vessel segments...
Segments are isolated for in vitro study (22, 24). Thus the tissue is exposed to experimental perturbation and is typically lost once activation has been implicated in mediating SMC depolarization. Therefore, the experimental approach is validated by evaluating arteriolar SMC [Ca$^{2+}$]$_i$ dynamics under physiological conditions.

**Functional Integrity of Aparenchymal Arterioles**

Arteriolar reactivity to changes in superfusate Po$_2$ is sensitive to experimental perturbation and is typically lost once segments are isolated for in vitro study (22, 24). Thus the increase in arteriolar SMC [Ca$^{2+}$]$_i$ during O$_2$-induced constriction supports the physiological nature of responses in vivo while confirming the integrity of aparenchymal arterioles after loading SMC with fura PE-3. In so doing, we present the first data for arteriolar SMC [Ca$^{2+}$]$_i$ responses to changes in Po$_2$.

Consistent with the observed increase in SMC [Ca$^{2+}$]$_i$ during 21% O$_2$ (results), an integral role for L-type Ca$^{2+}$ channel activation has been implicated in mediating SMC depolarization and arteriolar constriction in response to elevated Po$_2$ (37). As illustrated in Fig. 2, SMC [Ca$^{2+}$]$_i$, cycled with arteriolar diameter (and tissue blood flow) during spontaneous vasomotion. Nifedipine (an L-type Ca$^{2+}$ channel antagonist) was found to dilate these arterioles and eliminate vasomotion (1). In turn, the relaxation of arteriolar SMC in response to ACh is associated with hyperpolarization (33, 38, 39). Thus the fall in SMC [Ca$^{2+}$]$_i$, as observed here (Fig. 3), can be explained by closure of L-type Ca$^{2+}$ channels (23, 28). In response to PE, the rise in SMC [Ca$^{2+}$]$_i$ preceding vasoconstriction (Fig. 5) is consistent with that shown for arteriolar SMC (13, 27, 36, 42) in vitro and can be explained by the activation of L-type [Ca$^{2+}$]$_i$ channels upon depolarization (29, 38).

Collectively, the present data are consistent with the control of arteriolar SMC in vivo through electromechanical coupling (23, 28). Furthermore, the frequency and amplitude of vasomotion are similar to those studied previously with respect to underlying electrophysiological events (1). The delay observed between electrical events and vasomotor responses (~3 s; Ref. 1) is slightly longer than the ~2-s lag found here between the rise (and fall) in SMC [Ca$^{2+}$]$_i$ and arteriolar constriction (or dilation). These temporal relationships further indicate that changes in SMC membrane potential govern the open probability of L-type Ca$^{2+}$ channels to control arteriolar diameter (23). Moreover, as shown for membrane potential of arteriolar SMC in vivo (1), the bidirectional changes in arteriolar SMC [Ca$^{2+}$]$_i$ confirm that such measurements are responsive in both directions.

**Selectivity of SMC Labeling and Ca$^{2+}$ Signaling**

In the arterioles studied here, the endothelium is circumvented by a monolayer of SMC, with respective cell layers readily distinguished according to longitudinal vs. circumferential orientation (26, 32, 38). In vitro studies have shown that respective cell type-specific [Ca$^{2+}$]$_i$ measurements from arterioles are made possible by using acetoxymethyl ester forms of the dye and restricting the loading solution to the luminal or abluminal surface of the vessel wall (13, 20, 27, 42). Thus delivery in the bath targets SMC and luminal perfusion targets EC. A complementary strategy for dye delivery has proven useful in selectively disrupting respective cell layers using photodamage in vitro (19) and in vivo (2, 4, 25).

For intravital studies of arteriolar EC, techniques similar to those used to flush blood from the lumen (see Methods) have been used to load Ca$^{2+}$-sensitive dyes (16, 17). Whereas constraining the dye to the luminal compartment minimizes exposure of other cells to the dye solution, restriction of the dye to the SMC is not possible in the intact tissue, because arterioles are typically embedded in the tissue parenchyma. By preparing aparenchymal segments to minimize nonspecific signals, the present study demonstrates selective dye loading of arteriolar SMC in vivo using local perfusion. Visual inspection confirmed that the distribution of fura PE-3 was restricted to circumferentially oriented SMC, as are the [Ca$^{2+}$]$_i$ dynamics.

The [Ca$^{2+}$]$_i$ signals recorded here decreased routinely after an ACh stimulus, as illustrated in Fig. 3 and reported for SMC in isolated vessels with intact EC (3, 13). In contrast, the [Ca$^{2+}$]$_i$ signal originating from EC should increase in response to ACh (3, 7, 9, 13, 36). Nevertheless, EC [Ca$^{2+}$]$_i$ has also
been shown to increase during constriction to PE and attributed to Ca\(^{2+}\)/H\(^{+}\) diffusion from SMC through myoendothelial gap junctions (13, 42). An alternate although unresolved explanation is that \(\alpha_2\)-adrenergoreceptors expressed on EC elicit a Ca\(^{2+}\)/H\(^{+}\) response in the endothelium (10, 36).

Despite compelling evidence for gap junctional coupling between SMC and EC in microvessels isolated for in vitro study (18, 26, 40, 41), myoendothelial coupling has not been manifest during intravital studies of arterioles in vivo, either in the hamster cheek pouch (2, 8, 38) or the mouse cremaster muscle (33). Perhaps the discrete nature of myoendothelial contacts in these microvessels (31) enables local regulation of heterocellular coupling in a manner that can be affected by experimental conditions. In light of the findings presented here, the ability to monitor Ca\(^{2+}\)/H\(^{+}\) dynamics from arteriolar SMC as well as EC both in vivo and in vitro should prove insightful with respect to resolving such controversies in light of differences in respective investigative strategies.

Superfusion of SNP, an NO donor acting independent of the endothelium, resulted in a transient decrease in SMC [Ca\(^{2+}\)]\(_i\) that returned toward baseline, despite the persistence of vasodilation. This behavior differs from that reported for small branches of the hamster femoral artery studied in vitro after precontraction with norepinephrine, where dilation occurred independent of changes in SMC [Ca\(^{2+}\)]\(_i\). Thus, although pharmacomechanical coupling is implied under both conditions, its time course and contribution may vary between vessels as well with experimental conditions. Further evidence for changes in Ca\(^{2+}\) sensitivity of arteriolar SMC comes from in vitro studies, where activation of protein kinase C with indolactam produced vasoconstriction with no change in [Ca\(^{2+}\)]\(_i\) (13, 27). Although signaling events underlying calcium sensitization were not studied here, the present methods should prove useful in investigating these signaling pathways in future studies.

Critique of Methods

Photometry. Under resting conditions across experiments, the [Ca\(^{2+}\)]\(_i\) reported here for SMC in hamster cheek pouch arterioles (~400 nM) is severalfold higher than that reported for SMC in arterioles isolated from the cremaster of the rat (~55 nM) (27) or hamster (~170 nM) (42). As in vitro measurements tend to overestimate [Ca\(^{2+}\)]\(_i\) (30), this difference is unlikely to be explained by our calibration experiments. It is possible that the level of tone differed or that higher loading with fura 2 may have buffered [Ca\(^{2+}\)]\(_i\) in these former studies. The substantial level of spontaneous vasomotor tone observed here is consistent with Ca\(^{2+}\)-dependent activation of contractile proteins in SMC. Furthermore, a resting SMC [Ca\(^{2+}\)]\(_i\) of ~400 nM allows for the substantial decrease (~130 nM) during vasodilation with ACh as well as the near-doubling of SMC [Ca\(^{2+}\)]\(_i\) during peak constriction (to lumen closure) in response to PE. Although the application of our in vitro calibrations (see METHODS) to calculations of SMC [Ca\(^{2+}\)]\(_i\) in vivo must be interpreted with caution, the \(K_d\) we determined in vitro (303 nM) for this study provides a reproducible reference for our experimental conditions.

Compared to photometry using conventional fluorescence microscopy, confocal imaging can provide greater temporal and spatial resolution of Ca\(^{2+}\) signals (5, 17). However, the shallow depth of field renders the preparation more sensitive to movement as regions of interest can leave the focal plane.
In contrast to optically sectioning a microvessel by using confocal imaging (5, 17), photometry with conventional optics acquires light from above and below the focal plane. Thus obtaining cell type-specific responses requires that the indicator be confined to cells of interest. An alternative approach involves discrete sampling of pixel intensities in fluorescent images of the arteriolar wall after luminal or abluminal dye labeling (9). It is possible that abluminal perfusion resulted in dye uptake into EC that was undetectable by eye but sufficient to attenuate the integrated signal detected by the photometer. Nevertheless, the present data illustrate that the use of a ratiometric dye provides an affordable approach to obtaining a robust signal that can resolve SMC [Ca\(^{2+}\)] dynamics in vivo. In turn, such measures provide information for distinguishing between vasomotor responses mediated by electromechanical (i.e., Ca\(^{2+}\)-dependent) coupling of contractile proteins vs. those mediated by changes in their Ca\(^{2+}\) sensitivity (34).

**Effect of blood.** We used fura PE-3 in the present study as cellular retention of dye and fluorescence signals were found to be brighter and longer lasting than that obtained with either fura 2 or bis-fura in our preliminary studies. With either ratiometric dye, as free [Ca\(^{2+}\)] increases, the fluorescence at 510 nm should increase during excitation at 340 nm and fall during excitation at 380 nm (35). With reference to the robust responses in SMC [Ca\(^{2+}\)], evoked by PE, we investigated how the presence of blood affected respective signals. As shown in Fig. 4, flushing blood from the vessel lumen increased both the 340-nm signal and the 380-nm signal by the same amount (~45%) with no effect on the fura PE-3 ratio. Indeed, Fig. 5 and Table 1 illustrate that, across experiments, neither SMC [Ca\(^{2+}\)], dynamics nor vasoconstriction to PE was affected by whether blood was present in the vessel lumen.

The effect of blood on respective signals is clearly resolved in Fig. 6. In the presence of blood flow, the 380-nm signal initially decreased and then increased above baseline as arterioles constricted, whereas the 340-nm signal increased rapidly at first and then more slowly. In contrast, with blood flushed from the lumen, there was a clear divergence of respective signals as predicted (35). Nevertheless, the fura PE-3 ratio and SMC [Ca\(^{2+}\)], were similar under both conditions, as was the vasomotor response. Thus we attribute the blood “artifact” (which is most apparent in the 380-nm signal) to the progressive emptying of blood from the vessel lumen during constriction with the corresponding elimination of light scattering and absorption by red blood cells. In vitro studies using fura 2 had indicated that a component of both signals can increase as a consequence of constriction presenting a larger tissue volume (i.e., dye content) in the optical sample (27). In accord with the

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**Table 1. Time course of arteriolar smooth muscle calcium and diameter responses to phenylephrine**

<table>
<thead>
<tr>
<th></th>
<th>[Ca(^{2+})] Onset</th>
<th>Constriction Onset</th>
<th>Peak [Ca(^{2+})]</th>
<th>Peak Constriction</th>
<th>Delay</th>
<th>Delay Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.6±0.1</td>
<td>1.6±0.1</td>
<td>2.0±0.2</td>
<td>7.7±0.5</td>
<td>1.1±0.1</td>
<td>5.7±0.4</td>
</tr>
<tr>
<td>Flush</td>
<td>0.3±0.1</td>
<td>1.5±0.1</td>
<td>2.1±0.3</td>
<td>6.9±0.3</td>
<td>1.2±0.1</td>
<td>4.8±0.4</td>
</tr>
</tbody>
</table>

Summary data for temporal aspects of arteriolar smooth muscle cell intracellular calcium concentration ([Ca\(^{2+}\)]) and diameter responses to phenylephrine (PE) microiontophoresis (1 μA, 1,000 ms) during blood flow (Blood) and while flushing blood from the vessel lumen (Flush). Data are means ± SE in seconds (blood: n = 8; flushed: n = 7). [Ca\(^{2+}\)], onset, time elapsed between PE stimulus and initial rise in [Ca\(^{2+}\)]; onset, time elapsed between PE stimulus and peak of arteriolar constriction; peak [Ca\(^{2+}\)], time elapsed between PE stimulus and peak [Ca\(^{2+}\)]; peak constriction, time elapsed between PE stimulus and peak constriction; delay, time elapsed between onset of [Ca\(^{2+}\)], rise and onset of arteriolar constriction; delay peak; time elapsed between onset of [Ca\(^{2+}\)], rise and peak of arteriolar constriction. There were no significant differences between respective conditions.

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**Fig. 6. Effect of blood on numerator and denominator fluorescence intensity during changes in arteriolar SMC [Ca\(^{2+}\)].** In response to PE. **A:** With blood present in the lumen, numerator (signal with excitation at 340 nm; ○) and denominator (signal with excitation at 380 nm; □) initially diverge as calculated [Ca\(^{2+}\)]. The denominator then reverses and overshoots (dashed rectangle) along with secondary increase in numerator as vessel constricts. **B:** With blood flushed from the lumen, divergence between numerator and denominator was maintained during the [Ca\(^{2+}\)], transient.

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**Fig. 4.** Effect of blood on respective signals as predicted (35). Nevertheless, the fura PE-3 ratio and SMC [Ca\(^{2+}\)], were similar under both conditions, as was the vasomotor response. Thus we attribute the blood “artifact” (which is most apparent in the 380-nm signal) to the progressive emptying of blood from the vessel lumen during constriction with the corresponding elimination of light scattering and absorption by red blood cells. In vitro studies using fura 2 had indicated that a component of both signals can increase as a consequence of constriction presenting a larger tissue volume (i.e., dye content) in the optical sample (27). In accord with the
present findings, the 340/380-nm ratio was found to be insensitive to this effect (27). These control experiments substantiate our ability to evaluate SMC [Ca\textsuperscript{2+}] dynamics in vivo and resolve the effect of blood on the optical properties of fura PE-3.

In conclusion, we present the first recordings of [Ca\textsuperscript{2+}] dynamics in arteriolar smooth muscle cells during the local control of tissue blood flow using conventional fluorescence microscopy and Ca\textsuperscript{2+} photometry. Experiments monitored [Ca\textsuperscript{2+}] responses to stimuli that act directly on SMC (O\textsubscript{2}, PE, and SNP) or indirectly via the endothelium (ACh). Our findings indicate that aparenchymal arteriolar segments retain functional integrity, including oxygen sensitivity, spontaneous vasomotion, after selective loading of SMC with the calcium-sensitive dye fura PE-3. Although the presence of blood flow in the arteriolar lumen attenuates fluorescence significantly, the ratiometric nature of fura PE-3 enables measurements of SMC

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