Low intravascular pressure activates endothelial cell TRPV4 channels, local Ca\textsuperscript{2+} events, and IK\textsubscript{Ca} channels, reducing arteriolar tone

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Endothelial cell (EC) Ca\textsuperscript{2+}-activated K\textsuperscript+ (IK\textsubscript{Ca}) channels in arteriolar endothelial cells (ECs) are activated by intrinsic spontaneous or receptor-mediated Ca\textsuperscript{2+} events, each leading to hyperpolarization of smooth muscle cells (SMCs) and vasodilation independent of nitric oxide—endothelial-dependent hyperpolarization (EDH) response. This hyperpolarization spreads both radially and longitudinally through the vascular wall via patent gap junctions to evoke local and conducted dilation, and it is central to cardiovascular function (1, 2).

EDH is the predominant endothelium-dependent mechanism in smaller “resistance” arteries and arterioles. The underlying hyperpolarization is generated by two subtypes of IK\textsubscript{Ca} channels found in the EC, but not SMC, membrane, the small (SK\textsubscript{Ca}, IK\textsubscript{Ca}\textsubscript{2.3}) and intermediate (IK\textsubscript{Ca}\textsubscript{3.1}) conductance forms that may be activated independently of each other (3). The physiological importance of independent activation is apparent from studies with K\textsubscript{Ca}3.1-deficient mice in which the mean blood pressure is raised by ∼7 mmHg, but further elevated by disrupting both K\textsubscript{Ca} channels (4). In mesenteric resistance arteries, IK\textsubscript{Ca} channels are focused within EC projections through the internal elastic lamina (IEL) termed myoendothelial junctions (MEJs). MEJs can contain gap junctions (MEGJs) coupling ECs to SMCs, and EDH can spread by direct electrical coupling and/or a diffusible factor (5, 6). The IK\textsubscript{Ca} channels enriched within MEJs can be activated by spontaneous inositol 1,4,5-trisphosphate (IP\textsubscript{3})-mediated Ca\textsuperscript{2+} events, discovered in unpresurized arteries and termed Ca\textsuperscript{2+} pulsars (7). Block of these intrinsic Ca\textsuperscript{2+} pulsars, or of IK\textsubscript{Ca} channels, depolarizes the adjacent SMC by ∼8 mV, suggesting that the two are functionally linked and continually suppress SMC membrane potential (7). Although ECs in pressurized resistance arterioles/arteries in vivo and in vitro generate spontaneous EC Ca\textsuperscript{2+} events, it is not known whether changes in intraluminal pressure influence these events and whether there is a functional consequence against myogenic tone (8–10).

Because the endothelium has a marked inhibitory influence on vascular tone in vivo, particularly by emitting hyperpolarization, it is puzzling that removal of this monolayer does not seem to alter the myogenic response per se (11, 12). Furthermore, changes in intraluminal pressure in arterioles appear not to alter global EC (Ca\textsuperscript{2+})\textsubscript{1} (13). However, the spatial and temporal resolution of [Ca\textsuperscript{2+}]\textsubscript{i} measurements has improved greatly since the early 1990s, revealing, for example, that SMCs and ECs generate elementary and spatially restricted Ca\textsuperscript{2+} events spontaneously. These rapid, subcellular events are not necessarily reflected by global [Ca\textsuperscript{2+}]\textsubscript{i}; e.g., localized Ca\textsuperscript{2+} sparks in cerebral artery SMCs suppress tone by activating BK\textsubscript{Ca} channels in the adjacent subcortex (14).

We therefore investigated the possibility that arteriolar intraluminal pressure might influence the generation of spontaneous Ca\textsuperscript{2+} events in the endothelium and thereby alter myogenic tone. Myogenic tone reflects SMC constriction that is related to the level of intraluminal pressure. Acute increases in pressure evoke vasodilation apparent at pressures of 30–40 mmHg and above, reflecting divergence of the active and passive pressure/diameter curves (15). Of direct relevance to the present study, in vivo measurement of intravascular pressure in rat cremaster muscle arterioles at rest revealed pressures of 70–80 mmHg (16). The myogenic response stabilizes local blood flow and capillary filtration pressure as blood pressure changes and provides a basal tone that can be enhanced or attenuated by nerve transmitters, autacoids, and hormones (15). Myogenic contraction relies in large part on SMC depolarization, so hyperpolarization originating from either cell type will exert a significant influence on tone (17).

Our data reveal a unique mechanism intrinsic to ECs, whereby low (<50 mmHg) intraluminal pressure activates TRPV4 channels to increase the frequency of EC Ca\textsuperscript{2+} events within MEJs. The increase is sufficient to activate EC IK\textsubscript{Ca} channels and, as a consequence, reverse or suppress myogenic tone. These observations extend the concept of MEJs as signaling microdomains and reveal a physiological role for these thin membrane structures.

**Results**
All experiments were performed using isolated, cannulated, and pressurized rat cremaster arterioles, with a passive internal diameter of 160–200 μm at 80 mmHg, a pressure at which they developed spontaneous myogenic tone.

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Effect of Intraluminal Pressure on EC and SMC Ca\textsuperscript{2+} Events and Myogenic Tone. We developed a method to simultaneously record EC and SMC Ca\textsuperscript{2+} events in adjacent cells using multiphoton microscopy at the arteriole midplane. By changing intraluminal pressure, we observed reciprocal changes in Ca\textsuperscript{2+} events within the two cell types. Decreasing pressure (80–5 mmHg) reduced the frequency of spontaneous Ca\textsuperscript{2+} events in the SMCs from 17.9 ± 1.2 to 7.9 ± 1.0 min\textsuperscript{−1}. At the same time, the frequency of Ca\textsuperscript{2+} events in the adjacent ECs increased from 1.9 ± 0.3 to 5.1 ± 0.8 min\textsuperscript{−1} (Fig. 1D and Movie S1).

Responses at the midplane were confirmed by lowering the plane of focus to view ECs at the bottom of the arteriole (Fig. 2). The majority of spontaneous EC Ca\textsuperscript{2+} events were nonpropagating (local) (5 mmHg, 73%; 80 mmHg, 75% of events), whereas the remaining subset propagated along the EC axis (waves). Of note, spontaneous EC Ca\textsuperscript{2+} events were not reflected by global Ca\textsuperscript{2+} measurements (Fig. S1 and Movie S2). To establish the pressure at which the activation of EC Ca\textsuperscript{2+} events changed, full myogenic response curves were performed. Step increases in intraluminal pressure (5–80 mmHg) were associated with passive distension of the vessel, and beyond 30 mmHg, myogenic tone developed in the vessel, and beyond 30 mmHg, myogenic tone developed in a pressure-dependent manner (Fig. 2D). The diameter of arterioles was similar at 5 mmHg (no tone, 64.2 ± 7.6 μm, n = 3) and 80 mmHg (myogenic tone, 72.2 ± 5.8 μm, n = 3), and localized, spontaneous EC Ca\textsuperscript{2+} events were present at each pressure. The frequency of ECs events was significantly increased at and below 50 mmHg, as was the percentage of active cells (5 mmHg, 61 ± 7% compared with 80 mmHg, 42 ± 5%; paired data, n = 14, P < 0.05).

A similar profile was observed in mesenteric resistance arteries (not myogenically active; Fig. S2B). The spontaneous EC Ca\textsuperscript{2+} events in cremaster arterioles did not synchronize between cells, and although Ca\textsuperscript{2+} events seemed to originate from the same subcellular region of an EC, in some cases they propagated (Fig. 2B).

TRPV4 Channels Underlie Increases in EC Ca\textsuperscript{2+} Events at Low Pressure. The TRPV4 channel agonist GSK1016790A (GSK, 30 nM) evoked local increases in EC Ca\textsuperscript{2+} at 5 and 80 mmHg (Fig. 3A). With 100 nM GSK, these events evolved into cell-wide Ca\textsuperscript{2+} waves, but this concentration visibly damaged the preparation (as also observed by ref. 18). As a consequence, regardless of GSK concentration used, each experiment was terminated after exposure to the agonist. GSK also evoked arteriolar dilation, an effect blocked by the TRPV4 channel antagonist RN1734 (30 μM; Fig. 3B). RN1734 also reduced the frequency of spontaneous EC Ca\textsuperscript{2+} events at low pressure, but not at 80 mmHg, although it did block increases to 30 nM GSK at both pressures (Fig. 3C–F) and reduced the EC-dependent vasodilation to ACh (Fig. S3). Similar effects were obtained with another TRPV4 antagonist (HC067047, 10 μM; Fig. S4) and in mesenteric arteries (Fig. S2C and D). RN1734 also blocked ATP-sensitive K\textsuperscript{+} channels, although this action did not account for the effects against EC Ca\textsuperscript{2+} events (Table S1).

Spatial Correlation Between EC Ca\textsuperscript{2+} Events and TRPV4 Channels. Spontaneous EC Ca\textsuperscript{2+} events were mainly focused within holes through the IEL (71 ± 1% of cases, 213/300 events, n = 3). In the same arterioles, 58 ± 6% of these events correlated with signal

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**Fig. 1.** Midplane imaging of spontaneous Ca\textsuperscript{2+} events in pressurized cremaster arteriolar ECs and SMCs. (A) Schematic representation of an arteriole (Left) showing the SMCs (light gray) wrapped circumferentially around the axially aligned ECs (dark gray, Center). Focusing at the midplane of an arteriole (blue box) allows both cell types to be imaged simultaneously (Right). (B and C) Midplane images of an intact arteriolar pressurized to either 5 (B) or 80 (C) mmHg (ECs left to right, SMCs in cross section). Regions of interest (ROIs) shown on the image correspond to the temporal fluorescence traces from either ECs (Upper) or SMCs (Lower). (Scale bar, 10 μm; Movie S1). (D) Summary data of events recorded simultaneously in both cell types. The pressure-dependent frequency of EC Ca\textsuperscript{2+} events (Upper) changed in the opposite direction to SMCs (Lower). Note that changes in fluorescence intensity do not directly relate to amplitudes in [Ca\textsuperscript{2+}], *P < 0.05, significantly different from 5 mmHg (n = 5). Images were acquired at ~3 Hz.
for TRPV4 channels (Fig. 4). In general, the expression of TRPV4 channels also corresponds to holes in the IEL in cremaster (Fig. 4A) and mesentery (Fig. S2A). Punctate, membrane-localized expression of TRPV4 channels was also evident in SMCs (Movie S3).

**TRPV4, IKCa, and SKCa Channels Cluster in Holes Through the IEL.** The 3D reconstruction of z-stack confocal sections through the wall of pressurized arterioles revealed dense clusters of both KCa3.1 and KCa2.3 channels within holes in the IEL (Fig. 5 and Movie S3).

**Blocking TRPV4 and IKCa Channels Increases Myogenic Tone.** Nitric oxide did not modulate myogenic tone at any pressure (Fig. S5A). In the presence of N-nitro-l-arginine methyl ester (l-NAME) to block NO synthase, inhibition of TRPV4 channels with 30 µM RN1734 significantly increased myogenic tone at low pressure (20 and 40 mmHg; n = 4), although this effect was intermediate to the increase in myogenic tone that developed after selective block of IKCa channels with 1 µM TRAM-34 (n = 3; Fig. 6). The ability of TRAM-34 to increase myogenic tone at pressures <40 mmHg was not mimicked by block of SKCa channels with apamin (n = 3), although the combined presence of TRAM-34 and apamin did increase myogenic tone slightly more than TRAM-34 alone (n = 6). When the endothelium was physically disrupted, myogenic tone at low pressures was unaffected by TRAM-34, apamin, and l-NAME (Fig. S5B).
Intraluminal perfusion of 10 μM xestospongin C significantly reduced the frequency of EC Ca\(^{2+}\) events at 3 mmHg and almost abolished events at 80 mmHg (Fig. 7B). In contrast, neither inhibition of phospholipase A\(_2\) (PLA\(_2\)) with 3 μM AACOCF\(_3\) (Fig. 7C), voltage-gated Ca\(^{2+}\) channels (VGCC) with 1 μM nifedipine (Fig. 7D), nor block of ryanodine receptors with 10 μM ryanodine (Fig. 7F) affected the frequency of EC spontaneous Ca\(^{2+}\) events. Removal of extracellular Ca\(^{2+}\) did not alter the EC spontaneous Ca\(^{2+}\) events at 80 mmHg but reduced the event frequency at 5 mmHg by ∼50% (Fig. 7E), a similar profile seen with the TRPV4 channel antagonist RN1734 (Fig. 3 E and F).

**Discussion**

Little is known about the functional importance of spontaneous Ca\(^{2+}\) events generated by vascular ECs in situ. We now show an intrinsic mechanism involving EC Ca\(^{2+}\) events in pressurized arteries and arterioles that activates hyperpolarization and dilation in response to low intraluminal pressure. The mechanism involves TRPV4 channels focused within MEJs that are activated by low pressure to increase local EC Ca\(^{2+}\) event frequency. Direct activation of TRPV4 channels leads to Ca\(^{2+}\) sparklets (18), and, indirectly, sparklets will stimulate other [Ca\(^{2+}\)]\(_i\)-sensitive Ca\(^{2+}\) signaling components [e.g., PLC, IP\(_3\) receptors (IP\(_3\)Rs)]. Whether direct or indirect, the increase in local [Ca\(^{2+}\)]\(_i\) activates adjacent...
EC IK_{Ca} channels, causing EC-dependent vasodilation at low pressures, reducing myogenic tone (Fig. S6). The fact that similar changes in frequency in response to pressure occur in mesenteric resistance arteries in the absence of myogenic tone suggests that the relationship is a general property of arterial ECs, providing that TRPV4 and K_{Ca} channels are present. As such, these direct and indirect contributions should now be considered when measuring the frequency and spatial location of spontaneous EC Ca^{2+} events in arteries and arterioles.

ECs in intact arteries and arterioles can generate spontaneous, localized, and asynchronous Ca^{2+} events that are universally ryanodine-insensitive, and, at least in part IP_{3}R-based, i.e., Ca^{2+} puffs/pulsars. The spontaneous events can propagate to generate Ca^{2+} waves across ECs (7–10, 19, 20). However, it is not clear whether there is any functional correlation for these spontaneous EC Ca^{2+} events, particularly in the absence of agonist stimulation. By studying isolated, intact arteries and arterioles under physiological intraluminal pressures, we observed spontaneous EC Ca^{2+} events predominantly in the vicinity of holes through the IEL and at a similar frequency to the recently defined Ca^{2+} pulsars in mouse mesenteric resistance artery ECs (4–6 mmHg, 50 mmHg) expressed en face arteries and ~2 min^{-1} in arteries pressurized to 80 mmHg (7). Our data now extend these seminal observations, showing a subpopulation of EC Ca^{2+} events activated specifically by intraluminal pressures <50 mmHg. Before the recent identification of Ca^{2+} sparklets by Sonkusare et al. (18), most of the events we observe would have been considered pulsars.

We also show that the EC Ca^{2+} events induced by low intraluminal pressure involve TRPV4 channels. ECs contain a range of different TRP channels (21), and in resistance arteries, TRPV4 channels are intimately involved in vasodilation to EC-dependent agonists (22, 23) and shear stress-induced dilation (24). Sustained activation to low pressure is a unique addition to this list. The Ca^{2+} events stimulated by low pressure can be identified by removing extracellular Ca^{2+} or by pharmacologically blocking TRPV4 channels (schematically depicted in Fig. S6). Interestingly, both mesenteric resistance arteries and cremaster arterioles displayed an increase in frequency of EC Ca^{2+} events at 5 mmHg. This increase required Ca^{2+} influx through TRPV4 channels, because TRPV4 antagonists suppressed the frequency at 5 mmHg to a rate similar to 80 mmHg, i.e., ~2 min^{-1}. This activity profile correlated with dense immunohistochemical signal for TRPV4 channels in the regions generating the EC Ca^{2+} events. TRPV4 expression was also apparent in some SMCs, but GSK evoked dilation, so any potential role is not clear.

Although the low-pressure-induced EC Ca^{2+} events appear primarily due to activation of TRPV4 channels, it was not possible to separate the contribution due to influx through TRPV4 channels from indirect activation of other [Ca^{2+}]_{i}-sensitive pathways resulting in Ca^{2+} influx and/or release. Together, all these routes can potentially generate cell-wide waves in the endothelium of pressurized cremaster arteries. Importantly, because TRPV4 channels are themselves [Ca^{2+}]_{i}-sensitive (25), sparklets may also propagate into waves via activation of adjacent TRPV4 channels.

How low intraluminal pressure might activate EC TRPV4 channels is not clear. Although TRPV4 channels can be activated by arachidonic acid derivatives (epoxyeicosatrienoic acids; ref. 26), this possibility could be ruled out because a PLA2 inhibitor had no effect on the EC Ca^{2+} events. The most likely explanation is that activation somehow follows a reduction in the luminaly generated radial compressive force experienced by the EC monolayer. TRPV4 channels are sensitive to changes in cell shape, as demonstrated by activation in response to increasing shear stress or as a result of hypotonic cell swelling (27, 28). In our experiments, EC Ca^{2+} events were decreased to a similar extent at 80 mmHg (as in vivo; ref. 16) in cremaster arterioles, whether or not the arterioles developed myogenic tone (with or without nifedipine present). These observations were consistent with our experiments using myogenically inactive mesenteric

![Fig. 6. EC TRPV4 and IK_{Ca} channels suppress the development of myogenic tone in cremaster arterioles. (A and B) At low intraluminal pressure, block of either TRPV4 channels with RN1734 (30 µM, n = 4; A) or IK_{Ca} channels with TRAM-34 (TR, 1 µM, n = 3; B) significantly increased the level of myogenic tone. (C) In contrast, block of SK_{Ca} channels with apamin (Ap) did not alter the pressure/diameter profile (100 nM, n = 3). The passive diameters obtained in Ca^{2+}-free buffer for each dataset are also shown. *P < 0.05, significantly different from control (100 µM l-NAME) at the same pressure (paired observations).](image)

**Fig. 7.** EC Ca^{2+} events involve IP_{3}R signaling and Ca^{2+} influx but not arachidoninc acid derivatives, VGCC, or ryanodine receptors. (A) EC spontaneous Ca^{2+} events in pressurized cremaster arterioles were completely abolished by the PLC antagonist U-73122 (3 µM) at 5 mmHg (n = 3) and 80 mmHg (n = 3). (B) IP_{3}R blockade with xestospongin C (Xest C, 10 µM) attenuated spontaneous EC Ca^{2+} event frequency at both 5 mmHg (n = 3) and 80 mmHg (n = 4). (C) Block of PLA_{2}, a liberator of arachidonic acid, with AACOCF_{3} (3 µM) did not alter (ns, not significant) EC Ca^{2+} event frequency at 5 mmHg. (D) Block of VGCC with nifedipine (1 µM) had no effect on Ca^{2+} event frequency at either 5 or 80 mmHg (n = 4) but prevented the development of myogenic tone. (E) Removal of extracellular Ca^{2+} (Ca^{2+}-free) reduced EC activity at 5 mmHg but not at 80 mmHg (n = 3). (F) Ryanodine (10 µM) abolished asynchronous propagated SMC Ca^{2+} events (waves) at both 5 and 80 mmHg intraluminal pressure in cremaster arteries (n = 3) but did not affect EC spontaneous Ca^{2+} activity in the same arteries (n = 3). *P < 0.05, significantly different from control at each pressure.
arteries and suggest that it is not tone per se that turns off EC Ca\textsuperscript{2+} events. In each case, the ECs would experience different tension, but the compressive effect of 80-mmHg intraluminal pressure would be relatively constant. If the cell shape assumed when radial compression is low does activate TRPV4 channels, the subcellular mechanism remains to be defined.

A link between low intraluminal pressure, EC Ca\textsuperscript{2+} events, and myogenic tone involving TRPV4 channels suggests that Ca\textsuperscript{2+} sparklets are of fundamental importance for vascular function. These elementary events are revealed by block of IP\textsubscript{3}-mediated Ca\textsuperscript{2+}-release and appear to operate cooperatively within a four-channel metastructure (18). As few as three TRPV4 channels per cell have been suggested as sufficient to activate EC IC\textsubscript{Ca} channels, leading to hyperpolarization and vasodilation (7, 18). Our data now show that low pressure is sufficient to increase EC Ca\textsuperscript{2+} event frequency by twofold to threefold and drive significant vasodilation. This mechanism is intrinsic to the arteriole, operating independently of the surrounding tissue. What is not clear is how Ca\textsuperscript{2+} sparklets might enhance puff/pulsar frequency, but this may simply reflect the known ability of Ca\textsuperscript{2+} to stimulate IP\textsubscript{3}Rs (29). Consistent with this suggestion, IP\textsubscript{3}Rs are densely clustered within the ECs or SMCs, or EC and SMCs. To simultaneously image EC and SMC Ca\textsuperscript{2+} events, a multiphoton laser (790 nm) was used to image the arterial wall at the midplane. Visible (488 nm) and near-infrared (800 nm) channels were used to image either EC or SMC Ca\textsuperscript{2+} events at the bottom plane of the arteries. Results are presented as frequency (events cell \textsuperscript{−}min \textsuperscript{−}1) or F\textsubscript{IP3}, calculated by dividing the fluorescence (F) by an average baseline fluorescence (F0). See SI Materials and Methods.

**Materials and Methods**

**Arterial Preparation.** Rat cromaster arteries were isolated and cannulated onto glass pipettes. After warming to 34°C, intraluminal pressure was raised to 80 mmHg, and arteries developed myogenic tone and fully dilated to the EC-dependent dilator ACh. See SI Materials and Methods.

**Measurement of EC and/or SMC Ca\textsuperscript{2+}, Changes in Pressurized Arteries.** Oregon Green 488 BAPTA-1 AM was loaded into ECs, SMCs, or EC and SMCs. To image EC Ca\textsuperscript{2+} events, a multiphoton laser (790 nm) was used to image the arterial wall at the midplane. Visible (488 nm) channels were used to image either EC or SMC Ca\textsuperscript{2+} events at the bottom plane of the arteries. Results are presented as frequency (events cell \textsuperscript{−}min \textsuperscript{−}1) or F\textsubscript{IP3}, calculated by dividing the fluorescence (F) by an average baseline fluorescence (F0). See SI Materials and Methods.

**Experimental Protocols and Drugs.** See SI Materials and Methods.

**Data Analysis.** In all cases results are summarized as the mean ± SEM of n arterioles, one per animal.

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Supporting Information

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SI Materials and Methods

Animal Procedures and Isolated Arterial Preparation. Cremaster arterioles. Animal use was approved by the University of Oxford Local Ethical Review Committee and by the UK Home Office, and conformed to the Animals (Scientific Procedures) Act 1986. Male Wistar rats (Charles River; weight: 240–280 g) were anesthetized with urethane (2.8 g/kg i.p.), after which the cremaster muscles were exteriorized (1), excised from the rat, and placed in cool (4 °C) MOPS buffer containing (mmol/L): 145 NaCl, 4.7 KCl, 2.0 CaCl$_2$, 1.17 MgSO$_4$, 2.0 MOPS, 1.2 Na$_2$HPO$_4$, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.75 NaOH (pH 7.40 ± 0.02). Following tissue removal, rats were killed following Schedule 1 procedures of the UK Animals (Scientific Procedures) Act 1986. Segments of the main intramuscular artery were dissected from the muscle as described (2, 3). Artery segments were cannulated with glass micropipettes (external diameter ~95 μm) and positioned in a 1.5-mL temperature-regulated chamber (Warner Instruments) on the stage of an inverted microscope (IX70 or IX81; Olympus) and continually superfused (2 mL·min$^{-1}$) with MOPS buffer. To avoid luminal flow, equal pressure was maintained across the vessel throughout experiments. Artery segments were warmed to 34 °C, gradually pressurized to 80 mmHg, longitudinally stretched (4), and allowed to develop spontaneous tone over a 20-min equilibration period. Following development of ~50% contraction/tone, arterioles were tested for leaks followed by exposure to 100 and 300 nM ACh; only arterioles dilating >95% of the Ca$^{2+}$-free passive diameter (maximal dilation) were used for experiments. Inner diameter was measured by using a calibrated line tool in Andor iQ (Andor Technology). Percent dilation is of maximal dilation from resting myogenic tone.

Mesenteric arteries. Male Wistar rats (Charles River; weight: 225–250 g) were killed by a Schedule 1 procedure of the UK Animals (Scientific Procedures) Act 1986. The mesentery was excised from the rat and placed in cool MOPS as described above. Segments of the third-order mesenteric artery were dissected from the surrounding fat and connective tissue as described (5). Third-order branches of rat mesenteric artery do not normally develop myogenic tone (5). Experiments were performed at 36–37 °C.

Measurement of EC and/or SMC [Ca$^{2+}$]. Changes in Pressurized Arterioles. Cell loading. For selective loading of the endothelium, pressurized vessels were perfused intraluminally (30 min) or bathed (120 min) at 34 °C to load the SMCs, in both cases with filtered (0.2 μm pore) MOPS-buffered solution containing Oregon Green 488 BAPTA-1 AM (OGB-1, 10 or 20 μM, respectively) and 0.0025% pluronic F-127. For simultaneous imaging of both cell types by multiphoton microscopy, ECs were loaded in lumen and bath) and HC 067047 (10 μM) or the selective inhibitor of IP$_3$ receptor (IP$_3$Rs), xestospongin C (10 μM). The contribution of voltage-gated Ca$^{2+}$ channels (VGCC) to EC Ca$^{2+}$ events was determined by using nifedipine (1 μM). Arterioles were incubated in the presence of ryanodine (10 μM) to assess effects of ryanodine receptor block on either EC or SMC Ca$^{2+}$ events. The contribution of arachidonic acid was examined by blocking phospholipase A$_2$ (PLA$_2$) with AACC003 (3 μM).

Immunohistochemistry. Immunohistochemistry was performed in pressurized arterioles as described (5). In brief, arterioles were fixed in 2% (wt/vol) paraformaldehyde for 10 min at 37 °C, washed with PBS, and incubated in blocking buffer (luminal and abluminal, 1% BSA and 0.1% Tween 20, pH 7.1) for 60 min at 37 °C, then overnight with primary antibody at 4 °C. Primary antibodies were as follows: 1:100 rabbit polyclonal anti-rat TRPV4 nm visible laser, the slightly higher laser intensity required caused irreversible cell damage, evidenced by focal contraction at the point of laser stimulation. To this end, all midplane experiments were performed by using a multiphoton laser, and all live cell experiments were performed with the minimal laser intensity possible to ensure contraction was not evoked.

Imaging EC or SMC Ca$^{2+}$ events. Fluorescence intensity from selectively loaded ECs or SMCs was visualized by lowering the focal plane to the EC (Fig. 2A) or SMC layers at the bottom of the pressurized artery. Images were obtained by using a 40x water immersion objective (40x0.9 NA objective; Olympus; excitation 488 nm, emission 515 nm) and captured with an iXon 887 EMCCD camera (Andor Technology) coupled to a Nipkow spinning disk confocal head (CSU22; Yokogawa), mounted on the trincocular head of the Olympus IX70 inverted microscope, and recorded using Andor iQ Software. The clip box was adjusted to allow acquisition at 8–10 Hz with at least 8–15 cells visible.

Ca$^{2+}$ image analysis. Data were analyzed by using Metamorph software (version 7.7.4.0; Molecular Devices). For midplane experiments, regions of interest were placed on three to five active SMCs and/or one or two ECs per run, and the frequency of Ca$^{2+}$ responses (events·cell$^{-1}$·min$^{-1}$) was recorded. For SMC imaging experiments, the frequency of Ca$^{2+}$ events for three to five active cells was averaged. Because of the variability of the SMC Ca$^{2+}$ events, intercellular variability was low, and thus a subset of cells was chosen for analysis. For EC imaging experiments, all of the cells in the field of view (8–15 cells) were analyzed for Ca$^{2+}$ event frequency. In all cases, only active cells were included in the average. Results are presented as frequency (events·cell$^{-1}$·min$^{-1}$) or F/F$_{0}$, calculated by dividing the fluorescence (F) by an average baseline fluorescence (F$_{0}$).

Experimental Protocols. Effect of intraluminal pressure on EC Ca$^{2+}$ events and diameter. After loading ECs with OGB-1, the vessel was equilibrated at 80 mmHg until myogenic tone developed. Intraluminal pressure was then stepwise increased between 5 and 80 mmHg. At each chosen pressure, the artery was allowed to reach steady-state diameter (~18 min) before determining the frequency of spontaneous Ca$^{2+}$ events in ECs of pressurized arterioles.

Effect of inhibitors and vasoactive agents on EC Ca$^{2+}$ events. The contribution of TRPV4 channels to EC Ca$^{2+}$ events was assessed by using the selective antagonists RN 1734 (30 μM, 45–60 min incubation in lumen and bath) and HC067047 (10 μM, ~70 min in bath) with the selective agonist GSK1016790A (GSK, 10–100 nM) used to demonstrate effectiveness of antagonist action. To determine the contribution of IP$_3$ to EC spontaneous Ca$^{2+}$ events, arterioles were luminaarily perfused with the PLC inhibitor U-73122 (3 μM) or the selective inhibitor of IP$_3$ receptors (IP$_3$Rs), xestospongin C (10 μM). The contribution of voltage-gated Ca$^{2+}$ channels (VGCC) to EC Ca$^{2+}$ events was determined by using nifedipine (1 μM). Arterioles were incubated in the presence of ryanodine (10 μM) to assess effects of ryanodine receptor block on either EC or SMC Ca$^{2+}$ events. The contribution of arachidonic acid was examined by blocking phospholipase A$_2$ (PLA$_2$) with AACC003 (3 μM).

Immunohistochemistry. Immunohistochemistry was performed in pressurized arterioles as described (5). In brief, arterioles were fixed in 2% (wt/vol) paraformaldehyde for 10 min at 37 °C, washed with PBS, and incubated in blocking buffer (luminal and abluminal, 1% BSA and 0.1% Tween 20, pH 7.1) for 60 min at 37 °C, then overnight with primary antibody at 4 °C. Primary antibodies were as follows: 1:100 rabbit polyclonal anti-rat TRPV4.
(aa 853–871; Sigma, T9075); 1:100 rabbit polyclonal anti-rat KeCa3.1 (aa 350–363; Alomone Laboratories, APC-064); 1:100 mouse monoclonal anti-human KeCa3.1 (third extracellular loop; Alomone Laboratories, ALM-051); and 1:100 rabbit polyclonal anti-human KeCa2.5 (aa 2–21, Alomone Laboratories, APC-025).

The following day, the bath solution was replaced with PBS, and the lumen was perfused with Alexa Fluor 488 secondary antibody (1:100 goat anti-rabbit IgG, Invitrogen, A-11008; or 1:100 chicken anti-mouse IgG, Invitrogen A-21200), and incubated for 2 h at room temperature. Nuclei and elastin (including the IEL) were stained with 15 μM propidium iodide and 200 nM Alexa Fluor 633 hydrazide (Molecular Probes, A-30634), respectively (5–7). Arterioles were excited at 488, 546, and 633 nm; the fluorescence emitted at 505–525, 560–620, and 655–755 nm was acquired through a water immersion objective (40x, NA 0.9, WD 0.15 mm; Olympus, 1,024 × 1,024 pixels) by using a laser scanning confocal microscope (FV1000; Olympus). z-stacks through the artery wall were obtained at 0.20-μm increments by using Fluoview Software (FV10-ASW 3.0; Olympus) and reconstructed in Imaris Software (Version 7.2.3; Bitplane).

**Correlation between spontaneous Ca\(^{2+}\) events, holes in the IEL, and TRPV4 expression.** In one set of experiments, arterioles were maintained at low pressure (5 mmHg), and spontaneous EC Ca\(^{2+}\) events and the IEL were imaged simultaneously at ∼3 Hz by using the 488- and 633-nm visible laser lines on an Olympus FV1000 inverted microscope. Arterioles were fixed at this low pressure, and immunohistochemistry for TRPV4 channels and imaging of the stained IEL were performed, maintaining the low pressure throughout. Landmarks in the stained IEL were used to match arteriolar cellular positions directly to compare Ca\(^{2+}\) event sites (ROIs) in live tissue and TRPV4 expression in fixed tissue. **Effect of inhibitors on myogenic tone development.** After initial equilibration at 80 mmHg, intraluminal pressure was dropped to 5 mmHg, then raised to 20, 40, 60, and 80 mmHg with an 18-min equilibration period between each pressure. These pressure response curves were repeated in the presence of L-NAME to block NO synthase, and then in the additional presence of RN 1734, TRAM-34, and/or apamin. RN 1734 (30 μM), TRAM-34 (1 μM), and apamin (100 nM) were used to determine the influence of TRPV4, IK\(_{\mathrm{Ca}}\), and SK\(_{\mathrm{Ca}}\), channels, respectively, on the development of myogenic tone.

**Drugs and Solutions.** Oregon Green 488 BAPTA-1 AM (O-6807) and pluronic F-127 (P300MP) were obtained from Molecular Probes. U-73122 (BML-ST391) and xestospongin C (BML-CA409) were obtained from Enzo Life Sciences. RN 1734 (3746) and HC 067047 (4100) were from Tocris Bioscience. Apamin (L8407) was from Latoxan. All of the other drugs were provided by Sigma-Aldrich. U-73122 was dissolved in chloroform, evaporated, and then dissolved in DMSO. Xestospongin C, TRAM-34, levomakalim, RN 1734, HC 067047, and GSK1016790A were dissolved in DMSO and then diluted in physiological buffer for experimentation (pH 7.4 at 37 °C), keeping the final DMSO concentration below 1:1,000 to avoid vehicle-associated artifacts. Glibenclamide was dissolved in ethanol. All other stock solutions were prepared in purified water. Inhibitors were added to the incubation solution, and arterioles were equilibrated for at least 15 min prior to obtaining responses if not mentioned specially.

**Data Analysis.** In all cases, results are summarized as the mean ± SEM of n arterioles, one per animal. Statistical comparisons were made using paired Student t test or one-way ANOVA with Bonferroni’s post test as appropriate, where P < 0.05 was considered significant.

Spontaneous subcellular EC Ca$^{2+}$ events are not reflected as global EC Ca$^{2+}$ changes in cremaster arterioles. (A) Ca$^{2+}$ changes were assessed in ECs in a pressurized (to 5 mmHg) cremaster arteriole. (Scale bar, 10 μm.) Regions of interest (ROIs) were drawn around three individual ECs (a–c; red, global Ca$^{2+}$) and three subcellular ROIs positioned over spontaneously active sites within the same ECs (blue, subcellular Ca$^{2+}$). (B) Time-series fluorescence plots clearly demonstrate that although whole-cell ROIs (red) can detect slight changes in fluorescence during local activity (black, up arrows), these small changes do not accurately or consistently reflect the localized subcellular events (red, down arrows). y- and x-axis labels apply to each time series fluorescence plot. See Movie S2 and superimposed IEL. Acquisition rate = ∼3Hz.
Fig. S2. Low intraluminal pressure increases EC Ca\textsuperscript{2+} event frequency and involves TRPV4 channels in rat mesenteric arteries that do not develop myogenic tone. (A) Confocal image of a pressurized mesenteric artery showing TRPV4 channel expression (yellow), elastin staining (including IEL, white), and nuclear staining (blue) (three 0.2-μm z-axis planes merged at the level of the IEL). Reconstructed z-stacks of the corresponding vertical plane (a) and horizontal plane (b) are indicated on the merged image in A. Note TRPV4 signal is primarily localized in holes through the IEL. B) The frequency of EC Ca\textsuperscript{2+} events at 5 mmHg was significantly greater than at 80 mmHg (n = 3). (C) GSK at a concentration of 30 nM stimulated a significant increase in EC Ca\textsuperscript{2+} event frequency (n = 5). (D) Summary data showing RN 1734 (30 μM) decreased the frequency of EC Ca\textsuperscript{2+} events at 5 mmHg (n = 3). *P < 0.05; significant difference from 5 mmHg (B) or control (C and D).

Fig. S3. The TRPV4 antagonist RN 1734 inhibits both vasodilation and EC Ca\textsuperscript{2+} event frequency in response to EC stimulation with ACh in cremaster arterioles. (A) Concentration-dependent vasodilation to ACh was significantly reduced by 30 μM RN 1734; n = 4. (B) Both spontaneous and 30 nM ACh-evoked increases in EC Ca\textsuperscript{2+} event frequency were reduced significantly by RN 1734 (30 μM; n = 3). *P < 0.05, significantly different from control (A) or L-NAME (B); §P < 0.05, significantly different from 30 nM ACh; ¶P < 0.05, significantly different from RN 1734.
Fig. S4. The TRPV4 channel antagonist HC 067047 inhibits spontaneous Ca\textsuperscript{2+} activity at 5 mmHg in rat cremaster arterioles. Summary data showing HC 067047 (10 μM) decreased the frequency of EC Ca\textsuperscript{2+} events at 5 mmHg and prevented responses to GSK (n = 3). *P < 0.05, significant difference from control, paired experiments.

Fig. S5. Effect of nitric oxide (NO) synthase inhibition and EC disruption on myogenic tone in cremaster arterioles. (A) Inhibition of NO synthase with L-NAME (100 μM) had no effect on the myogenic pressure–diameter curve (n = 6). (B) K\textsubscript{Ca} channels act through an endothelium-dependent mechanism to regulate development and maintenance of myogenic tone at low pressures. In EC denuded arterioles, TRAM-34 (TR; 1 μM, IK\textsubscript{Ca} channel blocker) and apamin (Ap; 100 nM, SK\textsubscript{Ca} channel blocker) did not increase myogenic tone at low pressures (5 and 20 mmHg) (n = 3). A slight but significant increase in myogenic tone was observed at 40 mmHg in the presence of TRAM-34 and apamin, but the decrease in diameter was markedly less than the same maneuver in endothelium-intact arterioles (Fig. 6). *P < 0.05, significantly different from control. ns, not significant.
Table S1. Actions of RN 1734 at K<sub>ATP</sub> channels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RN 1734</th>
<th>Glib</th>
<th>GSK</th>
<th>Glib + GSK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilation to LVK, % max</td>
<td>97.4 ± 1.1 (n = 4)</td>
<td>2.2 ± 2.2 (n = 3)</td>
<td>1.2 ± 1.2 (n = 4)</td>
<td></td>
<td></td>
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<tr>
<td>EC Ca&lt;sup&gt;2+&lt;/sup&gt; events, min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>4.6 ± 0.4 (n = 3)</td>
<td>2.2 ± 0.1 (n = 4)</td>
<td>4.0 ± 0.2 (n = 3)</td>
<td>8.2 ± 0.7 (n = 3)</td>
<td>8.2 ± 0.7 (n = 3)</td>
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Experiments showed RN 1734 (30 μM) blocked dilation to the K<sub>ATP</sub> channel opener levomakalim (LVK; 3 μM) in a manner similar to glibenclamide (Glib; 5 μM). Arterioles were pressurized to 80 mmHg, and dilation was assessed from the resting tone. This appeared to be a nonselective effect because glibenclamide did not affect the spontaneous or GSK1016790A (GSK; 30 nM)-evoked EC Ca<sup>2+</sup> events at low pressure (5 mmHg) or the myogenic tone curve (5 mmHg: control 61.3 ± 5.1, Glib 58.2 ± 4.5 μm; 80 mmHg: control 70.4 ± 9.6, Glib 63.5 ± 9.9 μm; n = 3). Thus, low pressure per se did not activate K<sub>ATP</sub> channels.

Fig. S6. Mechanisms linking low pressure, EC TRPV4 channels, and vasodilation. ECs and SMcs are separated by an IEL (see Inset for orientation). ECs send membrane projections through holes in the IEL where they may form heterocellular, MEJs that contribute to intercellular communication. IP<sub>3</sub>Rs, TRPV4, IK<sub>Ca</sub> and SK<sub>Ca</sub> channels are highly focused in this EC signaling “projection” microdomain. Low intraluminal pressure (1) activates Ca<sup>2+</sup> influx through TRPV4 channels (2, pink). This activates EC K<sub>Ca</sub> channels (3, black), hyperpolarizing the ECs, then via MEJs (4) and diffusible endothelium-derived hyperpolarizing factors (5) the surrounding SMcs to cause vasodilation (6). In addition, PLC activity (7) liberates IP<sub>3</sub> and DAG. IP<sub>3</sub> activates IP<sub>3</sub>Rs (8), releasing Ca<sup>2+</sup> from the endoplasmic reticulum (ER) and both IP<sub>3</sub> and DAG activate downstream targets (9) that include TRPV4 channel complexes. Increases in Ca<sup>2+</sup> can activate adjacent [Ca<sup>2+</sup>]<sup>-sensitive proteins (*) amplifying Ca<sup>2+</sup> events and thus vasodilation. IP<sub>3</sub>- and TRPV4-mediated K<sub>Ca</sub> activation (IK<sub>Ca</sub> being most functionally relevant) occurs at low pressures (<50 mmHg), whereas IP<sub>3</sub>-mediated events predominate at higher pressures (to 80 mmHg).

Movie S1. Simultaneous imaging of arteriolar ECs and SMCs using multiphoton microscopy. A movie of Oregon-Green BAPTA-1 loaded ECs (oriented from left to right) and SMCs (oriented perpendicular to the ECs) imaged at the midplane of an intact cremaster arteriole pressurized to 5 mmHg is shown. The first frame of the movie is a representative averaged image of the ECs and SMCs, with ROIs as indicated. The movie loops twice, with the ROIs appearing at the beginning of the second loop. Time-series plots of the calcium events in those regions of interest appear above (ECs) and below (SMCs) the movie. The movie speed is 12× real-time. (Scale bar, 10 μm.) y- and x-axis labels apply to both time-series fluorescence plots. Images were originally acquired at 0.327 s per frame.

Movie S2. EC Ca^{2+} events in cremaster arterioles at 5 mmHg. Imaging of Oregon Green BAPTA-1 loaded ECs at the bottom plane of the arteriolar wall at 5 mmHg reveals local and propagating Ca^{2+} events. The movie loops twice and is played back at 12× real-time. The IEL (gold) is superimposed on the ECs, allowing for visual alignment of EC Ca^{2+} events with holes in the IEL. (Scale bar, 10 μm.) Images were originally acquired at 0.35 s per frame.

Movie S2
Movie S3. TRPV4, IK\textsubscript{Ca}, and SK\textsubscript{Ca} channels are expressed within holes in the IEL. Pressurized cannulated arterioles were fixed and stained for TRPV4 (Left), IK\textsubscript{Ca} (Center), or SK\textsubscript{Ca} (Right) channel expression. Expression of each channel is indicated in yellow (corresponding labels above each arteriole), with elastin in white and nuclear staining in blue. Note the localization of TRPV4, IK\textsubscript{Ca}, and SK\textsubscript{Ca} channels primarily within holes through the IEL. The x-y plane shows ECs orientated left to right; the vessel is rotated through 45° to show the y-z plane and individual X-stack images to visualize the alignment of channel expression with holes through the IEL. The arterioles are rotated back to the x-y plane, and individual Z-stack images shown through the vessel wall from outside to inside the lumen. (Scale bar, 15 \mu m, corresponding to all images.) Data are representative of results observed in three arterioles.

Movie S3