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Smooth Muscle Ca^{2+}-Activated and Voltage-Gated K^+ Channels Modulate Conducted Dilation in Rat Isolated Small Mesenteric Arteries

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Abstract

Objective—To assess the influence of blocking smooth muscle large conductance Ca^{2+}-activated K^+ channels and voltage-gated K^+ channels on the conducted dilation to ACh and isoproterenol.

Materials and Methods—Rat mesenteric arteries were isolated with a bifurcation, triple-cannulated, pressurized and imaged using confocal microscopy. Phenylephrine was added to the superfusate to generate tone, and agonists perfused into a sidebranch to evoke local dilation and subsequent conducted dilation into the feed artery.

Results—Both ACh– and isoproterenol-stimulated local and conducted dilation with similar magnitudes of decay with distance along the feed artery (2000 μm: ~15% maximum dilation). The gap junction uncoupler carbenoxolone prevented both conducted dilation and intercellular spread of dye through gap junctions. IbTx, TEA or 4-AP, blockers of large conductance Ca^{2+}-activated K^+ channels and voltage-gated K^+ channels, did not affect conducted dilation to either agonist. A combination of either IbTx or TEA with 4-AP markedly improved the extent of conducted dilation to both agonists (2000 μm: >50% maximum dilation). The enhanced conducted dilation was reflected in the hyperpolarization to ACh (2000 μm: Control, 4 ± 1 mV, $n = 3$; TEA with 4-AP, 14 ± 3mV, $n = 4$), and was dependent on the endothelium.

Conclusions—These data show that activated BK_{Ca} and K_V-channels serve to reduce the effectiveness of conducted dilation.

Keywords

conducted response; K^+ channels; hyperpolarization

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INTRODUCTION

Vasomotor responses within arteries and arterioles are coordinated by rapid integration of membrane potential changes between vascular cells. In small resistance arteries and arterioles, agents acting to hyperpolarize smooth muscle cells either directly or secondarily to stimulation of endothelial cells, evoke a local dilation that can spread upstream to evoke remote dilation. These distant responses are termed “conducted” or “spreading” dilation. In rat mesenteric arteries, local and conducted hyperpolarization has been observed to both ACh and levcromakalim, agonists shown to act specifically at either endothelial or smooth muscle cells, respectively [41]. In this artery, ACh acts by increasing endothelial cell Ca^{2+} to stimulate Ca^{2+}-activated K^+ (K_{Ca}) channels, specifically small (SK_{Ca}, $K_{Ca}2.3$) and intermediate conductance (IK_{Ca}, $K_{Ca}3.1$) channels that are confined to the endothelium [11,14,16,17,40,44]. In contrast, levcromakalim directly activates ATP-sensitive K^+ (K_{ATP}) channels that are restricted to the smooth muscle cells in this artery [41,47], causing endothelium-independent hyperpolarization and relaxation [7,36,41,47]. Stimulation of β-adrenoceptors with isoproterenol has also been shown to activate K_{ATP} channels in rat mesenteric arteries [21,23], and as expected, dilation can occur via K_{ATP} channel-independent pathways [48]. Nevertheless, isoproterenol stimulates conducted dilation which is dependent on K_{ATP} channel activation [23].

Measurements of smooth muscle membrane potential at various distances upstream from the site of agonist or current delivery are rare [1,6,8,20,50,51], but have been used to estimate the cable length constant (λ) for a particular vessel or tissue, helping to quantify the decay of hyperpolarizing current with distance. The length constant can be affected by resistances of both the intercellular gap junctions and the membrane itself [4,25,28]. In general, it is accepted that the resistance provided by MEGJs is higher than in those connecting endothelial cells, and that the resistance between smooth muscle cells is also relatively high [4,10,28,45]. In rat mesenteric arteries, the resistance of MEGJs has been estimated to be around 70 MΩ [39] and the measured input resistance of endothelial cells in this artery is approximately a quarter of this value [47]. In rat mesenteric arteries, this low resistance endothelial cell pathway has been shown to be, and modeled as, a conduit for the longitudinal spread of hyperpolarizing signal [28,41,49] as it is in the microcirculation [19,24].

In rat mesenteric arteries, application of α_1-adrenoceptor agonists such as phenylephrine stimulate depolarization and an increase in smooth muscle cell Ca^{2+} [7,14,22,26,33,46] thereby activating large conductance (BK_{Ca}) channels [5,11,12,46] and voltage-gated K^+ channels (K_V channels) that are extensively expressed in these cells [31,35,52], but not endothelial cells [44]. Overall, phenylephrine has been shown to reduce smooth muscle cell input resistance, which in an endothelium-denuded preparation, changes from around 23–15 MΩ [36]. In parallel, phenylephrine may also modulate gap junction permeability by changes in voltage and Ca^{2+} [34,38], and via signaling intermediates such as PKC [18,38]. Thus, inhibition of one or more of these processes may potentially modulate the extent to which hyperpolarization can evoke local and conducted dilation responses. Evidence for the effect of K^+ channel blockers on rat mesenteric artery input resistance is scarce, although in unstimulated endothelium-denuded arteries, charybdotoxin (inhibits BK_{Ca}, and some K_V

channels) and glibenclamide (inhibits K_{ATP} channels) together increased smooth muscle input resistance by almost fourfold [45].

Since evoked conducted dilation relates to both the extent of patent gap junctions and ion channels, we sought to investigate whether we could enhance the magnitude and distance of conducted dilation in rat mesenteric arteries by blocking the major smooth muscle cell K^+ channels activated during contraction in this artery, *viz.* K_{Ca} and K_V channels. We used triple-cannulated rat mesenteric arteries and a variety of agonists known to act at K^+ channels expressed either on the endothelial or smooth muscle cells. Our data show for the first time that opening muscle K^+ channels in a resistance artery with 3–4 layers of smooth muscle can markedly limit the extent of conducted vasodilation.

MATERIALS AND METHODS

Rat Mesenteric Artery Isolation and Cannulation

Male Wistar rats (200–250 g, Charles River, UK) were killed by cervical dislocation and exsanguination according to requirements detailed under Schedule 1 of the Animals (Scientific Procedures) Act 1986 and monitored by the Home Office (UK) and local ethical committee. The mesentery was removed and placed in cold MOPS buffer containing (mM): NaCl 145.0, KCl 4.7, $CaCl_2{\cdot}2H_2O$ 2.0, $MgSO_4{\cdot}7H_2O$ 1.17, MOPS 2.0, $NaH_2PO_4{\cdot}H_2O$ 1.20, glucose 5.0, pyruvate 2.0, EDTA 0.02, NaOH 2.75, adjusted to pH 7.40 ± 0.02. Third-order branches (± fourth-order bifurcation) of the superior mesenteric artery were dissected, isolated, and cannulated at each end, as previously described [32,41,49]. Once pressurized, the presence of small side-branches was ascertained by a lack of diameter change following occlusion of all perfusion inflows. The MOPS-buffered solution was heated to 36.5 ± 0.2°C in all experiments, and arteries were all pressurized by a gravity-fed inflow and outflow system. Two open 5 mL syringes connected to the perfusion pipettes were attached to either side of a vertical belt. This assembly was secured to a block that was moved along a vertical track and held in position at the required height above the artery. All arteries were pressurized to 70 mmHg, and were submaximally contracted with phenylephrine (0.5–2 μM) to generate a consistent level of tone between treatments and preparations. Endothelial cell viability was assessed as >95% dilation to 1 μM ACh. All experiments were performed in the presence of the selective inhibitor of NO synthase, L-NAME (100 μM, Control).

Conducted Dilation to Luminally Perfused Agonists

In this series of experiments, three cannulation pipettes were used as previously described [49]. The three ends of an isolated artery were cannulated and mounted in a heated, 2 mL chamber (RC-27 chamber, PH-6 platform; Warner Instruments, Hamden, CT, USA) and continuously superfused at 2 mL/min with heated MOPS solution. The upstream end of the artery (feed artery) and one side of the bifurcation (Branch 2) were attached to the gravity-fed pressurizing syringe reservoirs. To avoid upstream flow of infused agonists, the upstream and downstream perfusion pressures through the feed artery were adjusted by rotating the belt, to generate luminal flow (7–9 cmH_2O gradient, 10–30 μL/min) while maintaining a constant average transmural pressure. In some experiments, the endothelium in the feed artery was damaged with an air bubble while maintaining endothelial cell viability in Branch

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1, as described previously [49]. In these arteries, the dilation to 1 μM ACh in Branch 1 remained unaffected, but was less than 10% of maximum in the feed artery, whilst retaining full dilation to 1 μM isoproterenol at all sites. Phenylephrine was added to the superfusion solution, and each agonist used to study conducted dilation into the feed artery was infused into one of the sidebranches (Branch 1) for at least two minutes at 20 μL/min using a BeeHive® syringe pump system (Bio-analytical Systems, Kenilworth, UK). In all experiments, the movement of perfusion solution was monitored by including 0.1 μM carboxyfluorescein in the agonist solution. Arteries were visualized using a laser scanning confocal microscope (excitation 488 nm, emission 505 nm, FV500-SU; Olympus, Japan) to enable simultaneous fluorescence and brightfield imaging, with a 4×/0.13 NA objective (UplanFl; Olympus, Japan), and images were recorded with Fluoview software (Fluoview, w/TIEMPO, version 5.0; Olympus Corporation, USA) at 1 Hz. There was a short delay in observing responses to luminal perfusion of agonists due to voiding the tubing and pipette dead-space volume.

For all measurements of conducted dilation, artery outer diameter was measured offline using motion analysis software (MetaMorph version 6.1; Universal Imaging Corporation, West Chester, PA, USA). This enabled simultaneous analysis of multiple, calibrated distances along the artery wall, and direct comparisons of local dilation to conducted dilation for a single application of agonist. The resolution of the system was 5 μm, equivalent to 1 pixel, ~1.5% of the maximum diameter of arteries. Fluorescence intensity was also measured offline simultaneously at multiple positions in the lumen of arteries, which was temporally matched to diameter measurements. Feed artery diameter was only measured if no upstream flow of fluorescent indicator was detected, as previously shown [49]. Values for diameter were measured from 0 to 2000 μm along the feed artery at 500 μm intervals. The 0-μm point was chosen to be at least 100 μm above the point were carboxyfluorescein fluorescence was observed as it passed around the bifurcation. It was considered that this point was not directly stimulated by agonists, so all measured diameter values along the feed artery are termed conducted dilation, whereas those within the branches were local dilation responses as they were directly stimulated by the agonists.

Sharp Microelectrode Electrophysiology

Hyperpolarization in pressurized arteries—Intracellular recordings of E_m were made using a custom-built cannulation setup orientated at ~45° to allow the positioning of the headstage perpendicular to the vessels. A Perspex plate was attached to the movable stage of an Olympus IX71 microscope, and to this two micromanipulators (NMN-25; Narishige, Tokyo, Japan) were fixed and held the cannulation pipettes. A third micromanipulator was added for experiments with triple-cannulated arteries. Arteries were continually superfused (4 mL/min) with heated solution (36.5 ± 0.5°C, SH-27B Inline Heater; Warner Instruments, Hamden, CT, USA) via a multi-barrel gravity-fed perfusion system. Intracellular recordings of E_m, were made using glass microelectrodes filled with 2 M KCl (giving tip resistances of 90–150 MΩ), and a Neurolog amplifier system (Digitimer Ltd, Welwyn Garden City, UK). Data were acquired using either Clampex version 8.0.2 (Axon, MDS Analytical Technologies, Sunnyvale, CA, USA) on a PC, or for triple-cannulated arteries, using a PowerLab 2/20 connected to an Apple Mac Mini running Chart version 5.5.6 (AD

Instruments Pty Ltd, Sydney, Australia). Impalements were performed manually using a third piezoelectric (PCS-5000 series) precision micromanipulator (Burleigh Instruments, New York, USA) placed on a stand to hold the headstage and microelectrodes at a 45° angle to impale smooth muscle cells above the midplane of the vessels in a region demonstrating typical vasoreactivity. Successful recordings were characterized by an abrupt deflection of signal on impalement of cells and an approximate return to pre-impalement values on removal of the microelectrode. Hyperpolarization of the resting membrane potential was elicited by the addition of isoproterenol (1 μM) or ACh (1 μM) to the superfusate. In experiments with the K_{ATP}-channel blocker, glibenclamide (10 μM), arteries were superfused for at least 15 minutes with the blocker alone, and the solution changed to one also containing the agonist. In triple-cannulated arteries, hyperpolarization to luminal perfusion of 1 μM ACh was recorded near the site 2000 μm upstream from the bifurcation. In these experiments nifedipine (1 μM) was added to the superfusion solution to reduce artery movement. Values are expressed as the mean ± standard error of the mean (SEM) of responses from the number of animals (*n*), where multiple responses per animal were averaged to give a single value.

Luminal Perfusion of Calcein AM to Assess Dye Coupling into the Feed Artery

The fluorescent indicator calcein AM (12.5 μM and 0.02% Pluronic F-127) was luminally perfused into Branch 1 of triple-cannulated arteries for 120 minutes in the continual presence of flow into the feed artery. By carefully maintaining sufficient flow into the feed artery, calcein AM did not pass into the feed artery during the entire loading protocol. In separate experiments, the gap junction uncoupler carbenoxolone (100 μM) was added to the bath for 45 minutes before commencing the calcein dye load. The passage of dye into the feed artery was compared in the two conditions.

Endothelial Cell Ca^{2+} Changes to Luminally Perfused Agonists

Endothelial cells of the feed and two branches of triple-cannulated arteries were selectively loaded with the calcium indicator Oregon Green® 488 BAPTA-1. Following equilibration of pressurized arteries, filtered (0.22 μm pore) MOPS buffer containing cell-permeant Oregon Green® 488 BAPTA-1 AM (10 μM) and 0.02% Pluronic F-127 was perfused through the artery lumen for five minutes, washed out, and allowed to de-esterify for at least 30 minutes. After excitation at 488 nm, the fluorescence emission intensity at 505–525 nm from endothelial cells was acquired at 1 Hz. In these experiments, ACh (3 μM) in MOPS buffer containing tetramethylrhodamine-labeled dextran (0.5 μM) was perfused into Branch 1 at 20 μL/min, and fluorescence intensity at 610 nm measured following excitation at 543 nm. This enabled simultaneous endothelial cell Ca^{2+} imaging and assessment of agonist flow to accurately measure distance from cells directly in contact with the agonist to within one cell. There was no crossover of fluorescence emission between channels. In experiments without phenylephrine tone (basal), cells were imaged toward the bottom of the artery using a 20×/ water immersion/0.5 NA objective (UMPlanFl; Olympus, Japan). In experiments where phenylephrine was added to generate tone it was not possible to accurately measure changes in endothelial cell Ca^{2+} during responses to ACh as the diameter and hence focal plane changed dramatically. To over-come this problem a 10×/0.3 NA objective (UPlanFl; Olympus, Japan) was used and the focal plane adjusted toward the midplane. In most Ca^{2+}

imaging experiments the artery moved during luminal perfusion into Branch 1, so fluorescence intensity within regions of interest were determined offline using Image SXM [2] using custom-written macros that tracked the chosen cells (Dr. Keith Brain, Oxford, UK). In all cases, only cells that did not move out of the focal plane, and that responded to bath addition of ACh (1 μM) were used.

Drugs

All chemicals were purchased from Sigma (Poole, UK) with the exception of IbTx (Latoxan, Valence, France), levcromakalim (GlaxoSmithKline, Harlow, UK), Calcein AM (C-3100), Oregon Green® 488 BAPTA-1 AM (O6807), Pluronic F-127 (P3000MP) and tetramethylrhodamine dextran (D3307, 3000 MW) were obtained from Molecular Probes (Invitrogen, Paisley, UK). All stock solutions were prepared in distilled water with the exception of levcromakalim, which was dissolved in ethanol; and IbTx, which was dissolved in MOPS-solution (and aliquots stored at −20°C). The pH of both the stock (20 mM) and working (150 μM) solutions of 4-AP were adjusted to 7.40 ± 0.02. Prior to use in experiments, all drugs were diluted in physiological buffer, and kept chilled (~4°C). All inhibitors were added to the superfusion solution and the artery exposed for a minimum of 10 minutes prior to obtaining responses.

Data Analysis

Results are summarized as the mean ± SEM of n arteries, one per animal. Statistical comparisons were made using the Mann–Whitney U test, where $p < 0.05$ was considered statistically significant. The dilation evoked by each agonist was calculated as the percentage of the maximum dilation from phenylephrine-contracted arteries (% dilation), 100% being the maximum diameter seen for each experiment. KCl (45 mM) and phenylephrine (10 μM) was added at the end of each experiment to obtain the maximum contraction (% contraction), 100% being the minimum diameter. During vasomotion, the diameter over at least 10 seconds was averaged. Fluorescence intensity (F) relative to the maximum fluorescence obtained during perfusion periods (F_{max}) were expressed as relative changes (F/F_{max}) for each luminal region of interest. Conducted dilation curves and bar graphs were prepared using Prism v5.0 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Conducted Dilation to Luminally Perfused Agonists

The maximum outer diameter of triple-cannulated arteries at 70 mmHg ranged from ~300 to 400 μm for the feed artery, and ~250–350 μm for Branch 1 (Table 1). All experiments were performed in the presence of NO synthase inhibitor L-NAME (100 μM). In general, the concentration of phenylephrine was adjusted such that contraction was ~70% of maximum contraction (Table 1), reaching an average outer diameter of ~220 μm along the feed artery. Previously it was shown that luminal perfusion with vehicle alone had no effect on arterial diameter, in spite of large increases in shear stress [49]. In this study, dilation was never observed during the mandatory period of up to a minute of luminal flow before the agonist reached the vessel ($n = 38$). As each agonist reached Branch 1, it evoked a local increase in diameter (ACh: 116 ± 6 μm, $n = 23$; isoproterenol: 126 ± 19 μm, $n = 9$; levcromakalim: 120

± 7 μm, $n = 6$). This dilation spread into the feed artery, to regions not directly stimulated by the agonists (0 μm position increase in diameter: ACh: 125 ± 6 μm, $n = 23$; isoproterenol: 127 ± 13 μm, $n = 9$; levcromakalim: 118 ± 8 μm, $n = 6$) and decayed with distance (500, 1000, 1500, 2000 μm position increases in diameter: ACh: 81 ± 4, 46 ± 4, 25 ± 4, 14 ± 3 μm, $n = 23$; isoproterenol: 71 ± 8, 47 ± 9, 40 ± 9, 35 ± 10 μm, $n = 9$; levcromakalim: 67 ± 5, 32 ± 3, 20 ± 2, 13 ± 3 μm, $n = 6$) (Figures 1 and 2A). The conducted dilation was synchronized in all regions of the artery with each agonist, and was not significantly different between agonists (Figure 2A). The level of phenylephrine tone (35–100% maximum contraction) did not affect the conducted dilation responses to ACh, isoproterenol or levcromakalim (Figure S1).

In rat mesenteric arteries, the dilation to isoproterenol and levcromakalim have been shown not to be endothelium-dependent, and both agonists have been shown to hyperpolarize rat mesenteric artery smooth muscle cells via activation of K_{ATP} channels [21,23,41]. Therefore, we assessed the effect of the K_{ATP} channel blocker glibenclamide against conducted dilation. Glibenclamide (10 μM) had no effect against either the local or conducted dilation responses to ACh (Figure 2B). In contrast, although glibenclamide had no effect against the local dilation to isoproterenol, it markedly reduced conducted dilation. Both the local and conducted dilation to levcromakalim were almost abolished by glibenclamide (Figure 2B). For the remainder of the experiments, isoproterenol was used in preference to levcromakalim because the latter was relatively slow to evoke dilation and to recover tone following washout.

Hyperpolarization to Agonists

The resting membrane potential of smooth muscle cells in pressurized arteries was −52.8 ± 0.6 mV ($n = 12$ from 44 cells). In some experiments, nifedipine (1 μM) was added to prevent tone and hence movement during dilation to agonists. This did not modify resting membrane potential (−53.6 ± 1.3 mV, $n = 11$ from 38 cells). Both ACh (1 μM) and isoproterenol (1 μM) stimulated hyperpolarization of ~10 mV (ACh: −16.1 ± 2.1 mV, $n = 6$, 13 cells; isoproterenol: −12.8 ± 1.8 mV, $n = 5$, 6 cells), but only the hyperpolarization to isoproterenol was sensitive to glibenclamide (10 μM, Figure 2C).

Effect of Carbenoxolone on Spread of Dye and Dilation

Dye coupling—The regions of direct dye contact in Branches 1 and 2 (Figure 3A) were strongly stained by calcein, which was apparent in smooth muscle, endothelial cells, as well as other cell-types, including nerves. Under control conditions, dye spread into the feed artery across at least five cell lengths ($n = 3$ arteries), which was fully blocked in the presence of carbenoxolone (Figure 3B).

Dilation—By adding carbenoxolone to the feed artery luminal perfusion solution, Branch 1 was not exposed the gap junction uncoupler, and the local dilation to ACh was not reduced. However, carbenoxolone very rapidly (observed within 10 minutes) prevented conducted dilation into the feed artery (Figure 3C). Conducted dilation to isoproterenol was not as readily reduced by luminal perfusion of carbenoxolone (even following 90 minutes exposure), but was markedly reduced following the addition of carbenoxolone to the

superfusion solution for 60–90 minutes (Figure 3C). Addition of 1 μM isoproterenol to the bath at the end of each carbenoxolone experiment fully dilated every position along the feed artery.

Effect of K^+ Channel Blockers on Conducted Dilation to Agonists

The present experiments were designed to test the role of BK_{Ca} and K_V channels in conducted dilation responses. The level of phenylephrine tone was matched as close as possible to control conditions and each K^+ channel blocker had varying effects on vasomotion (Table 2). When vasomotion was observed, changes in diameter were always simultaneous along the feed artery and two branches. Neither IbTx (100 nM) nor 4-AP (150 μM) had an effect on the conducted dilation response to ACh or isoproterenol when assessed against 80% dilation at 0 μm. TEA (1 mM) significantly increased the dilation at the upstream sites, and tended to augment the conducted dilation to isoproterenol (Figure 4). In the combined presence of IbTx together with 4-AP, the magnitude of conducted dilation to both ACh and isoproterenol was markedly and significantly increased, such that the entire artery was nearly fully dilated in every experiment (0, 500, 1000, 1500, 2000 μm position increases in diameter: ACh: 123 ± 25, 120 ± 23, 103 ± 21, 92 ± 20, 85 ± 22 μm, $n = 5$; isoproterenol: 121 ± 6, 105 ± 23, 104 ± 25, 92 ± 20, 93 ± 23 μm, $n = 3$) (Figure 5). The combination of TEA and 4-AP gave similar results (Figure 5B). In the presence of combined blockers, the agonists often overcame the vasomotion (Figure 5A), but when oscillations in diameter occurred during the conducted dilation response, they were always synchronized within the three branches.

Importance of the Endothelium for Conducted Dilation to Agonists

Following luminal perfusion of an air bubble into the feed artery, the response to bath application of 1 μM ACh in the feed artery was markedly reduced (1000 μm site, 12 ± 6% maximum dilation) whereas the dilation in Branch 1 was near maximal (92 ± 7% maximum diameter, $n = 3$). Responses to 1 μM isoproterenol remained unaffected at both sites (1000 μm site 98 ± 1%; Branch 1, 99 ± 1% maximum diameter, $n = 3$). Therefore, we were able to stimulate local dilation in Branch 1 to both agonists, and found that the dilation did not spread beyond 500 μm upstream into the feed artery either under control conditions, or in the presence of TEA and 4-AP (Figure 5B).

Conducted Hyperpolarization to ACh

In the presence of 100 μM L-NAME, 1 μM nifedipine and 2 μM phenylephrine, the membrane potential of triple cannulated arteries was −38.3 ± 6.2 mV ($n = 6$). Luminal perfusion of ACh evoked 3.5 ± 0.6 mV hyperpolarization at ~2000 μm upstream in the feed artery ($n = 3$). We were unable to record the hyperpolarization to ACh in either Branch 1 or at the 0 μm site due to movement of the artery upon turning on the agonist delivery syringe pump. However, we were able to monitor the appearance of carboxyfluorescein into Branch 1, which did not pass into the feed artery (Figure 6). In the additional presence of TEA (1 mM) and 4-AP (150 μM) the membrane potential was −41.7 ± 6.0 mV ($n = 6$). The presence of nifedipine prevented oscillations in diameter to phenylephrine, and the membrane potential also did not oscillate ($n = 6$). In the presence of the K^+ channel blockers, luminal

perfusion of ACh evoked a significantly greater increase in membrane potential ~2000 μm upstream from the bifurcation (13.8 ± 3.4 mV, $n = 4$) (Figure 6).

Effect of ACh on Endothelial Cell $[Ca^{2+}]_i$ in Pressurized Triple Cannulated Arteries

The possibility that Ca^{2+} waves passed between endothelial cells was investigated in the triple-cannulated arteries. Following luminal perfusion with Oregon Green® BAPTA-1, endothelial cells were selectively loaded in all three branches. The focal plane and objective magnification were optimized for each type of experiment, and it was found that only in the absence of tone could a 20× objective be used to assess individual endothelial cells very clearly (Figure 7A). Instead a 10× objective was used to monitor changes in endothelial cell Ca^{2+} during changes in diameter (Figure 7B). By simultaneously imaging the Ca^{2+} and agonist-delivery fluorescence indicators, it was possible to see within one cell (100 μm) which cells were directly stimulated by luminally perfused ACh (3 μM). This level of control enabled for the first time in cannulated arteries a clear assessment of intercellular Ca^{2+} waves from a stimulated to unstimulated cell.

In the absence of tone, endothelial cells in direct contact with ACh increased fluorescence intensity (R1, Figure 7A). The adjacent upstream endothelial cell also responded, albeit to a less extent (R2, Figure 7A). However, in no cases were increases in fluorescence observed beyond this single cell upstream (Figure 7A,C). In the presence of phenylephrine the same pattern was observed (Figure 7B,C). In either the absence or presence of tone, incubation with TEA (1 mM) in combination with 4-AP (150 μM) did not unmask an inter-endothelial cell Ca^{2+} wave ($n = 3$, not shown).

DISCUSSION

The aim of the present study was to investigate the influence of gap junctions and K^+ channels on conducted dilation in phenylephrine pre-contracted isolated rat mesenteric arteries. Agonists acting via different pathways and cell types each evoked local and conducted dilation when infused into a sidebranch of triple-cannulated arteries, and when responses were normalized, the decay over distance was equal with each agonist, whatever cell type they stimulated. The decay was markedly enhanced by a gap junction uncoupler and reduced following blockade of smooth muscle K^+ channels. In all cases, the endothelium appeared to be the conduit for longitudinal conduction. These data show that BK_{Ca} and K_V channels can modulate the capacity for conducted dilation.

It is well established that endothelium-dependent hyperpolarization of smooth muscle cells evokes conducted dilation in both vessels of the microcirculation and larger resistance arteries. However, it is less clear whether direct hyperpolarization of the vascular smooth muscle can evoke comparable responses. Herein in arteries pre-contracted with phenylephrine, we show that levcromakalim, acting specifically to activate smooth muscle cell K_{ATP} channels [41,47], stimulates conducted dilation that when normalized to 80% maximum dilation at the 0 μm site, decays over distance upstream to the same extent as similarly expressed responses to the endothelium-dependent agonist ACh. In addition, isoproterenol acting at β-adrenoceptors mimics the responses to both dilator agonists, as do our previous data demonstrating conducted dilation to purines acting at endothelial receptors

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[49]. Together these data suggest that there is no polarity with regard to the cell-type activated to initiate conducted dilation, an experimental finding that is entirely consistent with modeling in this artery [28]. Hyperpolarization secondary to opening SK/IK$_{Ca}$ or K$_{ATP}$ channels found specifically on each cell type stimulates both endothelial and smooth muscle cell hyperpolarization and dilation, which then spreads to the upstream sites in an endothelium-dependent manner [41,49] (Figure 8).

We have previously shown that both ACh and levcromakalim can stimulate both local- and distal-conducted hyperpolarization of rat mesenteric artery smooth muscle cells in intact, pinned out preparations [41]. Herein we show that isoproterenol also hyperpolarizes smooth muscle cells in pressurized arteries via K$_{ATP}$ channels, confirming previous work in pinned arteries [21] and with wire myography [23]. Others and we have previously shown that K$_{ATP}$ channels appear confined to smooth muscle cells in rat mesenteric arteries [41,47]. In freshly isolated endothelial cells from rat mesenteric arteries, levcromakalim was unable to stimulate hyperpolarization, whereas in ACh hyperpolarized cells by ~30 mV [41]. Therefore, hyperpolarization to isoproterenol, as to levcromakalim, is most likely initiated in smooth muscle cells. Unlike levcromakalim, isoproterenol evokes robust K$_{ATP}$ channel-independent dilation, as a consequence of other pathways stimulated following β-adrenoceptor stimulation. Herein this was observed as the glibenclamide-insensitive local dilation to isoproterenol in Branch 1, and was not unexpected [23,48]. However, glibenclamide did, as expected, block the local dilation to levcromakalim, and prevented conducted dilation to both agonists, confirming the central role hyperpolarization has in evoking conducted dilation. Data with isoproterenol in the presence of glibenclamide, are similar to responses with both ACh in the presence of carbenoxolone and the NO-donor DEA NONOate, in that local dilation, and associated small increases in flow and shear stress in the triple-cannulated arteries, is not sufficient in itself to evoke significant conducted dilation [49]. This is consistent with studies in the microcirculation, where agonists stimulating dilation without hyperpolarization, such as sodium nitroprusside [13], did not evoke conducted dilation [9,15,29,37] or increases in blood flow [29].

The importance of gap junctions in enabling the spread of dye and dilation was confirmed with carbenoxolone. Our data show that carbenoxolone prevents dye coupling between endothelial cells. Interestingly the longitudinal spread of calcein dye was less evident in smooth muscle cells, suggesting the pathway for inter-endothelial cell dye transfer is more favorable, as shown for two other low molecular weight dyes in arterioles [30]. We have previously shown that when calcein was added directly to the lumen of arteries, a carbenoxolone-sensitive dye transfer to smooth muscle was observed [27]. When added directly to the luminal perfusate, carbenoxolone blocked conducted dilation to ACh and reduced that to isoproterenol. The additional presence of carbenoxolone in the superfusion further reduced conducted dilation to isoproterenol. We did not pursue this further, but this likely reflects the cell type (smooth muscle *cf* endothelial cells) and K^+ channels activated by isoproterenol. Together with the dye experiments, these data support a role for gap junctions in enabling conducted dilation, consistent with current spread.

Blockers of BK$_{Ca}$ and K$_V$ channels augmented the contraction to phenylephrine, supporting a role for these channels in modulating contraction in this artery [11,35]. In terms of

vasomotion, the relatively rapid, small amplitude vasomotion observed in the presence of L-NAME was not markedly affected by the selective blocker of BK_{Ca} channels, IbTx, but was usually affected by either TEA or 4-AP alone, suggesting that K_V channels play a more dominant role in this rapidly coordinated but slow response.

Inhibition of one K^+ channel type alone had little to no effect on conducted dilation. Referring back to the glibenclamide experiments, although the initiation of hyperpolarization to both isoproterenol and levcromakalim was prevented, thereby precluding spread of hyperpolarizing current and ensuing dilation, the hyperpolarization to ACh remained unaffected. Therefore, we could show that K_{ATP} channels alone did not affect conducted dilation to ACh. This may reflect low level activation in the presence of phenylephrine in this artery, consistent with an inhibition of K_{ATP} channels by α-adrenoceptor agonists [3]. Neither block of either BK_{Ca} channels with IbTx or K_V channels with 4-AP had an effect on conducted dilation. However, TEA alone had a small effect to improve conducted dilation to ACh, likely reflecting the less selective actions of TEA, which can act at both K_V and BK_{Ca} channels. In contrast, either TEA or IbTx combined with 4-AP consistently and markedly augmented conducted dilation to both ACh and isoproterenol.

The ability of ACh and isoproterenol, endothelium-dependent and independent agonists, to evoke conducted dilation relies on the endothelium as the conduit. Therefore, hyperpolarization originating in either the endothelium (SK/IK_{Ca} channels) or smooth muscle cells (K_{ATP} channels) can pass longitudinally along the endothelium and then back to the smooth muscle (likely via MEGJs) at the upstream sites. This suggests that the hyperpolarizing current can pass bidirectionally through the MEGJs, and that perhaps the resistances of these junctions are not limiting for conducted dilation. This was further investigated in the presence of the BK_{Ca} and K_V channel blockers where it may be expected that any coupling via smooth muscle would be seen more readily. However, even in these arteries the endothelium appears crucial for conducted dilation responses. So whether the gap junctions between smooth muscle cells are too few and/or have high resistances requires further investigation.

Our sharp microelectrode measurements of conducted hyperpolarization to ACh are consistent with less loss of hyperpolarizing current in the presence of BK_{Ca} and K_V channel blockers. The concentration of ACh in Branch 1 was consistent between treatments, and although we could not record membrane potential at the “local” site or at 0 μm in the feed artery, we clearly and consistently observed a larger amplitude hyperpolarization to ACh ~2000 μm upstream from the bifurcation in the presence of K^+ channel blockers. The presence of nifedipine was essential to maintain impalements following addition of ACh in the presence of phenylephrine. Our ongoing studies show that nifedipine prevents oscillations in membrane potential and tension to phenylephrine in the presence of L-NAME, IbTx and 4-AP, but does neither prevent depolarization to phenylephrine nor hyperpolarization to ACh (H Takano, unpublished data). Thus, although we cannot quantitatively or temporarily link our measurements of hyperpolarization to dilation, qualitatively the data are consistent.

This remarkable observation strongly suggests that open BK_{Ca} and K_V channels in smooth muscle prevent the longitudinal spread of hyperpolarizing current necessary for dilation. So in an artery with 3–4 layers of smooth muscle cells as the mesenteric artery, hyperpolarization stimulated within a small bifurcation is a sufficient signal to initiate near full dilation along the entire feed artery, beyond 2 mm upstream. This is further than would be predicted by passive decay, one possible explanation being that interendothelial cell Ca^{2+} waves underlie the responses. However, we were unable to unmask waves either in the absence or presence of phenylephrine evoked arterial tone. In these experiments, we showed rises in Ca^{2+} at the site of ACh delivery in Branch 1, and the spread of this signal to one to two endothelial cells upstream, but certainly no further, even over an extended period of two minutes stimulation of the endothelium. This is consistent with predictions based on previous data in rat mesenteric arteries [28] but contrasts with studies in the microcirculation [42,43].

In summary, in rat mesenteric arteries contracted with phenylephrine to generate tone, dilation stimulated by agonists acting at either smooth muscle K_{ATP} channels or endothelial SK/IK_{Ca} channels spreads along the vessel length to stimulate conducted dilation, which decays dramatically over 2 mm. Blockade of BK_{Ca} and K_V channels markedly improves conducted dilation to the same agonists, which now decays markedly less over the 2 mm distance. Thus, the density of open voltage- and or Ca^{2+}-activated K^+ channels within vascular beds may limit the extent of conducted dilation, and therefore play a key role in regulating arterial conducted responses and therefore blood flow. Our data further confirm a key role for the endothelium as a conduit for conducted dilation responses, and support bidirectional electrical coupling through MEGJs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Abbreviations used

4-AP	4-aminopyridine
BK_{Ca}	large conductance K_{Ca}
IbTx	iberiotoxin
IK_{Ca}	intermediate conductance K_{Ca}
K_{ATP} channel	ATP-sensitive K^+ channel
K_{Ca} channel	Ca^{2+}-activated K^+ channel;
K_V channel	voltage-gated K^+ channel

L-NAME	L-nitroarginine methyl ester
MEGJ	myoendothelial gap junction
SK_{Ca}	small conductance KCa
TEA	tetraethylammonium
MOPS	3-(N-morpholino)propanesulfonic acid

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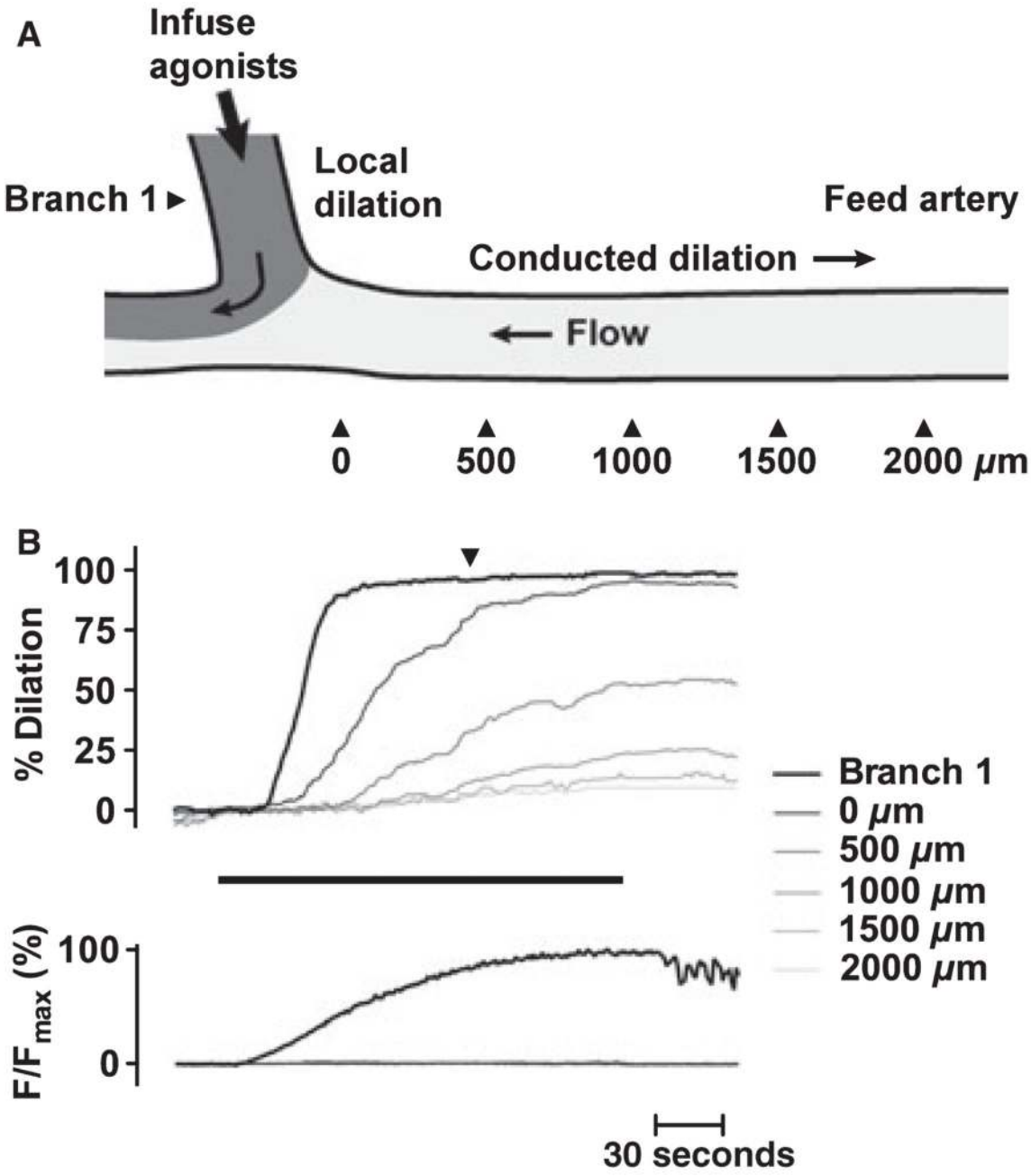

Figure 1.

Conducted dilation response to luminal perfusion of isoproterenol in triple-cannulated arteries. (**A**) Using a third pipette, one branch at an arterial bifurcation (Branch 1) was cannulated, through which perfusate containing an agonist and fluorescent marker was infused. (**B**) Simultaneous traces of arterial dilation (upper panel) and relative fluorescence (F/F_{max} in Branch 1, lower panel) in response to infusion of 1 μM isoproterenol and 0.1 μM carboxyfluorescein into Branch 1. The arrowheads in **A** indicate the positions of diameter and fluorescence measurement in Branch 1 and upstream from the bifurcation into the feed

artery (0–2000 μm). The bar indicates the period of infusion, L-NAME (100 μM) and phenylephrine (0.5 μM) were present in the superfusion solution, resulting in tone ~60% of the maximum contraction. Note the delay in dilation at 0 μm compared to Branch 1. The arrowhead in **B** indicates the time at which values for 80% dilation were used for summary data.

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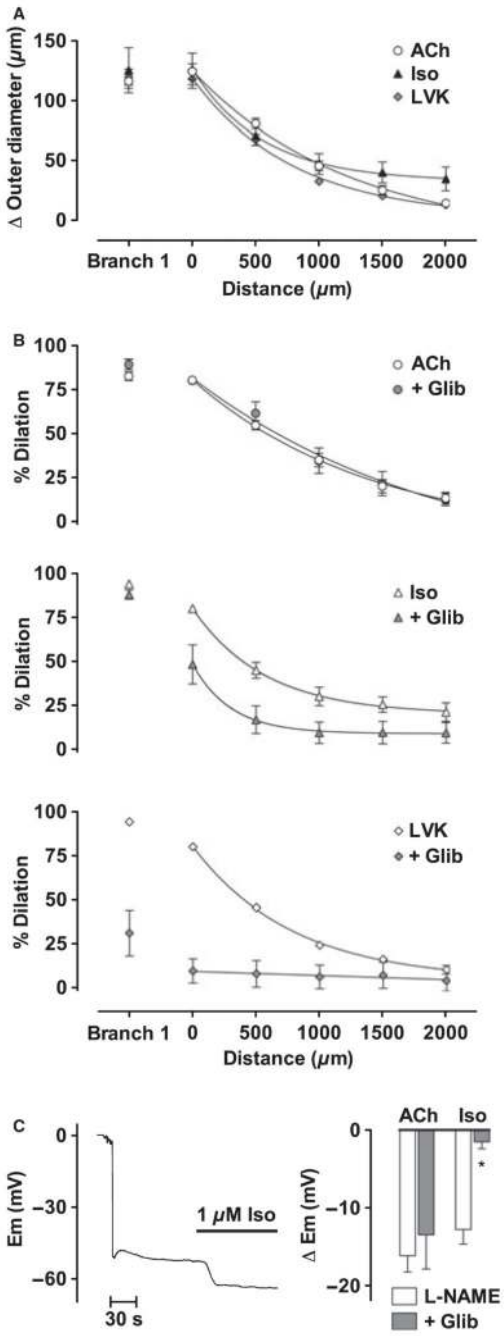

Figure 2.

Dilation and hyperpolarization to agonists. (**A**) In triple-cannulated arteries, the dilation at 0 μm in the feed artery was matched to ~80% of maximal diameter for ACh (1–3 μM, $n = 23$), isoproterenol (Iso, 1 μM, $n = 9$), and levcromakalim (LVK, 3 μM, $n = 6$), and responses simultaneously measured in Branch 1 and at upstream sites along the feed artery (0–2000 μm). The increases in diameter were comparable between agonists at all positions along the artery, each evoking similar conducted dilation. (**B**) The K_{ATP} channel blocker glibenclamide (10 μM) abolished the conducted dilation to LVK, was less effective against

isoproterenol, and was ineffective against ACh. Note that the values presented as increases in diameter in **A** are now presented as % dilation. (**C**) In double-cannulated arteries, a typical trace of a membrane potential recording showing hyperpolarization to 1 μM isoproterenol (left panel, bar indicates period of exposure to Iso). From resting membrane potential both ACh (1 μM, $n = 6$) and isoproterenol (1 μM, $n = 5$) hyperpolarized smooth muscle cells by >10 mV, but only the hyperpolarization to isoproterenol ($n = 3$) and not ACh ($n = 3$) was sensitive to glibenclamide (right panel). L-NAME present in all experiments.

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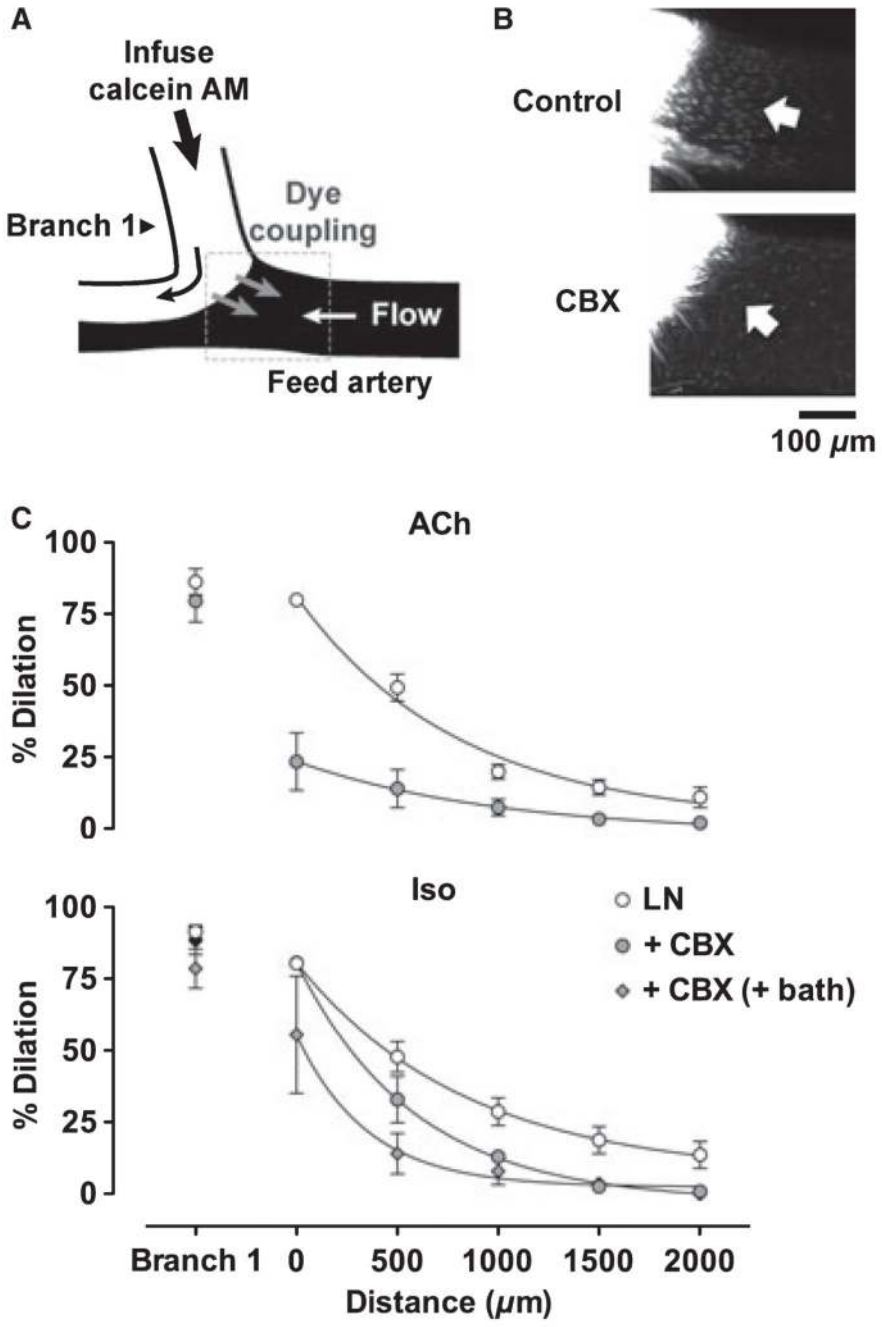

Figure 3.

Effect of carbenoxolone on spread of dye and dilation in triple-cannulated arteries. (**A**) Perfusate containing calcein AM was infused into Branch 1 in the continued presence of flow through the feed artery. The dashed lines correspond to the approximate region of the vessel seen in the micrographs in **B**. (**B**) Under control conditions (left) the dye spread to at least five endothelial cells upstream. In the presence of the gap junction uncoupler carbenoxolone (CBX, 100 μM) the dye spread was effectively abolished (right). Arrow heads indicate endothelial cell nuclei clearly stained by calcein. Note that in the presence of

CBX, the nerves, including cell bodies, were labeled. Each representative of three experiments. (**C**) In triple-cannulated arteries, the conducted dilation to ACh under control conditions (ACh, 1–3 μM, $n = 5$) was almost abolished by luminal perfusion of CBX into the feed artery (100 μM, $n = 5$). Responses were simultaneously measured in Branch 1 and at upstream sites along the feed artery (0–2000 μm). Note that the dilation in Branch 1 remained unaffected. Although conducted dilation to isoproterenol (1 μM, Iso) was reduced following 30–40 minutes luminal perfusion of CBX, the presence of CBX in the superfusion solution (+ bath) was required to block conducted dilation (60–90 minutes, bottom panel). L-NAME (LN, 100 μM) and phenylephrine (0.5–1 μM) were present in the superfusion solution, with tone matched to ~70% of the maximum contraction.

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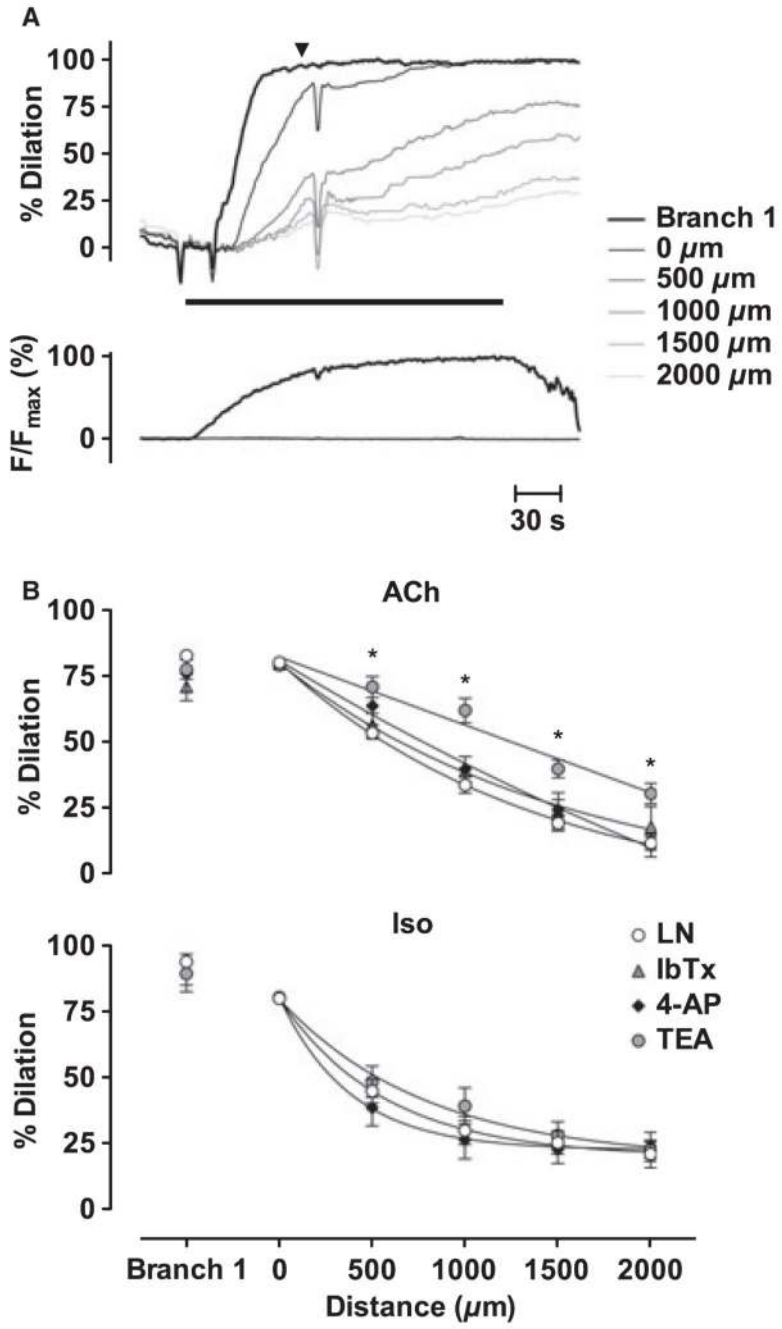

Figure 4.

Effect of K^+ channel blockers on conducted dilation response to luminal perfusion of agonists in triple-cannulated arteries. (**A**) Simultaneous traces of arterial dilation (upper panel) and relative fluorescence (F/F_{max} in Branch 1, lower panel) in response to infusion of 1 μM isoproterenol and 0.1 μM carboxyfluorescein into Branch 1. The bar indicates the period of infusion. L-NAME (LN, 100 μM), tetraethylammonium (TEA, 1 mM), and phenylephrine (0.5 μM) were present in the superfusion solution, resulting in tone ~60% of the maximum contraction, and low frequency vasomotion. The arrowhead indicates the time

at which values for 80% dilation were used for summary data. (**B**) In the presence of TEA, the conducted dilation at the time when 80% dilation was observed at 0 μm in the feed artery was augmented in response to ACh (1–3 μM, $n = 7$) but not to isoproterenol (Iso, 1 μM, $n = 7$). The other K^+ channel blockers iberiotoxin (IbTx, 100 nM) and 4-aminopyridine (4-AP, 150 μM) had no effect against ACh or isoproterenol when 80% dilation was observed at 0 μm ($n = 4–5$). Same control (LN alone) data as in Figure 2. *Significantly different from control.

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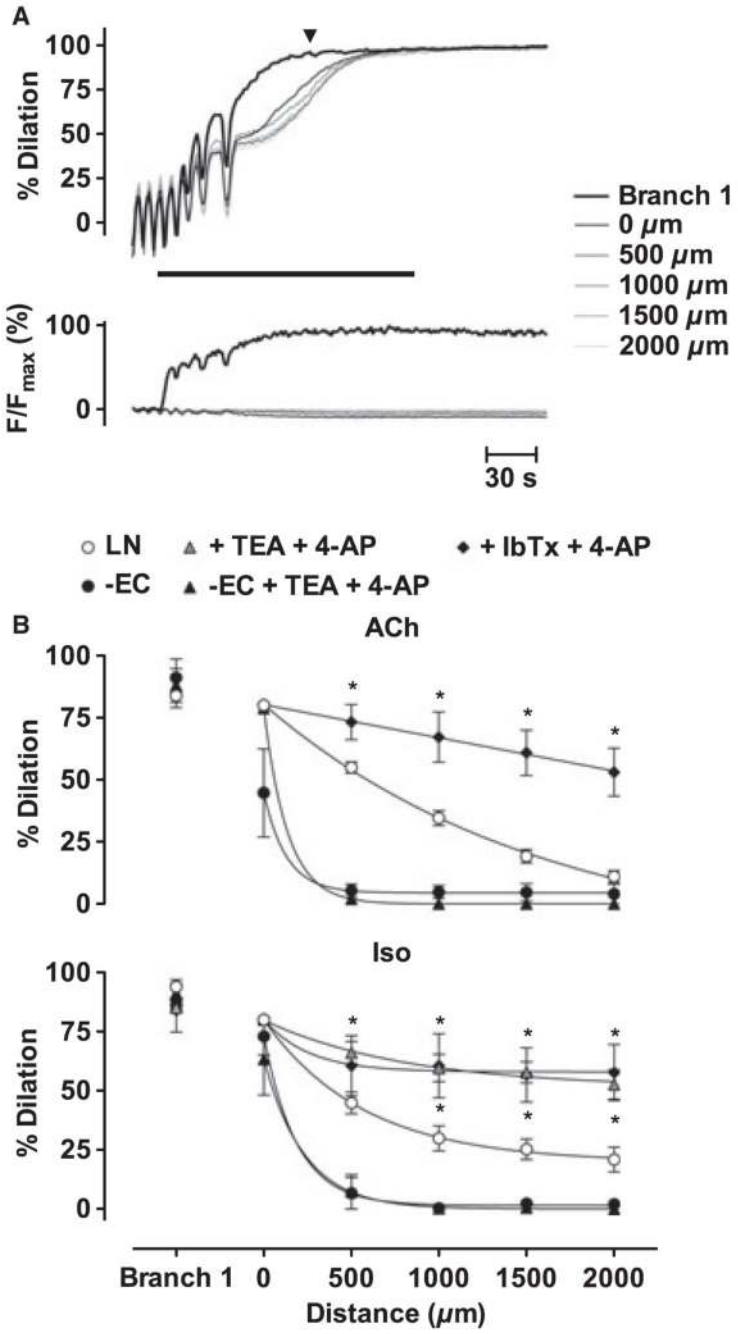

Figure 5.

Effect of combined K^+ channel blockers on conducted dilation response to luminal perfusion of agonists in triple-cannulated arteries. (**A**) Simultaneous traces of arterial dilation (upper panel) and relative fluorescence (*F*/*F*max in Branch 1, lower panel) in response to infusion of 1 μM isoproterenol and 0.1 μM carboxyfluorescein into Branch 1. The bar indicates the period of infusion. L-NAME (LN, 100 μM), tetraethylammonium (TEA, 1 mM), 4-aminopyridine (4-AP, 150 μM) and phenylephrine (1 μM) were present in the superfusion solution, resulting in tone ~70% of the maximum contraction, and high frequency

vasomotion. Note that the entire feed artery fully dilated to isoproterenol. The arrowhead indicates the time at which values for 80% dilation were used for summary data. (**B**) In the presence of either TEA with 4-AP, or iberiotoxin (IbTx, 100 nM) with 4-AP the conducted dilation at the time when 80% dilation was observed at 0 μm in the feed artery was markedly augmented in response to both ACh (1–3 lM, $n = 5$) and isoproterenol (Iso, 1 μM, $n = 3$–4). Same control (LN) data as in Figure 2. L-NAME present in all experiments. *Significantly different from control. The conducted dilation to both ACh (3 μM) and isoproterenol (1 μM) was effectively abolished beyond the bifurcation in arteries with damaged endothelial cells in the feed artery (-EC, $n = 3$ for each).

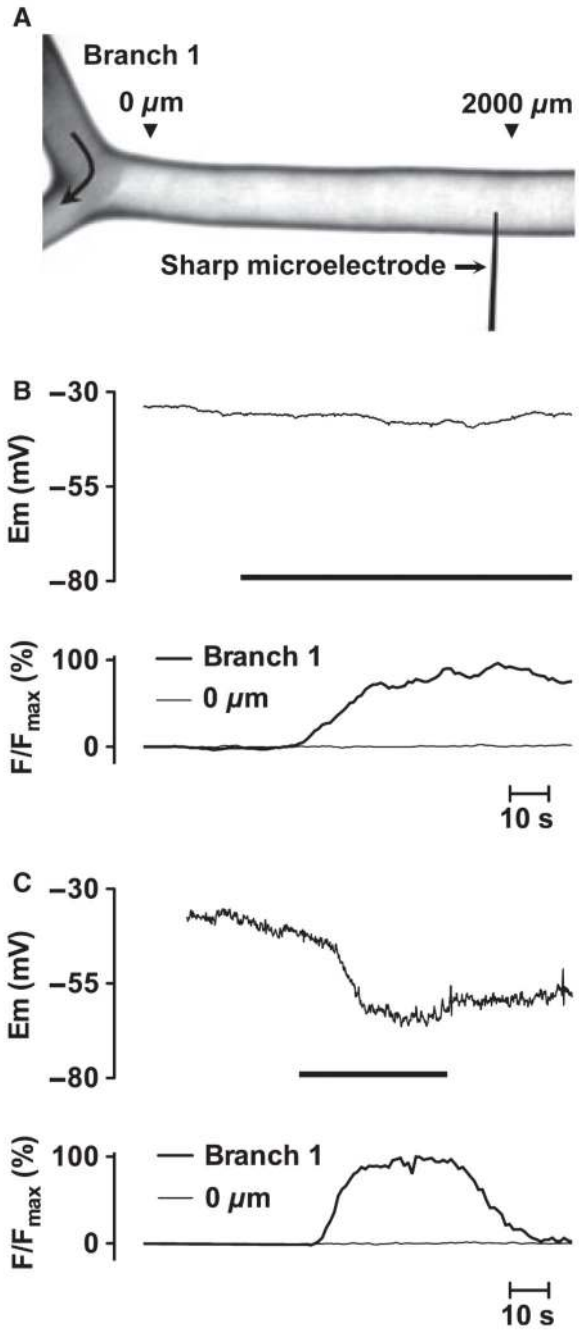

Figure 6.

Effect of combined K^+ channel blockers on conducted hyperpolarization to luminal perfusion of ACh in triple-cannulated arteries. (**A**) Experimental micrograph showing the experimental setup for recording membrane potential near the 2000 μm site upstream from the bifurcation. The position of the microelectrode has been traced for clarity and the plane of focus was at the microelectrode tip. ACh (1 μM) was luminally perfused into Branch 1 together with carboxyfluorescein (dark gray) and confined to Branch 1 and 2 by continuous luminal perfusion of the feed artery. All experiments were performed in the presence of 2

μM phenylephrine and 100 μM L-NAME. Nifedipine was also present to reduce movement of the artery. Representative traces of membrane potential (upper panels) and fluorescence (F/F_{max}, %) in Branch 1 and the 0 μm site in the feed artery (lower panels) show that under control conditions (**B**), only modest conducted hyperpolarization to ACh was observed; whereas in the presence of tetraethylammonium (TEA, 1 mM) and 4-aminopyridine (4-AP, 150 μM) the conducted hyperpolarization to ACh was enhanced (**C**). See Results for summary data.

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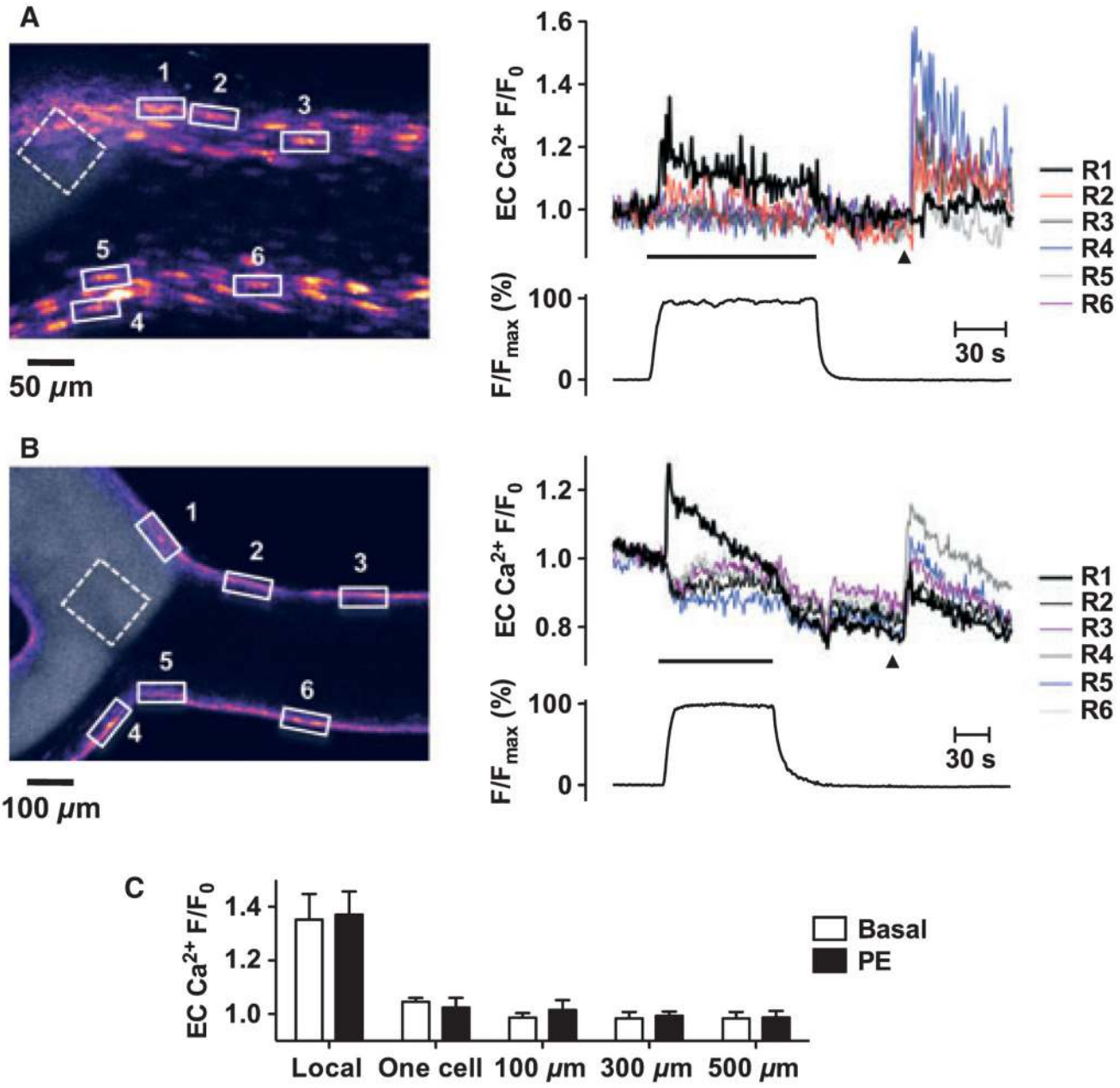

Figure 7.

Changes in intracellular Ca^{2+} in endothelial cells following luminal perfusion of ACh in triple cannulated arteries. Arteries were luminally loaded with the Ca^{2+} indicator Oregon Green® 488 BAPTA-1 (pseudo-color) to monitor changes in endothelial cell Ca^{2+} and rhodamine-dextran (0.5 μM, inverted grayscale) was included in the agonist solution (3 μM ACh). (**A**) A representative experiment showing simultaneous traces of changes in Oregon Green® 488 BAPTA-1 (EC Ca^{2+}) and rhodamine fluorescence (right panels). The numbered regions around single endothelial cells in the confocal micrograph (left) correspond to the relative changes in endothelial cell fluorescence intensity (EC Ca^{2+} F/F_0), and the intensity within the dashed box corresponds to the delivery of agonist and dye solution (F/F_{max}). Only

the cells within R1 and R2 showed a detectable increase in fluorescence during the period of ACh perfusion (indicated by bar), whereas all cells responded to the subsequent bath addition of ACh (1 μM at arrowhead). (**B**) As for **A**, a representative experiment but in the presence of phenylephrine tone, and the 488 nm laser intensity was doubled and tended to bleach the dye signal. (**C**) Values for relative changes in EC Ca^{2+} from each position along the artery within each experiment were averaged to provide summary data in the absence (n = 3–5) and presence (n = 3–7) of phenylephrine tone.

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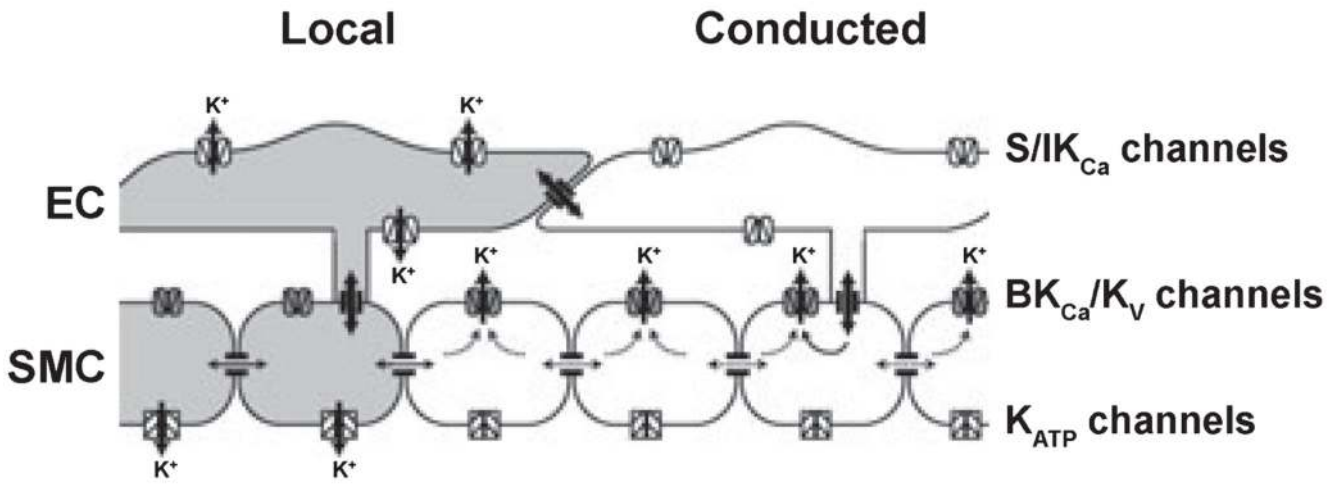

Figure 8.

Summary of the pathways stimulated during conducted dilation. Phenylephrine added to the superfusion solution reaches all cells, depolarizing and increasing Ca^{2+} in smooth muscle cells, activating BK_{Ca} and K_V channels. Patch clamp data [44] show that these channels are not present in endothelial cells. Addition of ACh to one end of the vessel (**Local**, indicated by gray shading) stimulates endothelial cell muscarinic receptors, increases endothelial cell Ca^{2+}, and activates SK_{Ca} and IK_{Ca} channels. In this vessel, these channels are only expressed on endothelial cells [14,40,44]. Addition of isoproterenol to the local site stimulates β-adrenoceptors, likely on both endothelial and smooth muscle cells. One of the signaling pathways linked to β-ARs is to activate K_{ATP} channels. However, these channels are not expressed in endothelial cells [41,47] so hyperpolarization originates from the smooth muscle cells. The KATP channels can be directly activated by levcromakalim (LVK), again limited to the local site. ACh, isoproterenol and LVK stimulate local and conducted dilation (**Conducted**), which decays with distance from the local site. The distance this dilation can spread upstream is enhanced following blockade of BK_{Ca} and K_V channels. In all instances the conducted dilation depends on endothelial cell integrity, suggesting that normally the inter-smooth muscle cell coupling is minimal and hyperpolarization can pass bidirectionally and effectively through myoendothelial gap junctions.

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Table 1.

Diameter of triple-cannulated arteries used for conducted dilation experiments

	Maximum	Minimum	Phenylephrine tone	Δ Diameter to dilator (80% at 0 μm)
Branch 1	307 ± 8	125 ± 6	172 ± 8 (75 ± 3%)	119 ± 6
0 μm	365 ± 7	148 ± 4	211 ± 7 (71 ± 3%)	124 ± 5
500 μm	360 ± 6	144 ± 3	210 ± 7 (70 ± 2%)	76 ± 3
1000 μm	360 ± 7	143 ± 4	219 ± 7 (66 ± 3%)	43 ± 3
1500 μm	362 ± 6	146 ± 3	223 ± 7 (64 ± 3%)	27 ± 3
2000 μm	361 ± 6	146 ± 3	229 ± 7 (61 ± 3%)	18 ± 3

Summary data for outer diameters in Branch 1 and along the feed artery at the positions used to measure dilation. The tone generated by phenylephrine is also expressed as the percentage of maximum contraction (% contraction, values in brackets). From this phenylephrine-stimulated tone, the increase in diameter in response to luminal perfusion of either ACh, isoproterenol or levcromakalim into Branch 1 is averaged over all the experiments; values equate to the time when the dilation at 0 μm was 80% of maximum diameter. Values are the mean ± SEM of 38 arteries; L-NAME present.

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Table 2.

Baseline tone in conducted dilation experiments

	Tone (% max)	*n*	Amplitude (μm)	Frequency (Hz)	*n′*
L-NAME	66 ± 3	38	6 ± 1	0.25 ± 0.01	16
+ IbTx	83 ± 7	5	8 ± 2	0.23 ± 0.02	4
+ 4-AP	67 ± 6	9	28 ± 5*	0.08 ± 0.03*	6
+ TEA	66 ± 4	14	18 ± 4*	0.13 ± 0.02*	12
+ IbTx + 4-AP	61 ± 4	8	45 ± 8*	0.13 ± 0.01*	8
+ TEA + 4-AP	79 ± 5	4	28 ± 6*	0.15 ± 0.01*	4

Summary data for the phenylephrine-stimulated tone (as a % maximum contraction, % max) in the feed artery of triple-cannulated arteries under control conditions and in the presence of K^+ channel blockers. Values are given for the 1000 μm position in the feed artery, and are qualitatively and temporarily representative of the entire feed artery and Branch 1. In the arteries that developed vasomotion, the amplitude and frequency of the oscillations in diameter are shown, and the changes in diameter were synchronized along the entire vessel. Values are the mean ± SEM of the number of arteries (*n*), and the number of arteries from *n* that developed vasomotion (*n′*). L-NAME, 100 lM; iberiotoxin (IbTx), 100 nM; 4-aminopyridine (4-AP), 150 μM; tetraethylammonium (TEA), 1 mM.