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Differential hyperpolarization to substance P and calcitonin gene-related peptide in smooth muscle versus endothelium of mouse mesenteric artery

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Abstract

Objective: We sought to define how sensory neurotransmitters substance P and calcitonin gene-related peptide (CGRP) affect membrane potential of vascular smooth muscle and endothelium.

Methods: Microelectrodes recorded membrane potential of smooth muscle from pressurized mouse mesenteric arteries (diameter, ~150 μm) and in endothelial tubes.

Results: Resting potential was similar (~ −45 mV) for each cell layer. Substance P hyperpolarized smooth muscle and endothelium ~ −15 mV; smooth muscle hyperpolarization was abolished by endothelial disruption or NO synthase inhibition. Blocking K_{Ca} channels (apamin + charybdotoxin) attenuated hyperpolarization in both cell types. CGRP hyperpolarized endothelium and smooth muscle ~ −30 mV; smooth muscle hyperpolarization was independent of endothelium. Blocking K_{Ca} channels prevented hyperpolarization to CGRP in endothelium but not smooth muscle. Inhibiting K_{ATP} channels with glibenclamide or genetic deletion of $K_{IR}6.1$ attenuated hyperpolarization in smooth muscle but not endothelium. Pinacidil (K_{ATP} channel agonist) hyperpolarized smooth muscle more than endothelium (~ −35 vs. ~ −20 mV).

Conclusions: Calcitonin gene-related peptide elicits greater hyperpolarization than substance P. Substance P hyperpolarizes both cell layers through K_{Ca} channels and involves endothelium-derived NO in smooth muscle. Endothelial hyperpolarization to CGRP requires K_{Ca} channels, while K_{ATP} channels mediate hyperpolarization in smooth muscle. Differential K^+ channel activation in smooth muscle and endothelium through sensory neurotransmission may selectively tune mesenteric blood flow.

Keywords

membrane potential; neurotransmitters; nitric oxide; sensory nerves

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CONFLICT OF INTEREST

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1 | INTRODUCTION

Perivascular nerves are integral to blood flow regulation. Activation of sensory nerves releases calcitonin gene-related peptide (CGRP) and substance P (SP).[1,2] Of these neurotransmitters, CGRP has been most widely studied and elicits vasodilation via binding to its receptors on vascular smooth muscle cells (SMCs) and endothelial cells (ECs).[3,4] Functional CGRP receptors are composed of three subunits: calcitonin receptor-like receptor (CRLR), receptor activity modifying protein 1 (RAMP1), and receptor component protein.[5] In SMCs from mesenteric,[6] renal,[7] and coronary[8] arteries, CGRP elicits vasorelaxation via protein kinase A-mediated activation of K^+ channels, with a primary contribution of ATP-sensitive K^+ (K_{ATP}) channels.[6,8–11] The ensuing efflux of K^+ leads to hyperpolarization, which reduces constitutive voltage-gated calcium channel (VGCC) activity and thereby attenuates SMC $[Ca^{2+}]_i$ to promote vasodilation.[12] CGRP can also elicit endothelium-dependent vasodilation through production of NO, which further hyperpolarizes SMCs.[13] While these effects of CGRP on membrane potential (V_m) of SMCs have been resolved, little is known of how CGRP may affect V_m of ECs.

How SP affects V_m of SMCs and ECs has received little attention. Nevertheless, endothelial disruption abolishes dilation of mesenteric arteries (MAs) to SP.[14,15] Binding to neurokinin receptors on ECs,[16] SP increases $[Ca^{2+}]_i$ to activate endothelial NO synthase,[15] with NO able to trigger SMC membrane hyperpolarization through Ca^{2+}-activated K^+ (K_{Ca}) and K_{ATP} channels.[17] While K_{Ca} channels have been implicated in hyperpolarization of cultured porcine coronary ECs to SP,[18,19] how SP may affect V_m in SMCs of pressurized resistance arteries or their native ECs has not been determined.

The objective of this study was to define how CGRP and SP effect V_m of SMCs and ECs in MAs freshly isolated from the mouse. Vessels were cannulated and pressurized with intact or denuded endothelium to evaluate EC-dependent and intrinsic SMC responses, with ECs studied in endothelial tubes following dissociation of SMCs. With both vasodilatory neurotransmitters released from perivascular sensory nerves, we hypothesized that CGRP and SP have similar actions on K_{Ca} and K_{ATP} channels in respective cell layers. Our findings illustrate that EC hyperpolarization to CGRP is mediated primarily by K_{Ca} channels, as is hyperpolarization of both ECs and SMCs to SP. In contrast, SMC hyperpolarization to CGRP is mediated by K_{ATP} channels. Such differences in how sensory neurotransmission affects V_m in respective cell layers of resistance arteries may be integral to precisely regulating local blood flow.

2 | MATERIALS AND METHODS

2.1 | Animal care and use

All protocols and experimental procedures were reviewed and approved by the Animal Care and Use Committee of the University of Missouri (Columbia, MO; Protocol #9220), were performed in compliance with all local, state, and federal guidelines for the humane care and use of animals, and in accord with the animal ethics guidelines for this journal. Experiments were performed on C57BL/6J male “wildtype” mice (n = 75, 27–32 g; 4–6 months old) purchased from Jackson Laboratorie (Bar Harbor, ME, USA). Complementary experiments

were performed on MAs from $K_{ATP}/K_{IR}6.1$ knockout mice ($K_{ATP}^{-/-}$, mice lacking *KCNJ8* gene on C57BL/6 background; $n = 6$) obtained from Dr. Michael Davis (University of Missouri; Columbia, MO).[20]

Mouse MAs provide an animal model which recapitulates aspects of human vascular behavior.[21] Mice were housed on a 12:12 h light-dark cycle at ~23°C with fresh water and food available *ad libitum*. Male mice were utilized to avoid sex differences as a confounding variable. Each mouse was anesthetized with ketamine and xylazine (100 and 10 mg kg^{-1}, respectively; intraperitoneal injection) for tissue harvest and killed by exsanguination.

2.2 | Materials

2.2.1 | Reagents—Acetylcholine (ACh, Cat. #A6625), glibenclamide (Cat. #G2539), N^G-nitro-L-arginine methyl ester (L-NAME; Cat. #N5751), norepinephrine (NE, Cat. #A7256), and pinacidil (Cat. #P154) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Apamin (Cat. #60772), CGRP (Cat. #20682), charybdotoxin (Cat. #28244), and SP (Cat. #24279) were acquired from AnaSpec (Fremont, CA, USA).

2.2.2 | Solutions—Isolated arteries and endothelial tubes were superfused with physiological salt solution (PSS; pH 7.4) containing (in mM): 140 NaCl (Fisher Scientific, Pittsburgh, PA, USA), 5 KCl (Fisher), 2 $CaCl_2$ (Fisher), 1 $MgCl_2$ (Sigma), 10 HEPES (Sigma), and 10 glucose (Fisher). During vessel dissection and isolation of endothelial tubes, the PSS contained 0.1% bovine serum albumin (#10856, USB Corp., Cleveland, OH, USA). During dissection, $CaCl_2$ was absent and 10^{-5} M sodium nitroprusside was added to relax SMCs. For dissociation of SMCs, 10^{-4} M $CaCl_2$ (Sigma) was included (required for enzyme activity) and sodium nitroprusside was omitted.

2.3 | Vascular preparations

The intestines were accessed via a laparotomy, excised, and pinned on to transparent silicone rubber (Sylgard 184; Dow Corning, Midland, MI, USA) then immersed in PSS within a dissection chamber maintained at 4°C. Individual MAs [inner diameter (ID), ~150 μm] supplying the small intestine were dissected and transferred to a tissue chamber (RC-27N, volume ~1 mL; Warner Instruments, Hamden, CT, USA) secured on an aluminum platform (length: 24 cm; width: 14.5 cm; thickness: 0.4 cm). Cannulation pipettes were pulled (P-97; Sutter Instruments, Novato, CA, USA) from glass capillaries (Cat. #64–1781, Warner; 0.94 mm ID, 1.2 mm outer diameter) and heat-polished (outer diameter, 100 μm). Each pipette was secured in a custom holder (Warner) held in a three-axis micromanipulator (DT3-100; Siskiyou Corp., Grants Pass, OR, USA) secured to the aluminum platform at each end of the tissue chamber. Pipettes were positioned in the chamber; individual MAs were cannulated and secured at each end with a strand of silk suture.[22,23]

2.3.1 | Endothelial disruption—To evaluate SMC responses independent of EC influences, the endothelium was disrupted by rubbing a small tungsten wire (diameter, 50 μm; Goodfellow, Huntington, UK) through the lumen of the vessel three times prior to cannulation.[22] Once secured and pressurized (as above), selective EC damage was confirmed by ~50% constriction to NE ($l.7 \times 10^{-7}$ M; EC_{50}) with loss of dilation to ACh

(10^{-5} M).[22] Dilation to ACh was calculated as: [(ID_{ACh}−IDNE)/(ID Ca2+ free−IDNE)] × 100%, where IDCa2+ free is maximal internal diameter in Ca^{2+}-free PSS, ID_{NE} is the internal diameter during constriction to NE, and ID_{ACh} is internal diameter during the addition of ACh. All endothelium-disrupted vessels studied in these experiments responded to NE but not ACh (vasodilation: intact = 76 ± 5%, endothelium-disrupted = 3 ± 6%).

2.3.2 | Endothelial tube isolation—Isolated MAs were placed into PSS containing 0.62 mg·mL^{-1} papain (P4762, Sigma), 1.0 mg·mL^{-1} dithioerythritol (D8255, Sigma), and 1.5 mg·mL^{-1} collagenase (C8051, Sigma) then incubated for 25 min at 33°C. Following incubation, the enzyme solution was replaced with dissociation PSS and arterial segments were transferred into the tissue chamber. Trituration pipettes were pulled from borosilicate glass capillary tubes [Cat. #1B100-4, World Precision Instruments (WPI), Sarasota, FL, USA], heat polished to a tip ID of ~120 μm and connected to a Nanoliter Injector (WPI) to control fluid movement. During visual observation at 200× magnification, arterial segments were gently triturated to remove SMCs.[24] Once isolated, each end of the endothelial tube was gently pressed against the bottom of the tissue chamber (a 24 × 54 mm coverslip) with a heat-blunted pipette (tip, diameter ~80 μm) and extended to approximate *in situ* length.[23,25]

Completed preparations were placed on an inverted microscope (Eclipse TS100; Nikon, Tokyo, Japan) mounted on a vibration-isolated table (Technical Manufacturing Corp., Peabody, MA, USA) and superfused at 4 mL·min^{-1} with PSS. An inline heater (SH-27B, Warner) and heating platform (PH6, Warner) coupled to a controller (TC-344B, Warner) maintained the superfusion solution temperature. Cannulated MAs were pressurized to 100 cm H_2O while being warmed to 37°C[22] for experiments; any vessels with leaks were discarded. Endothelial tubes were studied at 32°C to maintain viability.[25] Pharmacological agents were added to the superfusion solution to achieve final concentrations in the vessel chamber.

2.4 | Intracellular recording

The V_m of SMCs of intact and endothelium-disrupted MAs and of ECs in endothelial tubes was recorded with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA) using microelectrodes pulled (P-97, Sutter) from glass capillary tubes (GC100F-10, Warner) and backfilled with 2 M KCl (tip resistance: ~150 MΩ). A Ag/AgCl pellet in the effluent PSS served as a reference electrode. The output of the amplifier was connected to a data acquisition system (Digidata 1322A, Molecular Devices) and an audible baseline monitor (ABM-3, WPI). Successful impalements were indicated by sharp negative deflection of V_m, shift in tone of the ABM, stable V_m for >1 min, depolarization (≥20 mV) to 0.1 M KCl (SMCs) or hyperpolarization (≥20 mV) to 10^{-5} M ACh (ECs),[23,26] recovery to resting V_m after KCl or ACh washout, and prompt return to ~0 mV upon electrode withdrawal. For intact vessels, SMCs are the first cell to be penetrated when approached from the bath; ECs comprise endothelial tubes. Data were acquired at 1 kHz on a personal computer using Axoscope 10.1 software (Molecular Devices). Following cell impalement, baseline V_m was recorded for 10 min to ensure stable electrode placement prior to pharmacological treatments. Experimental protocols were completed within 90 min. Each protocol was

performed on a separate vessel preparation with 1 SMC or 1 EC continuously recorded (Figure 1). One neurotransmitter was studied in each preparation.

2.5 | Pharmacology

Increasing concentrations of CGRP (10^{-10}–10^{-6} M) or SP (10^{-10}–10^{-6} M) were added in half log molar increments, each for 5 min, with continuous recording of V_m. Responses to SP and CGRP were then evaluated in the presence of L-NAME (10^{-4} M) in separate preparations. Responses were also evaluated in the presence the K_{ATP} channel antagonist, glibenclamide (10^{-6} M), which is selective for K_{ATP} over other K^+ channels including K_V, K_{Ca}, and K_{IR}.[12] We have previously shown that 10^{-6} M glibenclamide prevents hyperpolarization to the K_{ATP} agonist pinacidil (10^{-5} M).[27] We also evaluated responses in the presence of charybdotoxin (10^{-7} M; inhibits BK and IK) + apamin (3×10^{-7} M; inhibits SK) to inhibit K_{Ca} channels in SMCs and ECs.[28–30] The K_{Ca} channels were inhibited simultaneously as they are each activated through a similar Ca^{2+}-dependent mechanism, and their inhibitors can overlap among channel subtypes.[30,31] All antagonists were equilibrated for 20 min prior to evaluating their effect on electrophysiological responses to SP or CGRP. For each reagent requiring a solvent, controls refer to experiments performed with its stated vehicle added to control PSS; the control solution for water-soluble compounds was PSS. Concentration-response curves to pinacidil (10^{-9}–10^{-5} M) were evaluated in the same manner as responses to CGRP. For analyses, data were sampled for 30 s once a stable V_m response was achieved at each concentration of agonist.

2.6 | Data and statistical analysis

Electrophysiological analyses included: resting V_m (mV) under control conditions; peak V_m (mV) = V_m during maximal response to SP, CGRP, or pinacidil; and change in V_m (ΔV_m) = peak response V_m – preceding baseline V_m. The order of experiments was randomized between control and treatment groups. Blinding of the investigator was not feasible as protocols required the operator to be aware of which agent(s) were added to the PSS and recordings were analyzed immediately after completion of the protocol. Data were analyzed using analysis of variance (Prism 5, GraphPad Software, La Jolla, Ca, USA). For each experimental condition, a repeated measures ANOVA was performed to determine whether the intervention had a significant effect on V_m compared to baseline. For comparisons between groups, two-way ANOVA was performed to compare effects of cell types and pharmacological interventions. When significant main effects were detected with ANOVA, post hoc comparisons were performed using Bonferroni tests. $p \leq 0.05$ was considered statistically significant. Summary data are presented as means ± SE. Results were obtained from $n = 5$ vessels in each group. Data supporting the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Mesenteric arteries are hyperpolarized by both sensory neurotransmitters

The baseline V_m was similar (~ −45 mV) between cell types in respective preparations (Table 1). During continuous recording of V_m, SP (10^{-10}–10^{-6} M) hyperpolarized SMCs of intact MAs in a concentration-dependent manner (Figure 2A,B). Following EC disruption,

SMCs were no longer hyperpolarized by SP (Figure 2C). SP also hyperpolarized ECs in a concentration-dependent manner but the change in V_m from baseline was less than observed for SMCs with intact endothelium (Figure 2D). The EC_{50} values for hyperpolarization to SP were not significantly different between SMCs of intact MAs vs. ECs of endothelial tubes (Table 1).

During continuous recording of V_m, CGRP (10^{-10}–10^{-6} M) hyperpolarized SMCs in a concentration-dependent manner and to a similar level irrespective of the endothelium (Figure 3). Hyperpolarization of ECs with CGRP was not different from its effect on SMCs. Further, the magnitude of hyperpolarization with CGRP was greater than SP in both cell layers (Table 1). Respective EC_{50} values for CGRP hyperpolarization were not significantly different between preparations (Table 1).

3.2 | Effects of NO on SMC hyperpolarization in response to CGRP

Substance P and CGRP can signal SMCs through NO.[1] To test for the contribution of NO to hyperpolarization, concentration-response curves were repeated for SMCs of intact MAs in the presence of the NO synthase inhibitor, L-NAME (10^{-4} M). Consistent with the effect of endothelial disruption (Figure 2), EC hyperpolarization to SP was eliminated during NO synthase inhibition (Figure 4A,B). To confirm the viability of SMCs in these preparations, addition of 0.1 M KCl following each L-NAME protocol consistently elicited depolarization (ΔV_m = 34 ± 5 mV; Figure 4A). In contrast to inhibiting SMC hyperpolarization to SP, L-NAME had no effect on hyperpolarization to CGRP (Figure 4C), nor did it alter EC_{50} values from those in Table 1.

3.3 | K_{ATP} channels do not contribute to hyperpolarization elicited by SP

To test whether K_{ATP} channels contribute to hyperpolarization by SP, the channel inhibitor glibenclamide was added at a concentration (10^{-6} M) that blocks hyperpolarization to the K_{ATP} channel opener pinacidil (10^{-5} M).[27] Inhibition of K_{ATP} channels had no significant effect on hyperpolarization to SP in either SMCs or ECs (Figure 5), nor did glibenclamide alter EC_{50} values from those in Table 1. Responses to SP with its vehicle (DMSO) in PSS did not differ significantly from PSS alone.

3.4 | K_{Ca} channels contribute to SMC and EC hyperpolarization in response to SP

The role of K_{Ca} channels in mediating hyperpolarization to SP was tested with the antagonists charybdotoxin (10^{-7} M) + apamin (3×10^{-7} M).[28,29] For SMCs, K_{Ca} channel inhibition attenuated hyperpolarization to SP (Figure 6A,B), although some hyperpolarization remained at concentrations of SP ≥10^{-7} M. In ECs, K_{Ca} channel inhibition nearly eliminated hyperpolarization (peak ΔV_m = −4 ± 1 mV) and significantly increased EC_{50} values (control = 7.4×10^{-9} M; charybdotoxin + apamin = 13.6×10^{-9} M; $p < 0.05$). K_{Ca} channel inhibition did not alter EC_{50} values in SMCs from those in Table 1.

3.5 | K_{ATP} channels contribute to SMC but not EC hyperpolarization elicited by CGRP

In the presence of glibenclamide (10^{-6} M), SMC hyperpolarization to CGRP was significantly attenuated (Figure 7), with a maximal ΔV_m of ~ −10 mV vs. ~ −30 mV under control conditions. Contrary its effect on SMCs (and to our hypothesis), EC

hyperpolarization to CGRP was maintained in the presence of glibenclamide. These findings were verified in $K_{ATP}^{-/-}$ mice (global deletion), where EC responses to CGRP were unaffected while SMC responses were significantly attenuated compare to those of wildtype mice (Figure 8). In SMCs from mice lacking K_{ATP} channels, there was a significant increase in the EC_{50} value to CGRP (wildtype = 10×10^{-9} M; $K_{ATP}^{-/-} = 20 \times 10^{-9}$ M; $p < 0.05$). The absence of K_{ATP} function in $K_{ATP}^{-/-}$ mice was confirmed by testing with the K_{ATP} channel activator pinacidil (10^{-5} M); from a baseline V_m of −45 mV, the ΔV_m was <5 mV.

To determine whether K_{ATP} channel activation can hyperpolarize SMCs and ECs, we stimulated intact MAs and endothelial tubes of wildtype mice with the K_{ATP} agonist pinacidil (10^{-9}–10^{-5} M). Pinacidil evoked concentration-dependent hyperpolarization of SMCs within intact MAs and of ECs within endothelial tubes (Figure 9). From a similar resting V_m (~ −45 mV), maximal hyperpolarization was nearly twofold greater in SMCs ($\Delta V_m = -33 \pm 2$ mV) vs. ECs ($\Delta V_m = -18 \pm 1$ mV). Furthermore, EC_{50} values for pinacidil were significantly greater in ECs (1.2×10^{-7} M) vs. SMCs (4.1×10^{-8} M; $p < 0.05$).

We next tested the role of K_{Ca} channels to mediate hyperpolarization to CGRP. Addition of charybdotoxin + apamin attenuated SMC hyperpolarization to CGRP by ~30% (Figure 10). K_{Ca} channel inhibition had a greater effect in ECs, nearly abolishing hyperpolarization to CGRP (peak $\Delta V_m = -3 \pm 1$ mV). To ensure that the observed lack of hyperpolarization was not due to nonviable EC tube preparations, responses to ACh (10^{-5} M) were verified at the end of each protocol following washout of the channel blockers ($\Delta V_m = -31 \pm 3$ mV; Figure 10A). Inhibition of K_{Ca} channels did not significantly alter EC_{50} values to CGRP in SMCs compared to those in Table 1.

4 | DISCUSSION

The present experiments define the electrophysiological responses of SMCs and ECs comprising mouse MAs during controlled exposure to CGRP and SP. The stability of our V_m recordings facilitated continuous, uninterrupted evaluation of complete concentration-response relationships of SMCs within intact MAs, endothelium-disrupted MAs, and of ECs within intact endothelial tubes following dissociation of SMCs. Our major findings (Figure 11) show that: 1) both sensory neurotransmitters hyperpolarize ECs and SMCs, with CGRP having ~twofold greater effect than SP; 2) SMC hyperpolarization to CGRP can occur independent of ECs while SMC hyperpolarization to SP is mediated through endothelium-derived NO; 3) for ECs, K_{Ca} channels are primary mediators of hyperpolarization to both SP and CGRP; and 4) K_{ATP} channels are primary mediators of SMC hyperpolarization to CGRP but have a negligible role in hyperpolarization of either cell layer to SP.

4.1 | Sensory neurotransmitters hyperpolarize vascular smooth muscle and endothelial cells

Substance P hyperpolarized SMCs within intact vessels and ECs within endothelial tubes; however, endothelial disruption prevented SMC hyperpolarization (Figure 2). These findings are consistent with endothelial denudation preventing hyperpolarization of SMCs to SP in unpressurized porcine coronary artery segments.[32] Substance P exerts its actions in the vasculature through neurokinin receptors,[16] and our previous findings confirm that

neurokinin 1 receptors are expressed on ECs, but not SMCs, in mouse MAs.[23,33] The present data are also consistent with neurokinin receptor expression on human aortic ECs.[16] The magnitude of EC hyperpolarization in endothelial tubes (~ −15 mV from a resting V_m of ~ −45 mV) was less than observed in cultured porcine coronary artery ECs (ΔV_m ~ −25 mV from resting V_m of ~ −30 mV).[18] Such differences may reflect a difference between vascular beds, animal species, and/or cultured EC monolayers vs. native EC preparations. Differences between our experimental conditions also encompass endothelial tubes being studied at 32°C to maintain viability[25] as compared to SMCs in intact MAs studied at 37°C. Finding that the inhibition of NO synthase prevented SMC hyperpolarization to SP in intact MAs (Figure 4) indicates that endothelium-derived NO mediates SMC hyperpolarization rather than SP acting directly on SMCs to elicit hyperpolarization. Although SP released from sensory nerves does not act directly on SMCs, electrical field stimulation of MAs releases SP that acts upon the endothelium.[23] Furthermore, the neurokinin receptors on ECs can also be activated by circulating SP.[34] Because we have not been able to consistently evaluate V_m of ECs within the intact MA, the present data cannot address the contribution of myoendothelial coupling between respective cell layers of the vessel wall.

In contrast to SP effecting SMC hyperpolarization indirectly via the endothelium, we show here that CGRP acts directly on each cell layer of the MA. Thus, neither endothelium-derived NO nor myoendothelial coupling through gap junctions[26] are required for SMC hyperpolarization to CGRP. These data are consistent with hyperpolarization observed in SMCs from rabbit MAs[6,11] and illustrate the similarity in response of ECs to SMCs to this sensory neurotransmitter. The ability of CGRP to hyperpolarize SMCs and ECs individually is also consistent with expression of CGRP receptor components RAMP1 and CRLR on both cell types.[22,23] Nevertheless, our findings raise the question of why ECs hyperpolarize to CGRP if the endothelium is unnecessary for SMC hyperpolarization. Cell-cell coupling through gap junctions along and between ECs and SMCs may coordinate the electrical and vasomotor response along the vascular wall to ensure that CGRP elicits SMC hyperpolarization and vasodilation irrespective of whether it is presented abluminally from perivascular nerves or intraluminally via the circulation. Additionally, diffusion of endothelium-derived NO can relax SMCs by reducing sensitivity to $[Ca^{2+}]_i$ independent of affecting V_m.[35]

4.2 | K_{Ca} channels mediate hyperpolarization of SMCs and ECs to SP

K_{Ca} channels are integral mediators of endothelium-dependent dilation.[12,36,37] They are tetrameric proteins formed of four α and β subunits and are unique among K^+ channels in their ability couple intracellular Ca^{2+} signals to membrane hyperpolarization. Based on their single-channel conductance, K_{Ca} channels are classified into SK, IK, and BK channels. Whereas SMCs preferentially contain BK channels, SK and IK channels are primarily located in ECs.[36] While K_{Ca} channels are established mediators of EC hyperpolarization to SP,[18,19,38] the channels mediating SMC hyperpolarization to SP had remained undefined. The present data show that, while inhibiting K_{ATP} channels had little effect on EC or SMC hyperpolarization to SP (Figure 5), blocking K_{Ca} channels attenuated hyperpolarization in both cell types (Figure 6). While K_{Ca} inhibition nearly eliminated hyperpolarization to SP in ECs, the remaining endothelium-dependent hyperpolarization in SMCs (from

Figure 2C) suggests that the endothelium activates additional ion channels on SMCs that contribute to hyperpolarization to SP. Because L-NAME prevented hyperpolarization to SP in SMCs (Figure 4), we suggest that K_{Ca} channels (likely BK) on SMCs are activated by endothelium-derived NO to mediate hyperpolarization, which can occur through cGMP- and PKG-dependent phosphorylation.[37,39] Alternatively, direct activation of BK channels by NO has been reported in vascular smooth muscle independent of guanylate cyclase signaling.[40] Because SK and IK channels can regulate NO synthesis in ECs,[30,41] it is possible K_{Ca} inhibition in ECs prevents SMC hyperpolarization by limiting NO production. Furthermore, because SK and IK channels contribute to endothelium-dependent hyperpolarization,[30,42] myoendothelial coupling may also contribute to SMC hyperpolarization elicited by SP.

4.3 | K_{ATP} channels mediate CGRP-dependent hyperpolarization of SMCs but not ECs

K_{ATP} channels are integral to the regulation of vasomotor tone.[43,44] While K_{ATP} channels in SMCs of systemic arteries are well documented,[6,8–11,45] ECs of the aorta, mesenteric, and coronary arteries are also reported to express K_{ATP} channels.[46–49] K_{ATP} channels are formed from octameric protein complexes composed of four inward rectifying subunits (K_{IR} 6.1 and 6.2), each associated with a larger sulphonylurea receptor (SUR).[50,51] The K_{IR}6.1 and SUR2B isoform is the primary form of the channel in arterial myocytes.[50,52] In mice lacking the K_{IR}6.1 subunit, SMC hyperpolarization to CGRP was greatly reduced, validating the role of K_{ATP} channels as mediators of CGRP-dependent hyperpolarization in MAs utilizing a genetic approach (Figure 8). However, K_{ATP} channels contribute only a portion of SMC hyperpolarization to CGRP in MAs (~10 mV remaining following inhibition or knockout). Because K_{Ca} channels have also been implicated in SMC hyperpolarization to CGRP,[10,53,54] we tested their contribution to SMC hyperpolarization in the intact mouse MA. As shown in Figure 10, inhibition of K_{Ca} channels reduced hyperpolarization to CGRP by ~10 mV, which approximates the portion of this response not mediated by K_{ATP} channels.

Despite the expression of K_{ATP} channels in ECs,[46–49] hyperpolarization to CGRP remained unaltered in endothelial tubes treated with glibenclamide and in ECs from $K_{ATP}^{-/-}$ mice (Figures 7 and 8). Therefore, we tested whether activation of K_{ATP} channels could elicit hyperpolarization in ECs. Stimulation of K_{ATP} channels with pinacidil evoked hyperpolarization of ECs (Figure 9). These findings indicate that while K_{ATP} channels are present in ECs of the mouse MA, they are not functionally coupled to CGRP receptors. In contrast, the combination of charybdotoxin and apamin nearly abolished the response to CGRP in ECs, pointing to K_{Ca} channels as the primary mediator of hyperpolarization to this neurotransmitter (Figure 10). Therefore, while CGRP elicits hyperpolarization in both cell layers, its receptors are functionally coupled to different classes of K^+ channels in SMCs vs. ECs.

5 | SUMMARY AND CONCLUSION

We have investigated the effect of CGRP and SP on the membrane potential of ECs and SMCs of MAs from C57BL/6J male mice. While both sensory neurotransmitters elicit hyperpolarization of respective cell layers, their signaling pathways differ. Whereas SP hyperpolarizes the endothelium through K_{Ca} channels, these channels in smooth muscle are

activated via NO. CGRP also hyperpolarizes both cell layers but does so via KCa channels in endothelium and KATP channels in smooth muscle.

Impaired sensory nerve function contributes to impaired vasomotor function in aging, diabetes, and hypertension,[22,55,56] under-scoring the importance of perivascular sensory nerves in both health and disease. Recent findings have revealed a novel role for sensory neurotransmitter dysfunction in MAs during inflammatory bowel disease, whereby aberrant SP signaling impedes CGRP-mediated vasodilation.[23] Based on the integral role of membrane potential in regulating arterial diameter and organ blood flow, resolving the determinants of electrophysiological responses in smooth muscle and EC layers of the vessel wall identifies selective targets for treating vascular disease associated with sensory neurotransmitter dysfunction.

6 | PERSPECTIVES

In mouse MAs, vascular smooth muscle is hyperpolarized by substance P via endothelium-derived NO activation of K_{Ca} channels and by CGRP via activation of K_{ATP} channels independent of the endothelium. The endothelium is hyperpolarized by both SP and CGRP through activation of K_{Ca} channels. New insight into differential signaling underlying hyperpolarization of the arterial wall advances the ability to selectively target conditions of sensory neurotransmitter dysfunction.

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Abbreviations:

ACh	acetylcholine
BK	large conductance K_{Ca} channel
CGRP	calcitonin gene-related peptide
DMSO	dimethylsulfoxide
EC	endothelial cell
ID	inner diameter
IK	intermediate conductance K_{Ca} channel
K_{ATP}	ATP-sensitive potassium channels
K_{Ca}	Ca^{2+}-activated potassium channels
L-NAME	N^G-nitro-L-arginine methyl ester

MA	mesenteric artery
NE	norepinephrine
NO	nitric oxide
PGP	protein gene product
PSS	physiological saline solution
SK	small conductance K_{Ca} channel
SMC	smooth muscle cell
SP	substance P
SUR	sulphonylurea receptor
VGCC	voltage-gated Ca^{2+} channel
V_m	membrane potential

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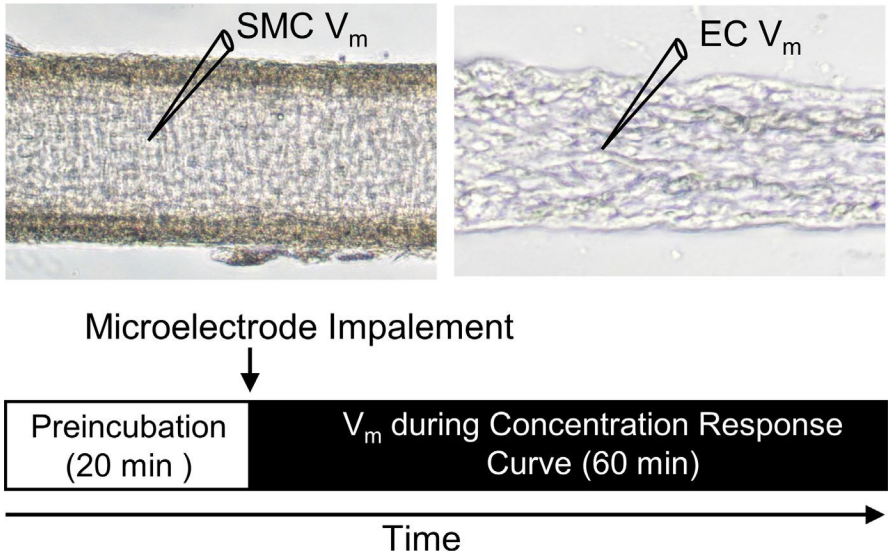

FIGURE 1.

Experimental design. Diagram illustrating experimental setup illustrating sharp microelectrode impalement for measurement of V_m in a SMC of an intact artery (left) or an EC in an endothelial tube (right). Following 20 min preincubation with a pharmacological agent or vehicle control, V_m was recorded during cumulative addition of SP or CGRP. Responses to each neurotransmitter and pharmacological agent were tested in separate preparations

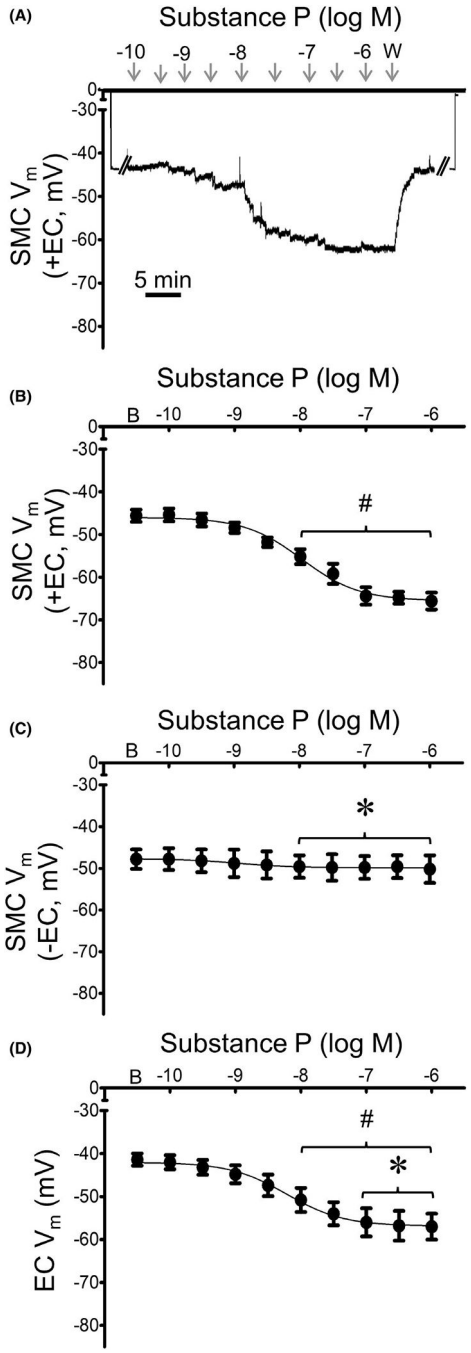

FIGURE 2.

Mesenteric artery SMCs and ECs are hyperpolarized by SP. (A) Representative recording of membrane potential (V_m) illustrates concentration-dependent hyperpolarization of a SMC by SP in an intact MA (+EC). From zero baseline, note abrupt change in V_m upon entry and exit from cell. (B) Summary data for V_m of SMCs in intact MAs (+EC) studied at 37°C to increasing [SP] (10^{-10}–10^{-6} M). (C) Summary data for V_m of SMCs of endothelium-disrupted MAs (−EC) studied at 37°C to SP as in B. (D) Summary data for V_m of ECs within endothelial tubes studied at 32°C to SP as in B. Values are means ± SEM for $n = 5$

per group. B = baseline V_m. W = washout in control PSS. $^{\#}p < 0.05$ vs. baseline (prior to application of SP) V_m. $^{*}p < 0.05$ vs. SMC (+EC)

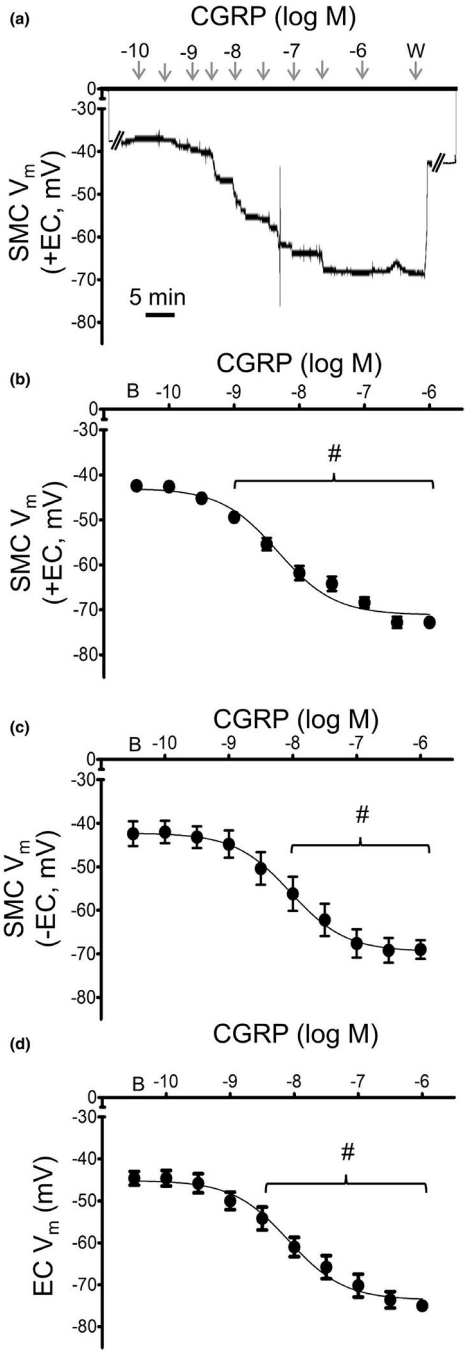

FIGURE 3.

Mesenteric artery SMCs and ECs are hyperpolarized by CGRP. (A) Representative recording of V_m illustrates hyperpolarization of a SMC by CGRP in an intact MA (+EC). (B) Summary data for V_m in SMCs of intact MAs (+EC) studied at 37°C to increasing concentrations of CGRP (10^{-10}–10^{-6} M). C) Summary data for V_m of SMCs of endothelium-disrupted MAs (−EC) studied at 37°C. (D) Summary data for V_m of ECs within endothelial tubes studied at 32°C. Values are means ± SEM for $n = 5$ per group. B

= baseline V_m. W = washout in control PSS. $^{\#}p < 0.05$ vs. baseline (prior to application of CGRP) V_m

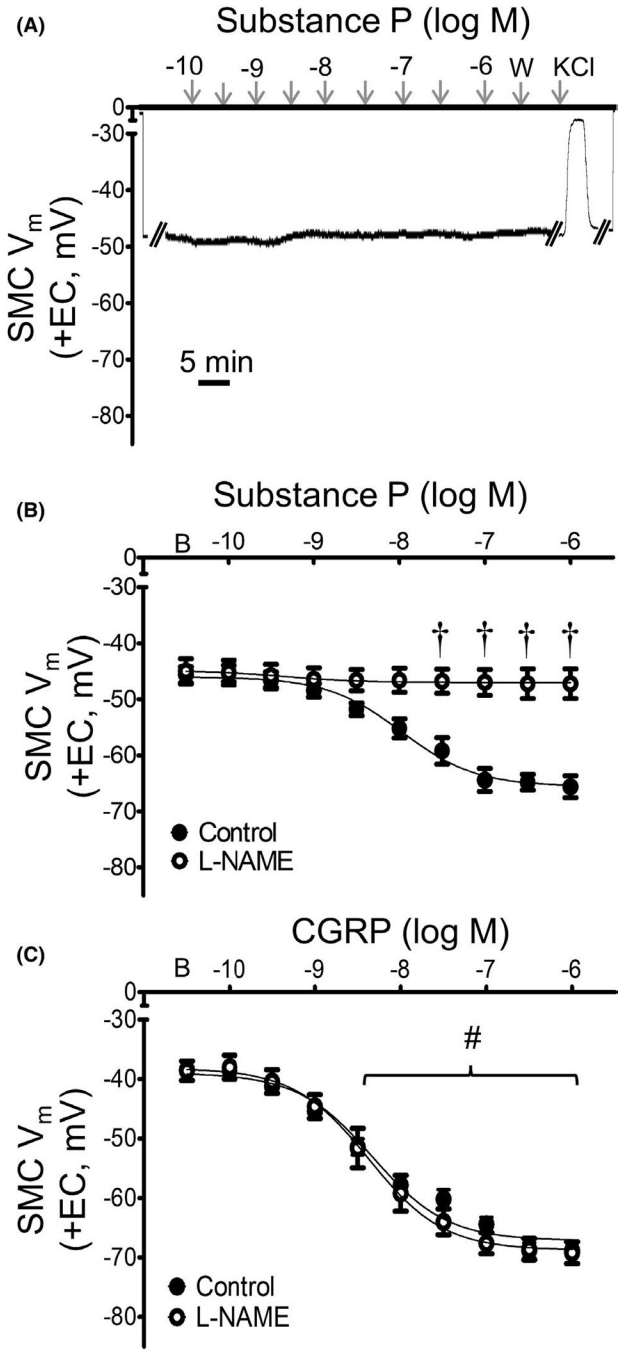

FIGURE 4.

SP hyperpolarizes SMCs through NO signaling. A) Representative recording of V_m demonstrates lack of response to SP of a SMC in an intact MA (+EC) in the presence of L-NAME (10^{-4} M); response to acute administration of KCl (5 min) confirms preparation viability. (B) Summary data for V_m responses to SP in SMCs from intact MAs studied at 37°C under control conditions (from Figure 1B) or in the presence of L-NAME. (C) Summary data for V_m responses to CGRP in SMCs from intact MAs in the absence (from Figure 2B) or presence of L-NAME (10^{-4} M). Values are means ± SEM for $n = 5$ per group.

B = baseline V_m. W = washout in control PSS. $^{\#}p < 0.05$ vs. baseline (prior to application of SP or CGRP) V_m during L-NAME. $^{\dagger}p < 0.05$ vs. control

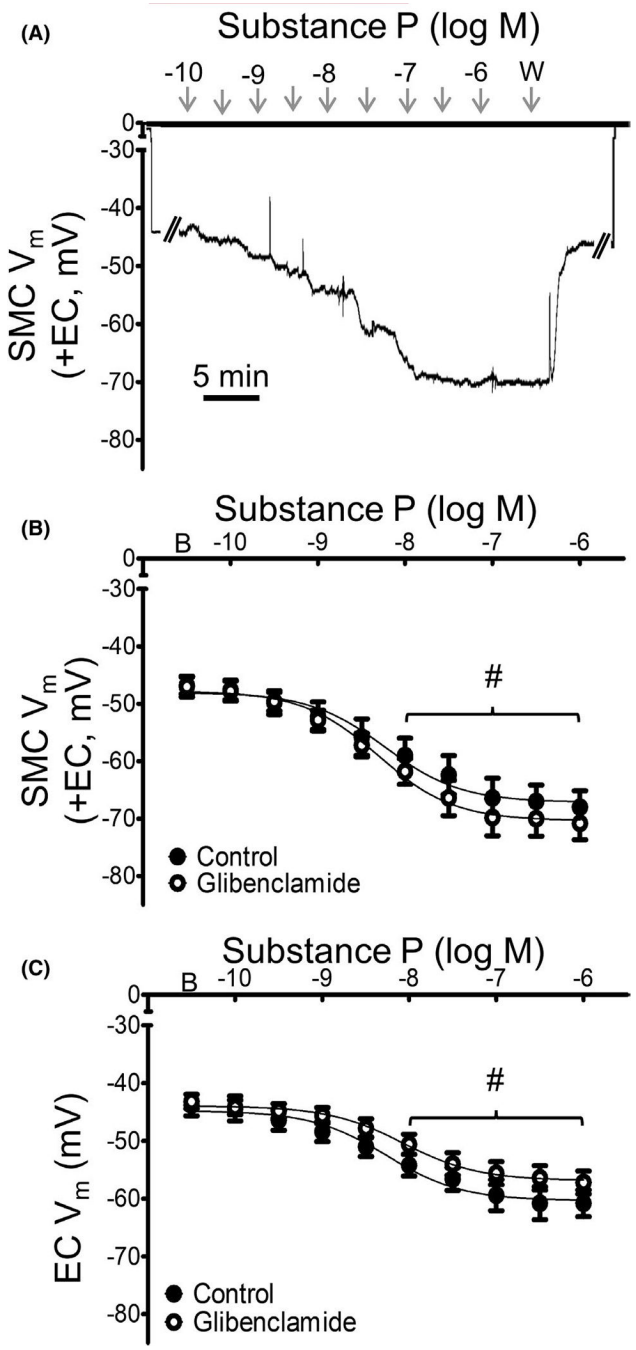

FIGURE 5.

Hyperpolarization to SP does not occur through K_{ATP} channels. (A) Representative recording of V_m in an intact MA (+EC) illustrates maintained SMC hyperpolarization to SP in the presence of glibenclamide (10^{-6} M). (B) Summary data for V_m responses to SP in SMCs from intact MAs (+EC) studied at 37°C under control (vehicle = 0.1% DMSO) conditions or during exposure to the K_{ATP} channel inhibitor glibenclamide. (C) Summary data for V_m responses to SP for ECs in endothelial tubes studied at 32°C in the absence or presence of glibenclamide. Values are means ± SEM for $n = 5$ per group. B = baseline

V_m. W = washout in control PSS. $^{\#}p < 0.05$ vs. baseline (prior to application of SP) V_m in preparations treated with glibenclamide

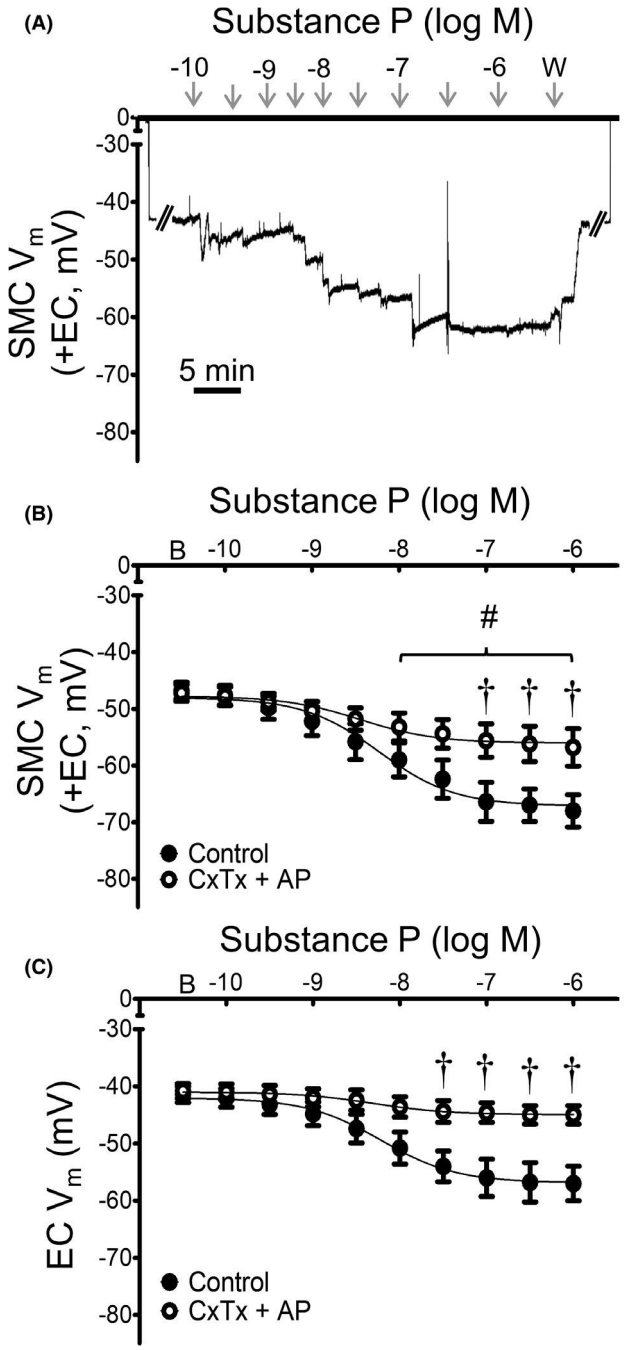

FIGURE 6.

Hyperpolarization to SP occurs through K_{Ca} channels. (A) Representative recording of V_m in an intact MA (+EC) demonstrates attenuation of SMC hyperpolarization to SP in the presence of K_{Ca} channel inhibitors charybdotoxin (ChTx; 10^{-7} M) + apamin (AP; 3×10^{-7} M). B) Summary data for V_m responses to SP in SMCs from intact MAs (+EC) studied at 37°C under control (from Figure 1) conditions and during K_{Ca} channel inhibition. (C) Summary data for V_m responses to SP for ECs within endothelial tubes studied at 32°C during the absence or presence of K_{Ca} channel inhibitors. Values are means ± SEM for n =

5 per group. B = baseline V_m. W = washout in control PSS. $^{\#}p < 0.05$ vs. baseline (prior to application of SP) V_m in vessels treated with ChTx + AP. $^{\dagger}p < 0.05$ vs. control

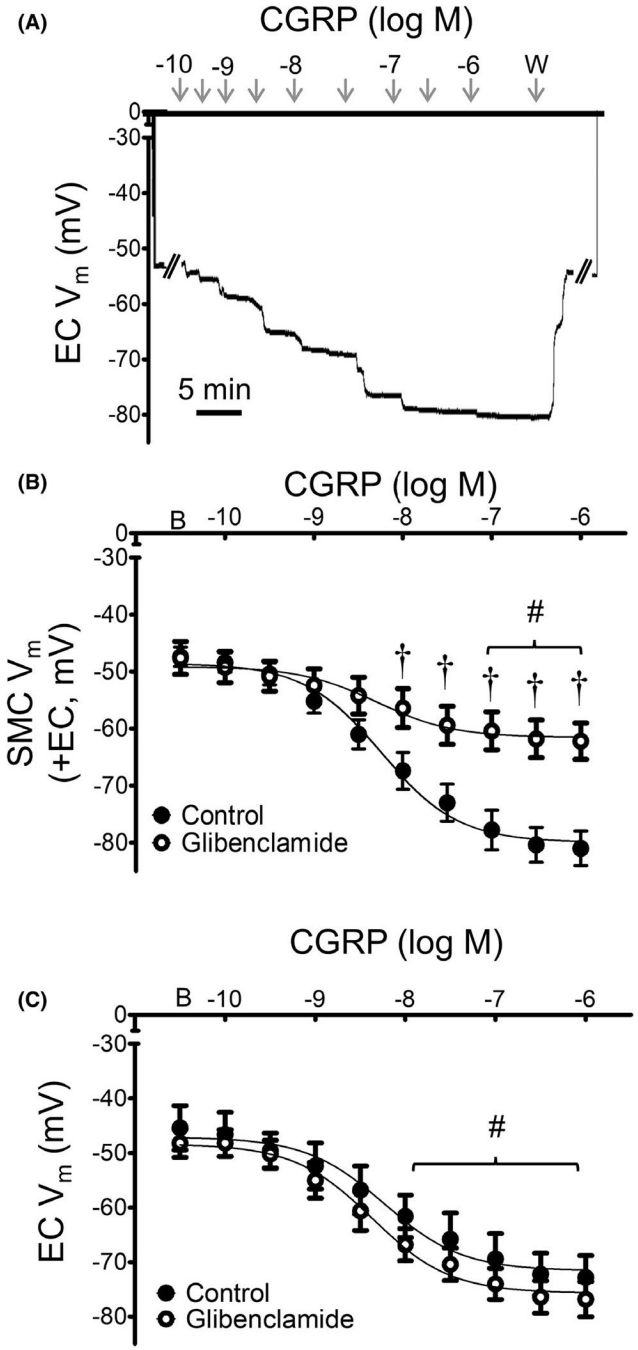

FIGURE 7.

Hyperpolarization to CGRP in SMCs, but not ECs, requires K_{ATP} channels. (A) Representative recording of V_m from a MA endothelial tube in the presence of glibenclamide (10^{-6} M) illustrates normal EC response to CGRP (10^{-10}–10^{-6} M). (B) Summary data for SMCs of intact MAs (+EC) studied at 37°C to CGRP in the absence or presence of glibenclamide or its vehicle. (C) Summary data for ECs within endothelial tubes studied at 32°C in response to CGRP in the absence or presence glibenclamide. Values are means ± SEM for $n = 5$ per group. B = baseline V_m. W = washout in control PSS. $^{\#}p < 0.05$

vs. baseline (prior to application of CGRP) V_m in vessels treated with glibenclamide. †$p < 0.05$ vs. control

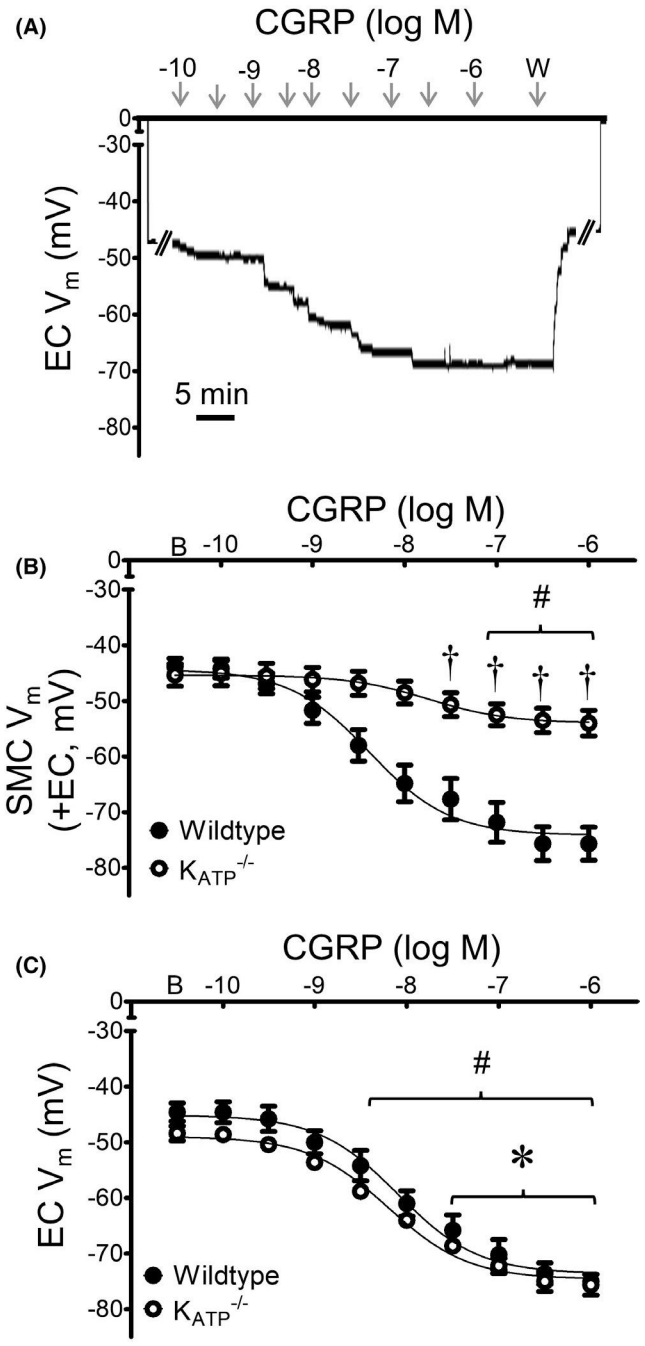

FIGURE 8.

Genetic deletion of K_{ATP} channels inhibits hyperpolarization to CGRP. (A) Representative recording of V_m from an EC within a MA endothelial tube of a mouse lacking K_{ATP} channels illustrates normal (wildtype) response to CGRP (10^{-10}–10^{-6} M). (B) Summary data for responses to CGRP in SMCs of intact MAs (+EC) studied at 37°C from $K_{ATP}^{-/-}$ vs. wildtype mice. (C) Summary data for responses to CGRP in endothelial tubes studied at 32°C from $K_{ATP}^{-/-}$ vs. wildtype mice. Values are means ± SEM for $n = 5$ per group. B = baseline V_m. W = washout in control PSS. #$p < 0.05$ vs. baseline (prior to application of

CGRP) V_m in $K_{ATP}^{-/-}$ mice. *$p < 0.05$ EC vs. SMC V_m in $K_{ATP}^{-/-}$ mice. $^{\dagger}p < 0.05$ vs. wildtype

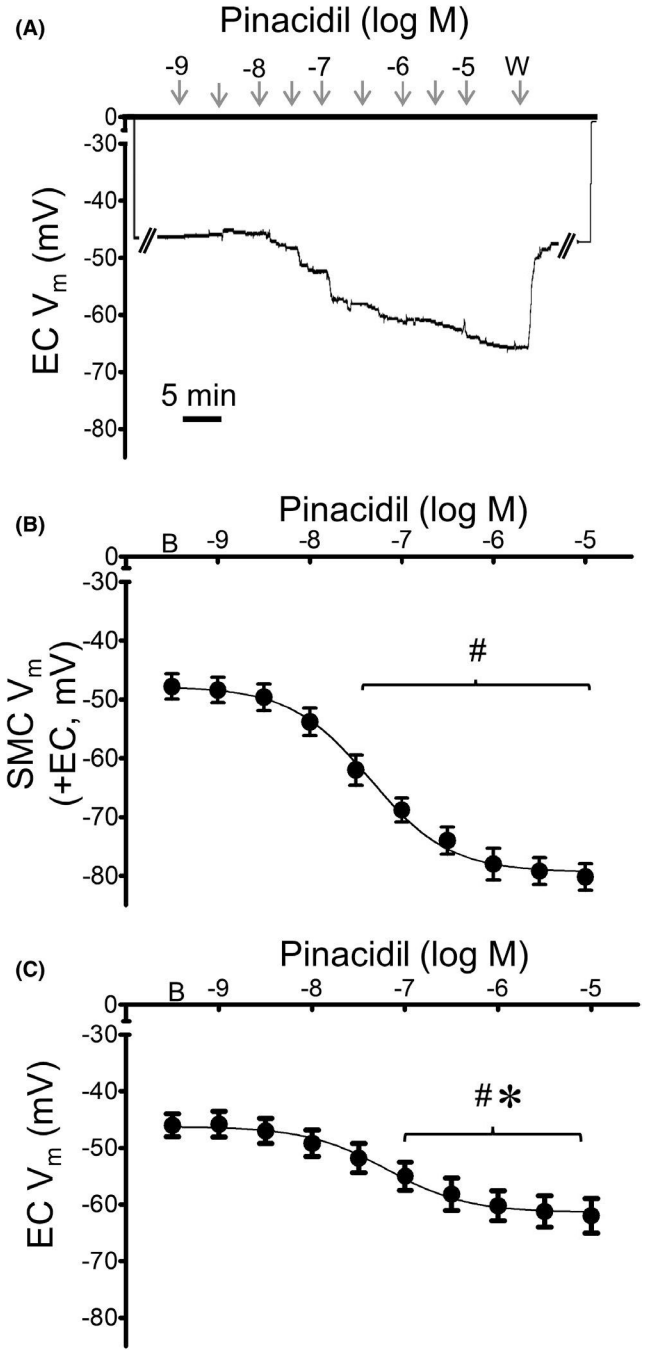

FIGURE 9.

K_{ATP} channel activation hyperpolarizes SMCs more than ECs. (A) Representative recording from an EC within a MA endothelial tube illustrates concentration-dependent hyperpolarization to pinacidil (10^{-9}–10^{-5} M). (B) Summary data from SMCs of intact MAs (+EC) studied at 37°C in response to increasing concentrations of pinacidil. (C) Summary data for ECs within endothelial tubes studied at 32°C in response to increasing concentrations of pinacidil. Values are means ± SEM for $n = 5$ per group. B = baseline V_m.

W = washout in control PSS. # $p < 0.05$ vs. baseline (prior to application of pinacidil) V_m. *$p < 0.05$ EC vs. SMC V_m

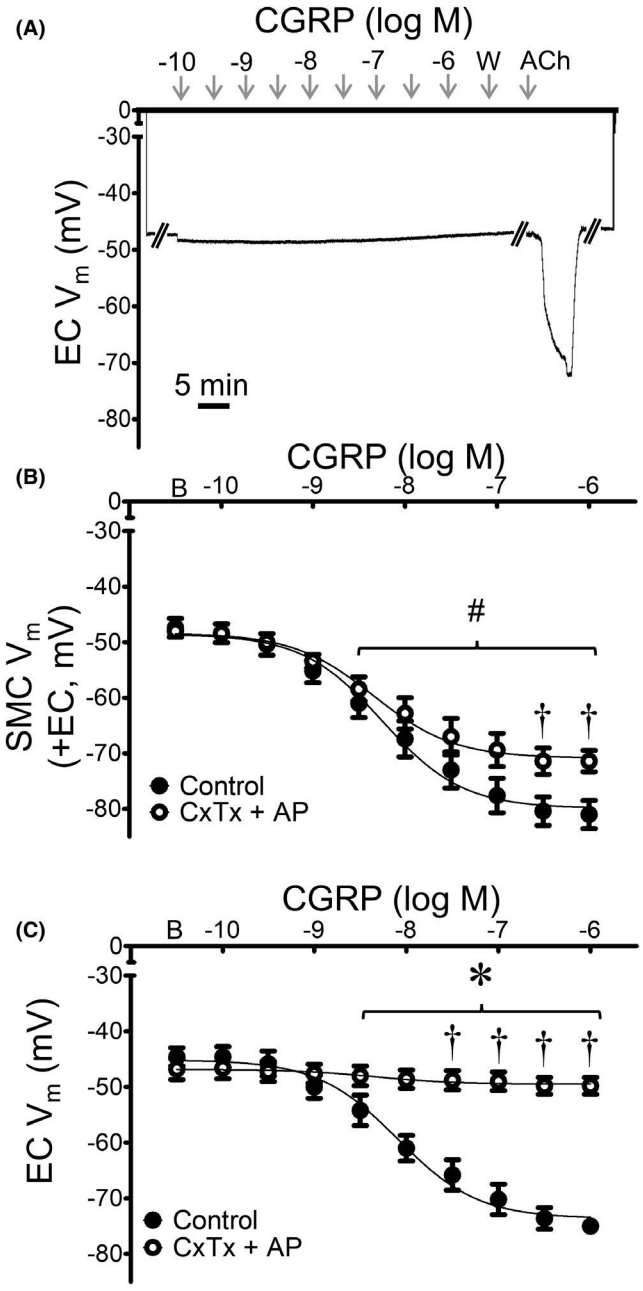

FIGURE 10.

K_{Ca} channels contribute to hyperpolarization of ECs more than SMCs in response to CGRP. (A) Representative recording of V_m from an EC within a MA endothelial tube demonstrates lack of hyperpolarization to CGRP in the presence of charybdotoxin (ChTx; 10^{-7} M) + apamin (AP; 3×10^{-7} M); note hyperpolarization to ACh (5 min) following washout, confirming EC viability. (B) Summary data for V_m responses to CGRP in SMCs from intact MAs (+EC) studied at 37°C under control conditions (from Figure 2) or during K_{Ca} channel inhibition. (C) Summary data for V_m responses to CGRP in ECs within endothelial tubes studied at 32°C in the presence or absence of K_{Ca} channel inhibitors. Values are means ± SEM for $n = 5$ per group. B = baseline V_m. W = washout in control PSS. $^{\#}p < 0.05$ vs.

baseline (prior to application of CGRP) V_m in vessels treated with ChTx + AP. $^{\dagger}p < 0.05$ vs. control. $^{*}p < 0.05$ EC vs. SMC V_m

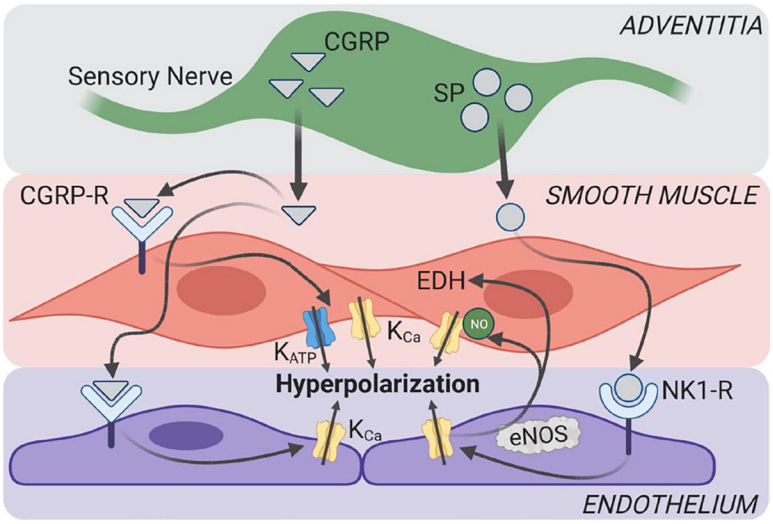

FIGURE 11.

Summary of findings. Schematic depicts perivascular neurotransmitter signaling in mouse mesenteric arteries. NO signaling mediates endothelium-dependent hyperpolarization (EDH) of SMCs to SP but not CGRP. K_{Ca} channels mediate hyperpolarization to SP in both SMCs and ECs. K_{Ca} channels are required for EC hyperpolarization to CGRP, while K_{ATP} channels mediate most SMC hyperpolarization to CGRP. CGRP-R, CGRP receptor; eNOS, endothelial nitric oxide synthase; NK1-R, neurokinin 1 receptor. Figure created using BioRender

TABLE 1

Membrane potential of ECs and SMCs at rest and in response to SP and CGRP

	Substance P				CGRP			
	Rest	**Max**	**ΔV_m**	**EC_{50}**	**Rest**	**Max**	**ΔV_m**	**EC_{50}**
SMC (+EC)	−46 ± 2	−66 ± 2	−20 ± 2	12.6	−42 ± 1	−73 ± 2	−30 ± 1 †	10.0
SMC (−EC)	−48 ± 2	−50 ± 2 *	−2 ± 1 *	NA	−43 ± 3	−69 ± 2	−27 ± 2 †	10.3
EC	−42 ± 1	−56 ± 2 *	−15 ± 3	7.4	−44 ± 2	−75 ± 1	−31 ± 1 †	7.8

Note: Summary data for resting V_m (Rest), V_m at maximum response (Max), ΔV_m (Max-Rest) and EC_{50} values ($\times 10^{-9}$ M) for hyperpolarization (mV) to SP and CGRP in ECs of endothelial tubes, SMCs of MAs with disrupted endothelium (−EC) and SMCs of endothelium-intact (+EC) MAs. Values are means ± SEM for $n = 5$ vessels per group.

* $p < 0.05$ vs. SMC (+EC).

† $p < 0.05$ vs. maximal response to SP.