Tuning Electrical Conduction Along Endothelial Tubes of Resistance Arteries Through Ca\(^{2+}\)-Activated K\(^{+}\) Channels

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**Rationale:** Electric conduction through gap junction channels between endothelial cells of resistance vessels is integral to blood flow control. Small and intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (SKCa/IKCa) initiate electric signals in endothelial cells, but it is unknown whether SKCa/IKCa activation alters signal transmission along the endothelium.

**Objective:** We tested the hypothesis that SKCa/IKCa activity regulates electric conduction along the endothelium of resistance vessels.

**Methods and Results:** Freshly isolated endothelial cell tubes (60 \(\mu\)m wide; 1–3 mm long; cell length, \(\approx 35 \mu m\)) from mouse skeletal muscle feed (superior epigastric) arteries were studied, using dual intracellular microelectrodes. Current was injected (\(\pm 0.1–3\) nA) at site 1 while recording membrane potential (\(V_m\)) at site 2 (separation distance=50–2000 \(\mu\)m). SKCa/IKCa activation (NS309, 1 \(\mu\)mol/L) reduced the change in \(V_m\) along endothelial cell tubes by \(\geq 50\%\) and shortened the electric length constant (\(\lambda\)) from 1380 to 850 \(\mu\)m (\(P<0.05\)) while intercellular dye transfer (propidium iodide) was maintained. Activating SKCa/IKCa with acetylcholine or SKA-31 also reduced electric conduction. These effects of SKCa/IKCa activation persisted when hyperpolarization (>30 mV) was prevented with 60 mmol/L [K\(^{+}\)]. Conversely, blocking SKCa/IKCa (apamin+charybdotoxin) depolarized cells by \(\approx 10\) mV and enhanced electric conduction (ie, changes in \(V_m\)) by \(\approx 30\%\) (\(P<0.05\)).

**Conclusions:** These findings illustrate a novel role for SKCa/IKCa activity in tuning electric conduction along the endothelium of resistance vessels by governing signal dissipation through changes in membrane resistance. Voltage-insensitive ion channels can thereby tune intercellular electric signaling independent from gap junction channels. (Circ Res. 2012;110:00-00.)

Key Words: conducted vasodilation • hyperpolarization • gap junctions • potassium channels • ion channels • membrane potential • conduction • vascular endothelium

Electric conduction through gap junction channels (GJCs) coordinates vaso motor responses in vascular resistance networks.\(^1\)–\(^3\) Through axial orientation and robust expression of GJC, endothelial cells (ECs) serve as the primary pathway for cell-to-cell conduction along the vessel wall,\(^4\)–\(^6\) with electric signals transmitted to smooth muscle cells (SMCs) via myoendothelial GJCs.\(^3\)–\(^7\)–\(^9\) Once initiated, hyperpolarization (and vasodilation) can travel along the resistance vasculature for millimeters.\(^10\)–\(^13\) As shown in exercising skeletal muscle, vasodilation originating downstream within the microcirculation can ascend the resistance network via conduction along the endothelium to encompass feed arteries upstream and thereby increase total blood flowing into the active muscle.\(^14\)

In accord with the cable properties of electric conduction, the distance that signals travel along the endothelium is determined by the length constant (\(\lambda = r_m/r_a\))\(^{1/2}\), where \(r_m\) = membrane resistance and \(r_a\) = axial resistance to current flow.\(^15\)\(^,\)\(^16\) With negligible resistance of cytoplasm, intercellular \(r_a\) is determined primarily by GJCs.\(^8\)\(^,\)\(^14\)\(^,\)\(^16\) Previous studies have focused on alterations of conducted vasodilation through manipulating the functional integrity of GJC\(^5\)\(^,\)\(^14\)\(^,\)\(^17\) or the expression profile of their connexin subunits.\(^18\)–\(^21\) Alternatively, changes in \(r_m\) may also provide a mechanism for governing electric conduction independent of manipulating GJCs. In turn, changes in \(r_m\) should reflect the activation of ion channels in the plasma membrane,\(^15\)\(^,\)\(^16\) which otherwise insulates the cell interior from the extracellular milieu. Remarkably, the effect of governing \(r_m\) through ion channel activation has not been studied in light of electric conduction along the endothelium of resistance vessels.

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The small (KCa 2.3; KCNN3)-and intermediate (KCa 3.1; KCNN4)-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (SKCa/IKCa) are abundantly expressed in EC membranes.\textsuperscript{22–24} On activation of SKCa/IKCa the efflux of K\textsuperscript{+} promotes hyperpolarization.\textsuperscript{22,25} Although this response may promote vasodilation and tissue perfusion,\textsuperscript{24} a reduction in rm along the endothelium could dissipate electric signals and thereby compromise electric conduction and impair ascending vasodilation. Given the prominent role of SKCa/IKCa in the initiation of EC hyperpolarization,\textsuperscript{9,24} we questioned whether activation of these ion channels affects electric conduction along the endothelium.

Understanding how the activation of SKCa/IKCa may govern electric conduction along the endothelium of intact vessels is limited by myoendothelial coupling to SMCs, perivascular nerve activity, and circulating vasoactive agents along with the shear stress exerted by blood flow. Further, the initiation of an electric signal to trigger conduction has typically consisted of an agonist (eg, acetylcholine [ACh])\textsuperscript{4,10,26} or electric field stimulation\textsuperscript{27,28} via micropipettes. However, such “local” stimuli can activate multiple cells simultaneously, with uncertainty in the precise origin and intensity of the actual stimulus. To avoid such confounding influences, we have freshly isolated EC tubes from feed arteries of mouse abdominal skeletal muscle.\textsuperscript{29,30} Intact EC tubes from these resistance vessels can exceed 3 mm in length, enabling the efficacy of electric conduction along the endothelium to be evaluated. Using sharp intracellular microelectrodes, current microinjection into a single EC alters membrane potential (Vm) independent of agonists or ion channel activation. By simultaneously recording Vm at defined distances from the site of current injection, we tested the hypothesis that SKCa/IKCa activity can “tune” electric conduction along the endothelium.

Findings reported in the present study are the first to show that activating SKCa/IKCa with either pharmacological agents (NS309, SKA-31) or a physiological agonist (ACh) impairs electric conduction along the endothelium of a resistance artery. Concomitantly, intercellular coupling through GJCs remains intact as confirmed by robust dye transfer between ECs. We further demonstrate that blocking constitutively open SKCa/IKCa enhances electric conduction. Thus, altering

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**Non-standard Abbreviations and Acronyms**

- Ap: apamin
- ACh: acetylcholine
- BKCa: large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels
- CA: conduction amplitude
- ChTX: charybdotoxin
- EC: endothelial cell
- GJCs: gap junction channels
- SKCa/IKCa: small- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels
- K\textsubscript{ATP}: ATP-regulated K\textsuperscript{+} channels
- K\textsubscript{IR}: inward-rectifying K\textsuperscript{+} channels
- K\textsubscript{v}: voltage-gated K\textsuperscript{+} channels
- NO: nitric oxide
- NS1619: 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one
- NS309: 6,7-dichloro-1H-indole-2,3-dione 3-oxime
- SMC: smooth muscle cell
- SKA-31: naptho[1,2-d]thiazol-2-ylamine
- V\textsubscript{m}: membrane potential

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The small (KCa 2.3; KCNN3)-and intermediate (KCa 3.1; KCNN4)-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (SKCa/IKCa) are abundantly expressed in EC membranes.\textsuperscript{22–24} On activation of SKCa/IKCa the efflux of K\textsuperscript{+} promotes hyperpolarization.\textsuperscript{22,25} Although this response may promote vasodilation and tissue perfusion,\textsuperscript{24} a reduction in rm along the endothelium could dissipate electric signals and thereby compromise electric conduction and impair ascending vasodilation. Given the prominent role of SKCa/IKCa in the
Tuning Endothelial Conduction Via $K_{Ca}$

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Tissue Preparation and Intracellular Recording**

Procedures were approved by the Institutional Animal Care and Use Committee and performed in accord with the National Research Council’s Guide for the Care and Use of Laboratory Animals (8th edition, 2011). Male C57BL/6 mice (bred at the University of Missouri; age, 3–6 months; n = 45) were anesthetized (pentobarbital; 60 mg/kg intraperitoneal injection). Abdominal muscles were resected and a midline incision was made from the umbilicus to just caudal to the renal hilus. After opening the peritoneum, the stomach was retracted and the mesentery was isolated. The greater omentum was retracted and the intestines were secured to the abdominal wall with sutures. The aorta and inferior vena cava were isolated and tied to prevent blood flow from the heart to the rest of the body. The abdominal musculature was resected and the peritoneum was closed with sutures. The aorta, inferior vena cava, and mesenteries were removed, leaving the midline EC tube with intact ECs. The midline EC tube was then placed into a 35 mm culture dish containing 10 mL of superfusion solution and Vm was confirmed to be the same between microelectrodes.

Entire EC tubes were exposed by adding agents to the superfusion solution and Vm was confirmed to be the same between microelectrodes.

Figure 1 illustrates the experimental approach and summarizes the analyses used. To study electric conduction over distance (Figures 2 and 3), implantation at site 1 was maintained while the microelectrode for recording Vm at site 2 was repositioned at the next distance (order randomized across experiments). As standard procedure for evaluating interventions, separation distance was maintained at 500 μm (Figures 4, 5, 6, 7, and 8) corresponding to 15 ECs placed end-to-end. Current was microinjected (±0.1–3 nA; 2-second pulse) at site 1 while recording Vm at site 2 under control conditions, during activation of $K_{Ca}$ with NS309, SKA-31, or ACh and during inhibition of $K_{Ca}$ with apamin (Ap+ChTX). Entire EC tubes were exposed by adding agents to the superfusion solution and Vm was confirmed to be the same between microelectrodes to ensure that the entire EC tube was isopotential before current injections.

**Data Analyses**

One EC tube was studied per mouse. Analyses included resting Vm (mV) under control conditions; change in Vm ($\Delta$ mV) = peak response Vm minus preceding baseline Vm; conductance amplitude (CA, mV/nA) = Vm at site 2 current injected at site 1; fraction of control

$r_{in}$ via opening and closing plasma membrane ion channels effectively tunes the conduction of electric signals along the endothelium.

#### Figure 2. Effect of distance and $SK_{Ca}/IK_{Ca}$ activation on electric conduction.

At defined separation distances between intracellular microelectrodes, continuous (paired) recordings at site 2 during current microinjection at site 1 were obtained under control conditions and during NS309 treatment to activate $SK_{Ca}/IK_{Ca}$. After each set of paired recordings, washout of NS309 (10–15 minutes) restored control Vm before recording at the next distance. A, Representative Vm recording at site 2 during ±0.1 to 3 nA microinjected at site 1 (distance=500 μm) before and during NS309. From control Vm of −25 mV, NS309 hyperpolarized ECs to −60 mV and decreased Vm responses. B, As in A, with separation distance = 1500 μm. Note reduction in control Vm responses compared with A, followed by loss of Vm responses during NS309. C, Summary data illustrating the effect of microelectrode separation distance on the change in Vm at site 2 (ΔVm2) under control conditions with 500 μm (black line) or 1500 μm (gray line) separation. With reduced slope at greater distance, ΔVm2 responses remained linear through the full range of current injected ($R^2$=1). With −1 nA current, absolute ΔVm2 decreased from 7.7±0.6 mV at 500 μm to 3.8±0.3 mV at 1500 μm. D, With separation maintained at 500 μm for the same EC tubes in C, activation of $SK_{Ca}/IK_{Ca}$ with NS309 (1 μmol/L) reduced CA to 3.1±0.3 mV. Summary data in C and D are mean±SEM; n=11. Note: C and D include (with permission: British Journal of Pharmacology, 2011) control data from 8 experiments presented in Figure 2B and 2C of Behringer et al.

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CA = CA during treatment/preceding control CA; conduction efficiency = CA at each distance/CA at 50 μm separation; length constant (λ) = distance over which the electric signal decayed to 37% (1/e) of the “local” value. Data were analyzed using analysis of variance with Bonferroni post hoc comparisons, regression analyses, and paired t tests. Differences were accepted as statistically significant, with \( P < 0.05 \).

Summary data are presented as mean±SEM. Values for \( n \) refer to the number of EC tubes studied under respective conditions.

Results

Effects of SK\(_{Ca}/IK_{Ca}\) Activation on Electric Conduction Over Distance

Resting \( V_m \) was \(-26±2 \text{ mV}\) at sites 1 and 2 independent of separation distance (\( n=11 \)). As shown in Figure 2, \( ΔV_m \) at site 2 increased in direct proportion to current injected irrespective of polarity. The amplitude of \( ΔV_{m2} \) for each current decreased when separation distance between microelectrodes was increased from 500 to 1500 μm (Figure 2A through 2C), yet the I-V relationship remained linear (Figure 2C). At 500-μm separation, the slope of the I-V relationship through ±0.1 to 3 nA was 7.5±0.5 mV/nA. When calculated for −1 nA, CA (500 μm) was not different (7.7±0.5 mV/nA; \( n=11 \)), substantiating −1 nA as a standard reference.

The \( ΔV_m \) (−37 mV) in response to 1 μmol/L NS309 corresponds to the peak hyperpolarization obtained with ACh (3 μmol/L). At each distance, opening SK\(_{Ca}/IK_{Ca}\) with 1 μmol/L NS309 reduced CA (Figure 2A and 2B), yet the I-V relationship remained linear (Figure 2D). Thus, NS309 decreased CA at 500 μm to nearly the same extent as did increasing distance to 1500 μm under control conditions (Figure 2C). After continuous paired recordings (control, NS309) at each distance, washout of NS309 (10–15 minutes) restored control \( V_m \) and CA (eg, CA at 500 μm: control, 7.7±0.4 mV/nA; NS309 washout, 7.9±0.4 mV/nA, \( n=16 \)), confirming the reversibility of SK\(_{Ca}/IK_{Ca}\) activation. The next separation distance was evaluated in the same manner.

During current injections, NS309 (1 μmol/L) reduced \( ΔV_{m2} \) at the “local” site by half and decreased \( λ \) by ∼40% (control: 1380±60 μm; NS309: 850±60 μm). At each distance, opening SK\(_{Ca}/IK_{Ca}\) with 1 μmol/L NS309 decreased CA (Figure 2A and 2B), yet the I-V relationship remained linear (Figure 2D). Thus, NS309 decreased CA at 500 μm to nearly the same extent as did increasing distance to 1500 μm under control conditions (Figure 2C). After continuous paired recordings (control, NS309) at each distance, washout of NS309 (10–15 minutes) restored control \( V_m \) and CA (eg, CA at 500 μm: control, 7.7±0.4 mV/nA; NS309 washout, 7.9±0.4 mV/nA, \( n=16 \)), confirming the reversibility of SK\(_{Ca}/IK_{Ca}\) activation. The next separation distance was evaluated in the same manner.

For the following experiments, current was injected at site 1 while \( V_m \) was recorded at site 2 with 500-μm separation maintained between microelectrodes. The stability of impalements enabled multiple treatments to be evaluated during continuous (paired) recordings. Responses were evaluated through the full range of current microinjection, with data presented for −1 nA.

Progressive Inhibition of Electric Conduction on Graded Activation of SK\(_{Ca}/IK_{Ca}\)

A question central to these experiments was: Can electric conduction be “tuned” according to the level of SK\(_{Ca}/IK_{Ca}\) activation? Current was injected at site 1 and \( V_m \) recorded at site 2 (500 μm) before and during increments in SK\(_{Ca}/IK_{Ca}\) activation with recovery between each treatment. Hyperpolarization began at 100 nmol/L NS309 and increased with [NS309] (Figure 4A; \( P<0.05 \)); maximal response at
10 μmol/L corresponded to $V_m = -81 \pm 1$ mV. Impairment of CA ($P < 0.05$) began at 300 nmol/L NS309 and CA decreased as [NS309] increased with conduction abolished at 10 μmol/L (Figure 4B and 4C). At 1 μmol/L, NS309 reduced CA by half (Figure 4B and 4C). In separate experiments, using SKA-31 to activate SK$_{Ca}$/IK$_{Ca}$ produced effects similar to NS309 though attenuated potency and efficacy (compare Figure 4D through 4F with Figure 4A through 4C). Nevertheless, for both pharmacological agents, a “threshold” $V_m$ associated with significant reduction in CA occurred at $-40$ mV (Figure 4A and 4D). These findings indicate that the efficacy of electric conduction along the endothelium can be tuned according to the level of SK$_{Ca}$/IK$_{Ca}$ activation.

**Maintenance of Dye Transfer During Inhibition of Conduction**

Impairment of electric conduction during SK$_{Ca}$/IK$_{Ca}$ activation is interpreted to reflect greater current dissipation along the endothelium. Thus, an essential question is: Does intercellular coupling persist when electric conduction is inhibited? We used dye transfer as a functional qualitative index of GJC patency between neighboring ECs.$^{4,7,10,27,30}$ An EC tube was exposed to the NS309 concentration that inhibited conduction (10 μmol/L) beginning 5 minutes before and throughout recordings. With 0.1% propidium iodide dye (MW=668.4 Da) in the microelectrode, fluorescent images were acquired after 30 minutes of continuous recording. Under control conditions (Figure 5A) and during hyperpolarization (to $-81 \pm 4$ mV) with inhibition of electric conduction (CA: 0.3±0.1 mV/nA), dye microinjected into one EC readily spread to neighboring ECs (Figure 5B; $n=3$). Thus, dye transfer during maximal activation of SK$_{Ca}$/IK$_{Ca}$ with NS309 was qualitatively similar to control, confirming that intercellular coupling through GJCs remained patent during absence of electric conduction. For reference, in EC tubes exposed to carbenoxolone (100 μmol/L) or β-glycyrrhetinic acid (40 μmol/L) to block GJCs, dye transfer was inhibited reversibly along with electric conduction.$^{30}$

**Inhibition of Conduction Independent of Hyperpolarization**

The activation of SK$_{Ca}$/IK$_{Ca}$ consistently hyperpolarized ECs, raising the question: Does loss of electric conduction during SK$_{Ca}$/IK$_{Ca}$ activation depend on hyperpolarization? Accord-
Inhibition of Conduction by ACh

To determine whether a physiological agonist that activates SK_{Ca}/IK_{Ca} exerts similar effects, EC tubes were superfused with ACh (3 \mu mol/L),^{30} During peak hyperpolarization, responses at site 2 decreased by \sim 70\% versus control (Figure 7). Exposure to ACh+60 mmol/L [K^+]_o confirmed that ACh inhibited CA (500 \mu mol) irrespective of hyperpolarization (Online Figure I). Thus, activating SK_{Ca}/IK_{Ca} with a classic vasodilator also impaired electric conduction irrespective of hyperpolarization.

Enhanced Conduction With SK_{Ca}/IK_{Ca} Blockade

Progressive activation of SK_{Ca}/IK_{Ca} impaired electric conduction in a graded manner (Figure 4). Thus, if a subpopulation of SK_{Ca}/IK_{Ca} was open at rest, then inhibition of these channels should enhance electric conduction. This prediction was tested by injecting current and recording \Delta V_m before and during exposure to Ap+ChTX. Exposure to these SK_{Ca}/IK_{Ca} blockers depolarized ECs (control: \sim 28 \pm 2 mV; Ap+ChTX: \sim 18 \pm 2 mV; n=6; P<0.05) and increased CA (500 \mu mol) by \sim 30\% (P<0.05; Figure 8). Further, the relationship between \Delta V_m at site 2 (500 \mu mol) and current injected at site 1 remained linear (with greater slope) through the entire range of current injection (\sim 15\%; n=6).

A summary of the treatment effects of the preceding interventions on electric conduction at a standard reference distance (500 \mu mol) is given in Online Table II.

Negligible Role for K_{ATP} or BK_{Ca} Channels

If manipulating SK_{Ca}/IK_{Ca} activity alters electric conduction, then complementary effects would be expected for other K^+ channels. We tested for such an effect of ATP-regulated K^+ channels (K_{ATP}) using levcromakalim (10 \mu mol/L; n=5). However, this K_{ATP} opener had no significant effect on resting \Delta V_m (\sim 27 \pm 1 mV) or CA (500 \mu mol; control, 8.4 \pm 1.1, levcromakalim: 8.6 \pm 1.1 mV/nA). Complementary experiments investigating a role for the large-conductance Ca^{2+} (and voltage)-sensitive K^+ channel (BK_{Ca}) using the activator NS1619 (10 or 30 \mu mol/L; n=4) were also without effect on resting \Delta V_m (\sim 27 \pm 2 mV) or CA (500 \mu mol; control, 8.7 \pm 1.3, NS1619: 9.3 \pm 1.1 mV/nA). These findings indicate that neither K_{ATP} nor BK_{Ca} are functionally expressed in EC tubes.

Negligible Role for Nitric Oxide

Endothelial cell hyperpolarization may also promote nitric oxide (NO) synthesis.\textsuperscript{31,32} We therefore tested whether inhibition of NO synthase altered the effect of SK_{Ca}/IK_{Ca} activation on electric conduction. Irrespective of treatment with the inhibitor N\textsuperscript{ω}-nitro-1-arginine (100 \mu mol/L), exposure to NS309 (1 \mu mol/L) or to ACh (3 \mu mol/L) suppressed CA (500 \mu mol) to the same extent (Online Figure II).

Discussion

This study has revealed the ability of membrane ion channel activation to tune the efficacy of electric conduction along the endothelium of resistance vessels. Using intact EC tubes freshly isolated from feed arteries of mouse skeletal muscle, findings illustrate that activation of SK_{Ca}/IK_{Ca} impairs axial signal transmission. This effect is explained by a fall in membrane resistance (r_m) that dissipates charge as current flows from cell to cell along the endothelium. During intracellular microinjection of propidium iodide, robust dye transfer between neighboring ECs confirmed that cell-cell coupling through GJCs was maintained in the absence of electric conduction. Thus, irrespective of SMCs or of changing cell-cell coupling through GJCs, dynamic changes in r_m can profoundly alter the efficacy of electric signal transmission along the endothelium of resistance vessels. Remarkably,
such tuning of electric conduction was governed by ion channels that are insensitive to voltage.

The Nature of Electric Conduction Along EC Tubes

Conduction amplitude was defined as the slope of the I-V relationship with responses routinely evaluated at ±1 nA; for example, within the linear range throughout our experiments. At a standard reference distance of 500 μm, our values of CA (≈8 mV/μA; Figure 2C and 2D and Figure 3A) compare favorably with values (≈7 mV/μA) per 0.8 nA calculated for the conduction of hyperpolarization along resistance arteries of hamster skeletal muscle. Moreover, hyperpolarizing and depolarizing signals conducted with similar efficacy along EC tubes as shown by the linear (ie, ohmic) changes in V_m throughout the range of currents injected (±0.1–3 nA) at each distance (Figure 2). Such behavior indicates low functional expression of voltage-sensitive ion channels and is consistent with the lack of effect of NS1619 on V_m or CA. In turn, the deviation from linearity observed during hyperpolarization with >1.5 nA in the presence of 60 mmol/L [K+]o (Figure 6C) can be explained by the reduction in EK. In contrast to the present findings, depolarizing currents injected into ECs (or SMCs) of feed arteries from hamsters evoked less than half of the absolute change in V_m than did hyperpolarizing current of equal intensity.7 Such deviation observed for intact vessels (versus the linearity of responses for EC tubes) may be attributed to the influence of SMCs through myoendothelial coupling,7,9 for example, through voltage-gated K⁺ channels (Kv) that may not otherwise be present in ECs.25

Whereas activating SKCa/IKCa with NS309 (1 μmol/L) reduced CA by 50% or more at each distance (Figure 3A), comparing absolute values does not resolve changes in the nature of electric conduction because the local response to current injection (ie, change in V_m) is reduced. Expressing responses at each distance relative to the local response provides a normalized index of “conduction efficiency” and illustrates significantly greater decay in electric conduction

Figure 6. Impaired electric conduction during SKCa/IKCa activation is independent of hyperpolarization. Continuous (paired) recordings of V_m at site 2 (V_m2) located 500 μm from current microinjections at site 1. A, V_m2 during ±0.1 to 3 nA before and during SKCa/IKCa activation with NS309 (1 μmol/L). During NS309, note hyperpolarization (to ~−60 mV) and decrease in V_m2 responses. B, As in A, before and during treatment with NS309+60 mmol/L [K⁺]o, to prevent hyperpolarization to NS309. Note decrease in V_m2 responses during NS309 similar to findings in A. Transient hyperpolarization near end of recording (↓) attributable to slower washout of NS309 versus KCl. a, b, and c correspond to small vertical arrows in A and B showing individual recordings of V_m2 during −1 nA injected at site 1 for a, control (ΔV_m = −6 mV); b, NS309 (ΔV_m = −3 mV); and c, NS309+60 mmol/L [K⁺]o (ΔV_m = −3 mV; note lack of hyperpolarization to NS309 with reduced hyperpolarization to −1 nA current). C, Summary data for ΔV_m2 responses to full range of current injection (±0.1-3 nA) during control and during 60 mmol/L [K⁺]o. *Significantly different from control, P<0.05. D, Summary data (mean±SEM) for CA at site 2 to −1 nA current injection at site 1 during control, during 60 mmol/L [K⁺]o, during NS309 (1 μmol/L), and during NS309 (1 μmol/L)+60 mmol/L [K⁺]o. *Significantly different from control (P<0.05). +Significantly different from 60 mmol/L [K⁺]o, P<0.05. Summary data (mean±SEM) in C and D are for n=7.
Tuning Electric Conduction Independent of GJC Regulation

Electric conduction along the endothelium is promoted by high $r_m$ preventing signal dissipation and low $r_a$ through GJCs between neighboring cells. Whereas GJCs have been a focus for regulating vascular conduction, the present study demonstrates that electric conduction along the endothelium can be progressively inhibited by graded activation of SKCa/IKCa (Figure 4). This interpretation is consistent with the robust expression and ionic conductance of both $K_{Ca2.3}$ (SKCa) and $K_{Ca3.1}$ (IKCa) channels estimated from patch-clamp studies of native ECs freshly isolated from mouse aortae and the established role of SKCa/IKCa in governing EC hyperpolarization.

From a typical resting $V_m$ of $\approx -25$ mV, incremental activation of SKCa/IKCa with NS309 approached $E_k$ and coincided with loss of conduction (Figure 4A). In a reciprocal manner, blocking SKCa/IKCa with Ap+ChTX depolarized EC tubes by $\approx 10$ mV, with a $\approx 30\%$ increase in CA (500 μm; Figure 8). This modest effect of SKCa/IKCa inhibition compared with SKCa/IKCa activation on electric conduction can be explained by a low percentage of the total SKCa/IKCa in EC membranes being open under control conditions.

The SKCa/IKCa opener SKA-31 had less potency and reduced efficacy compared with NS309 (Figure 4A and 4D). Nevertheless, the apparent threshold of channel activation required to significantly reduce CA corresponded to a $V_m$ of $\approx -40$ mV for both agents (0.3 μmol/L NS309, 3 μmol/L SKA-31; Figure 4). Hyperpolarization may also promote the production of NO, which may in turn alter $r_m$ or cell-to-cell coupling through acting on GJCs. Nevertheless, the inhibition of electric conduction by NS309 was maintained when hyperpolarization was prevented (Figure 6) and when NO synthesis was inhibited (Online Figure II). Importantly, and similar to the direct activation of SKCa/IKCa by NS309 and SKA-31, CA (500 μm) was reduced $\approx 70\%$ during indirect activation of these channels by ACh (Figure 7). Further, as seen with NS309, preventing hyperpolarization to ACh (Online Figure I) or inhibiting NO synthesis (Online Figure II) did not alter the ability of ACh to suppress electric conduction. Thus, the modulation of $r_m$ (and tuning of electric conduction) along the endothelium may reflect a more generalized response to physiological agonists that signal through G protein–coupled receptors, irrespective of NO.

The present findings support the hypothesis that opening ion channels in the plasma membrane impairs electric conduction along the endothelium. As dye transfer remained intact during exposure to NS309 when conduction was abolished (Figure 4), loss of conduction cannot be attributed to closure of GJCs. In turn, despite the lack of effect for levcromakalim or NS1619, we infer that the activation or inhibition of other ion channels that are functionally expressed in membranes of electrically coupled cells can have effects consistent with those shown here for SKCa/IKCa. Consistent with this inference are recent findings from rat mesenteric arteries, where spreading vasodilation in response during SKCa/IKCa activation when compared with control (Figure 3B). However, it is important to evaluate the effect of SKCa/IKCa activation when conduction is initiated from a similar local change in $V_m$. We therefore compared responses to $-2$ nA current during NS309 with responses to $-1$ nA under control conditions. With local responses ($\Delta V_m$) not different between conditions, the decay in $V_m$ over distance remained significantly greater during SKCa/IKCa activation (Figure 3C and Online Table 1).

Along the entire distance (2000 μm) studied, responses decayed by 4.4% per 100 μm. This value is consistent with $\approx 5\%$ per 100 μm reported for the decay of electric conduction along the endothelium of retinal arterioles. Further, the consistency of such behavior suggests that the biophysical properties of EC tubes determined here can be applied to the endothelium of vessels in other resistance networks. Indeed, $\lambda$ determined in the present study for electric conduction ($\approx 1.4$ mm; Figure 3) is consistent with control values reported for intact guinea pig arterioles ($\approx 1.1–1.6$ mm) and hamster retractor feed arteries (1.2 mm). A limitation of the present study is the lack of SMCs, which precludes resolution of how SKCa/IKCa activation may influence electric signaling through myoendothelial GJCs. Nevertheless, even with SMCs present in earlier studies, the similarity in values for $\lambda$ across preparations and laboratories supports the role of the endothelium as the primary cellular pathway for axial current flow. In turn, minimal charge loss through myoendothelial gap junctions is explained by the high input resistance of SMCs.
to ACh (or isoproterenol) was enhanced when K+ channels (BKCa and Kv) were inhibited in SMCs that were electrically coupled to ECs. Hyperpolarization can activate other K+ channels (eg, inward rectifying, KIR) to increase Vm and enhance electric conduction. Nevertheless, it is unlikely that such a role would be manifest in EC tubes, given the linearity of the I-V relationship (Figure 2) and a resting Vm (25 mV) above that associated with the negative slope conductance of KIR. Further, given the same decrease in CA to NS309 or ACh when hyperpolarization was prevented (Figure 6D and Online Figure I, respectively), it appears unlikely that that activation of KIR contributes to electric conduction along the endothelium. However, this conclusion does not preclude a role for KIR expressed in SMCs to contribute to conducted vasodilation along intact vessels.

**Summary and Conclusions**

Electric signaling underlies the correspondence between changes in Vm and diameter along the resistance vasculature. Previous studies have focused on the role of intercellular coupling through GJCs in determining the efficacy of electric conduction, particularly as it applies to conducted vasodilation along arterioles and ascending vasodilation during exercise. Paradigms for studying conducted responses have typically involved stimulating a vessel segment (or the tissue in which vessels are embedded) while monitoring vasomotor responses at sites remote from the region of stimulation. However, there is ambiguity in defining the number of cells activated, the precise stimulus intensity at the signal origin, and the specific role(s) of respective cell layers. In the present study, the endothelium of resistance arteries was isolated as an intact tube to eliminate the influence of blood flow or surrounding cells and tissue. Intracellular microelectrodes enabled electric conduction to be initiated from a point source (single cell) within the electric syncytium, using prescribed current pulses while monitoring Vm at defined distances. In turn, selective activation (or inhibition) of SKCa/IKCa along the endothelium...
resolved how electric conduction can be tuned through changes in membrane resistance while individual cells remain coupled to each other through GJCs.

This study presents 2 novel findings. First, electric conduction can be finely tuned through the activity of voltage-insensitive membrane ion channels. Thus, our experiments are the first to demonstrate an integral role for SKCa/IKCa activation in governing EC signaling that is distinct from their established role of initiating EC hyperpolarization\(^9,24\) (Figure 9). Second, SKCa/IKCa can define the spatial domain of electric signaling along the endothelium (Figure 9). The SKCa/IKCa have gained recognition as therapeutic targets in light of their ability to initiate hyperpolarization and promote vasodilation to increase tissue blood flow.\(^24,41,42\) The present findings imply that selectively targeting ion channels for therapeutic intervention should account for integrated effects throughout resistance networks as well as local effects on vasomotor tone.

Acknowledgments
Dr William F. Jackson provided helpful comments during the initial stages of this study.

Sources of Funding
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Disclosures
None.

References
Novelty and Significance

What Is Known?

- The endothelium conducts electric signals along resistance vessels to coordinate relaxation of smooth muscle cells.
- Gap junctions provide a low-resistance pathway for current to flow between endothelial cells.
- Small and intermediate Ca\(^{2+}\)-activated K\(^+\) channels (SK\(\text{Ca}/I\text{K}\text{Ca}\)) are highly expressed in endothelial cells and initiate hyperpolarization.

What New Information Does This Article Contribute?

- Electric conduction of hyperpolarization and depolarization along the endothelium produces equivalent (but opposite) changes in membrane potential.
- Irrespective of gap junctions or charge polarity, opening voltage-insensitive ion channels dissipates electric signals along the endothelium.
- Activation of SK\(\text{Ca}/I\text{K}\text{Ca}\) channels effectively “tunes” the ability of the endothelium to conduct electric signals.

The endothelium is recognized as the principal pathway for initiating vasodilation through SK\(\text{Ca}/I\text{K}\text{Ca}\) activation and for the conduction of hyperpolarization in resistance networks. In regulated conduction vasodilation, attention has focused on the role and manipulation of intercellular gap junctions to alter the resistance to current flow between cells. In contrast, we studied whether altering the electric resistance of endothelial plasma membranes via activation or blockade of ion channels can regulate electric conduction. Our new findings demonstrate that irrespective of charge polarity, electric conduction along the endothelium is impaired during SK\(\text{Ca}/I\text{K}\text{Ca}\) activation and is enhanced by SK\(\text{Ca}/I\text{K}\text{Ca}\) blockade. At the same time, neighboring cells remain well coupled to each other through gap junctions. We suggest that impaired tissue blood flow during endothelial dysfunction may reflect enhanced ion channel activation (i.e., “leaky” plasma membranes) that impairs coordinated control of vascular resistance. Therapeutic interventions designed to improve tissue blood flow by altering ion channel activation should take into account the effects on electric conduction in resistance networks.
Tuning Electrical Conduction Along Endothelial Tubes of Resistance Arteries Through Ca
2+-Activated K+ Channels

Erik J. Behringer and Steven S. Segal

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Tuning electrical conduction along endothelial tubes of resistance arteries through Ca\(^{2+}\)-activated K\(^+\) channels

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This Supplement contains:

- Detailed Methods with References
- Supplemental Tables I and II
- Supplemental Figures I and II
Detailed Methods

Animal care and use.
All procedures were approved by the Animal Care and Use Committee of the University of Missouri and performed in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed on a 12:12-h light-dark cycle at ~23 °C with fresh water and food available ad libitum. Experiments were performed on C57BL/6 males bred at the University of Missouri (age, 3–6 months). Each mouse was anesthetized using pentobarbital sodium (60 mg/kg, intraperitoneal injection) and abdominal fur was removed by shaving. Following surgical procedures, the anesthetized mouse was euthanized with an overdose of pentobarbital via cardiac puncture.

Solutions.
Physiological salt solution (control PSS) was used to superfuse EC tubes [(in mmol/L): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10 Glucose]. During SMC dissociation to produce EC tubes, PSS contained 0.1% Bovine Serum Albumin (USB Corp.; Cleveland, OH; USA). During dissection to prepare EC tubes, CaCl₂ was absent and 0.01 mmol/L sodium nitroprusside (SNP) was added to PSS (dissection PSS) to relax SMCs. During dissociation of SMCs, SNP was replaced with 0.1 mmol/L CaCl₂ (dissociation PSS). All reagents were obtained from Sigma-Aldrich (St. Louis, MO; USA) unless otherwise indicated.

Surgery and microdissection.
A ventral midline incision was made from the sternum to the pubis. While viewing through a stereo microscope (SMZ800, Nikon; Tokyo, Japan), fat and connective tissue superficial to the sternum were removed to expose the proximal ends of abdominal muscle feed arteries (superior epigastric artery) bilaterally. To maintain blood in the lumen and thereby facilitate visualization during dissection, each AFA was ligated along with its adjacent vein using 6-0 silk sutures (Ethicon; Somerville, NJ; USA). Abdominal muscles were removed bilaterally and placed in chilled (4 °C) dissection PSS. A muscle was pinned onto transparent silicone rubber (Sylgard 184, Dow Corning; Midland, MI; USA) and the vessel segment (length: ~2 cm) was dissected free from surrounding tissue. Residual blood was flushed from the vessel lumen by cannulating one end with a pipette made from heat-polished borosilicate glass capillaries (G150T-4, Warner Instruments; Hamden, CT; USA) with an outer diameter of 50-80 μm and connected to a static column (height, ~10 cm) of dissection PSS.

Endothelial cell tube isolation and superfusion.
As described,¹,² feed arteries were cut into segments (length, ~3 - 5 mm) and placed into dissection PSS containing 0.62 mg/ml papain, 1.0 mg/ml dithioerythritol, 1.5 mg/ml collagenase then incubated for 30 min at 34 °C. Following partial digestion, the PSS containing enzymes was replaced with dissociation PSS and vessel segments were transferred to a 100 x 15 mm Petri dish and gently triturated to remove SMCs using borosilicate glass capillary tubes [1.0 mm outer diameter (OD)/ 0.58 mm ID; World Precision Instruments (WPI), Sarasota, FL; USA] that were pulled (P-97; Sutter Instruments; Novato, CA; USA) and heat-polished (tip ID: 80-120 μm). Following removal of SMCs (confirmed by visual inspection at 200X magnification), an EC tube was transferred to a tissue chamber (RC-27N, Warner) secured on an aluminum platform (width: 14.5 cm, length: 24 cm, thickness: 0.4 cm) containing a micromanipulator (DT3-100, Siskiyou Corp.; Grants Pass, OR; USA) at each end that held a blunt fire-polished micropipette (OD, 60-100 μm) to secure the tube against the bottom (coverslip) of the tissue chamber. The entire preparation was secured on an inverted microscope (Eclipse TS100, Nikon) mounted on a vibration-isolated table (Technical Manufacturing Corp., Peabody, MA; USA) and superfused.
at 4 ml/min with PSS. The temperature of the chamber was regulated using an inline heater (SH-27B, Warner) and heating platform (PH6, Warner) coupled to a temperature controller (TC-344B, Warner). Temperature was increased over 30 min to 32 °C where intact preparations were studied for up to 4 hr. Pharmacological agents were added to the superfusion solution, thereby exposing the entire EC tube to the treatment. Preliminary experiments ejecting blue dye from micropipettes positioned within the chamber confirmed that superfusion flow was laminar in the axial direction of EC tubes.

**Intracellular recording.**
Membrane potential ($V_m$) in EC tubes was recorded with an Axoclamp amplifier (2B; Molecular Devices; Sunnyvale, CA; USA) using microelectrodes pulled (P-97; Sutter) from glass capillary tubes (GC100F-10, Warner) and backfilled with 2 mol/L KCl (tip resistance, ~150 MΩ). For experiments testing dye transfer between cells through gap junctions, microelectrodes were backfilled with 0.1% propidium iodide dissolved in 2 mol/L KCl. An Ag/AgCl pellet was placed in effluent PSS to serve as a reference electrode. The output of the amplifier was connected to an analog-to-digital converter (Digidata 1322A, Molecular Devices; Sunnyvale, CA; USA) with data recorded at 1000 Hz on a Dell personal computer using Axoscope 10.1 software (Molecular Devices). For dual simultaneous intracellular recordings, a second amplifier (IE-210, Warner) was integrated into the data acquisition system. Current ($\pm 0.1-3$ nA, 2 s) was delivered using the Axoclamp electrometer driven by a function generator (CFG253, Tektronix; Beaverton, OR; USA). For current injection, an EC was penetrated at a site located ~150 μm from the downstream (with respect to the direction of PSS superfusion) end of where EC tube was pinned (referred to as Site 1) while recording $V_m$ from an EC at Site 2, which was located at a defined separation distance (50-2000 μm) from Site 1 with reference to a calibrated eyepiece reticle while viewing at 20X magnification. For all experiments (except for length constant determinations), separation distance between microelectrodes was standardized at 500 μm, which corresponds to the distance of ~15 ECs placed end-to-end.

Successful impalements were indicated by sharp negative deflection of $V_m$, stable $V_m$ for >1 min, hyperpolarization ($\geq$ 20 mV) to 1 μmol/L NS309 (I\textsubscript{K\textsubscript{Ca}}/SK\textsubscript{Ca} activator; Tocris; Bristol, UK), recovery to resting $V_m$ after NS309 washout and return to ~0 mV upon withdrawal from the cell. Correspondence between current injection at Site 1 and $V_m$ responses at Site 2 indicated simultaneous intracellular current injection and $V_m$ recording. Following dye microinjection, using a filter set for rhodamine with illumination provided from a 50W Hg lamp, images of propidium iodide fluorescence were acquired using a 40X objective (Nikon Fluor40; NA: 0.75) and focused onto a cooled CCD camera (MicroFire, Optronics; Goleta, CA; USA).

**Pharmacology.**
NS309 and SKA-31 (Tocris; Bristol, UK) were used to evoke hyperpolarization in endothelial tubes by direct activation of SK\textsubscript{Ca}/IK\textsubscript{Ca} channels. Alternatively, the physiological agonist acetylcholine (ACh) chloride was used to indirectly activate SK\textsubscript{Ca}/IK\textsubscript{Ca} channels through G\textsubscript{q} protein coupled muscarinic receptors. To evaluate the potential effects of nitric oxide synthesis during NS309 or ACh treatment, $N^\omega$-Nitro-L-arginine (L-NNA) was applied during select experiments alone (10 min pre-treatment) and subsequently in combination with either NS309 or ACh. To test for the presence of large-conductance Ca$^{2+}$-activated K$^+$ channels (BK$\text{Ca}$) and K$\text{ATP}$ channels within the endothelium, NS1619 and levocromakalim were applied, respectively. The compounds NS309, SKA-31, NS1619 and levocromakalim were dissolved in DMSO and diluted to final working concentrations in PSS on the day of an experiment. Final concentration of DMSO was < 1%. Vehicle controls with DMSO ($\leq$ 2%) in PSS had no effect on $V_m$ or electrical coupling (n=3). Apamin (Alomone; Jerusalem, Israel) and charybdotoxin (Alomone...
Jerusalem, Israel or Anaspec; Fremont, CA) were dissolved and diluted into superfusion PSS in combination to block SKCa/IKCa channels.\textsuperscript{13}

Data analysis.
One EC tube was studied per mouse. Analyses included: 1) Resting \(V_m\) (mV) under Control conditions; 2) Change in \(V_m\) (\(\Delta mV\)) = peak response \(V_m\) – preceding baseline \(V_m\); 3) Conduction Amplitude (CA, mV/nA) = \(V_m\) at Site 2 / current injected at Site 1. With linearity of I-V relationships (see Results), -1 nA was used as a standard current for evaluating CA; 4) Fraction of Control CA = CA during treatment / preceding control CA; 5) Conduction Efficiency = CA at each separation distance / CA at 50 µm separation; 6) Length constant (\(\lambda\)) = distance over which the electrical signal decayed to 37\% (1/e) of the ‘local’ value. The shortest separation distance was 50 µm to ensure that both microelectrodes were not in the same EC while recording as close to the signal origin as possible; the length of individual ECs in these freshly-isolated tubes is typically 35-40 µm\textsuperscript{2}. Fraction of control CA was defined as the CA during NS309 or SKA-31 at respective concentrations divided by CA under control conditions. Linear regression (\(\Delta V_m\) at Site 2 versus current injection at Site 1), curve fitting (estimates of \(\lambda\)) were performed using GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA; USA). Statistical analyses included repeated measures Analysis of Variance with Tukey or Bonferroni post-hoc comparisons, linear regression and paired Student’s -tests (GraphPad Prism). Differences between treatments were accepted as statistically significant with \(P < 0.05\). Summary data are presented as means ± S.E.
References

13. Ledoux J, Bonev AD, Nelson MT. Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels in murine endothelial cells: Block by intracellular calcium and magnesium. J Gen Physiol. 2008;131:125-135.
Supplemental Table I.

<table>
<thead>
<tr>
<th>Distance (µm)</th>
<th>Control ( \Delta V_{m2} ) (mV)</th>
<th>NS309 ( \Delta V_{m2} ) (mV)</th>
<th>NS309 ( \Delta V_{m2} ) (mV)</th>
<th>NS309 ( \Delta V_{m2} ) (mV)</th>
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<tr>
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<tr>
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<td>-1.2 ± 0.3*</td>
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Spatial decay of electrical conduction increases during \( SK_{Ca}/IK_{Ca} \) activation with 1 µmol/l NS309. The standard current pulse microinjected at Site 1 to evaluate a change in membrane potential at Site 2 (\( \Delta V_{m2} \)) at distances of 50-2000 µm was -1 nA (Control, Column 2). Treatment with NS309 reduced \( \Delta V_{m2} \) to -1 nA at each distance (Column 3). To achieve the same \( \Delta V_{m2} \) at 50 µm in the presence of NS309 required twice the current (-2 nA; Column 4); note greater signal loss at 500-2000 µm vs. Control (these data are complementary to Figure 3C). Raising microinjection current to -3 nA during NS309 treatment exceeded Control \( \Delta V_{m2} \) responses at 50 and 500 µm while further illustrating enhanced signal dissipation by 2000 µm during \( SK_{Ca}/IK_{Ca} \) activation. *Significantly less than Control \( V_{m2} \) responses to -1 nA for the respective distance indicated, \( P < 0.05 \) (\( n = 11 \) at 50 - 1,500 µm; \( n = 7 \) at 2,000 µm).
### Supplemental Table II.

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<td>ΔVₘ₂ (mV)</td>
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**Effect of SKCa/IKCa activation on electrical conduction at a constant reference distance.**

These experiments evaluated the change in Vₘ from rest (ΔVₘ₂ = resting Vₘ - peak response Vₘ) at a constant distance (500 µm) from the site of current microinjection and correspond to Figures 4, 6 and 7. Note that ΔVₘ₂ Treatment responses to -1 nA are significantly less (P < 0.05) than ΔVₘ₂ Control responses during each intervention. During 1 µmol/L NS309 (either alone or with 60 mmol/L KCl) and for 10 µmol/L SKA-31, doubling current to -2 nA increased ΔVₘ₂ responses to approximate Control values. During 30 µmol/L SKA-31 and 3 µmol/L ACh, tripling current to -3 nA increased ΔVₘ₂ to approximate Control values but still could not restore ΔVₘ₂ responses to 10 µmol/L NS309 or 100 µmol/L SKA-31. *Significantly less than Control ΔVₘ₂ in response to -1 nA, P < 0.05.

Note: During SKCa/IKCa blockade with apamin + charybotoxin, ΔVₘ₂ responses to -1 nA increased (P < 0.05) from -6.5 ± 0.9 to -8.1 ± 0.9 mV, n=6; see Fig. 8B) and less current (-0.8 nA) was required to evoke ΔVₘ₂ responses (-6.5 ± 0.7) similar to those evoked by -1 nA under Control conditions.
Supplemental Figure I. Acetylcholine inhibits electrical conduction without hyperpolarization. Data are from continuous $V_m$ recordings at Site 2 located 500 μm from current microinjection. **A**, Representative recording illustrating responses to ±0.1-3 nA before and during 3 μmol/L ACh + 60 mmol/L $[K^+]_o$. Note loss of $V_m$2 responses during 3 μmol/L ACh + 60 mmol/L $[K^+]_o$. **B**, Summary data (means ± S.E.; n=4) for Conduction Amplitude to -1 nA. Plateau $V_m$ during ACh + 60 mmol/L $[K^+]_o$ was -20 ± 1 mV. *Significantly different from Control, P < 0.05. Conduction Amplitude recovered to 7.5 ± 0.5 mV/nA (n=4) upon washout of ACh + 60 mmol/L $[K^+]_o$. 
Supplemental Figure II. Impaired electrical conduction during SK$_{Ca}$/IK$_{Ca}$ activation is maintained during inhibition of nitric oxide synthase. Data represent continuous (paired) recordings of $V_m$ at 500 µm from site of current microinjection. A, Responses to ±0.1-3 nA before and during 1 µmol/L NS309. Note diminished responses during NS309. B, As in A with NS309 + 100 µmol/L N’-Nitro-L-arginine (L-NNA). C, Summary data for Conduction Amplitude under the initial Control conditions (Control 1), during NS309 alone, following washout of NS309 (Control 2; note reversibility) and during NS309 + L-NNA. The reduction in $V_m$ responses to current injection during NS309 + L-NNA was not different from NS309 alone. *Significantly different from Control, P < 0.05 (n = 4).

In separate experiments (n=3), complementary results were obtained when comparing ACh (3 µM) alone or in combination with 100 µmol/L L-NNA (also paired continuous recordings) as follows: Conduction Amplitude (mV/nA) at 500 µm separation: Control 1, 7.9 ± 1.4; ACh, 1.9 ± 0.4; Control 2, 7.7 ± 0.7; ACh + L-NNA: 2.0 ± 0.3. Across experiments, treatment with 100 µM L-NNA to inhibit nitric oxide synthase began at least 10 min before addition of NS309 or ACh.