Ca^{2+}-activated K^+ Channels in Murine Endothelial Cells: Block by Intracellular Calcium and Magnesium

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The intermediate (IK_{Ca}) and small (SK_{Ca}) conductance Ca^{2+}-sensitive K^+ channels in endothelial cells (ECs) modulate vascular diameter through regulation of EC membrane potential. However, contribution of IK_{Ca} and SK_{Ca} channels to membrane current and potential in native endothelial cells remains unclear. In freshly isolated endothelial cells from mouse aorta dialyzed with 3 μM free $[Ca^{2+}]_i$ and 1 mM free $[Mg^{2+}]_i$, membrane currents reversed at the potassium equilibrium potential and exhibited an inward rectification at positive membrane potentials. Blockers of large-conductance, Ca^{2+}-sensitive potassium (BK_{Ca}) and strong inward rectifier potassium (K_{ir}) channels did not affect the membrane current. However, blockers of IK_{Ca} channels, charybdotoxin (ChTX), and of SK_{Ca} channels, apamin (Ap), significantly reduced the whole-cell current. Although IK_{Ca} and SK_{Ca} channels are intrinsically voltage independent, ChTX- and Ap-sensitive currents decreased steeply with membrane potential depolarization. Removal of intracellular Mg^{2+} significantly increased these currents. Moreover, concomitant reduction of the $[Ca^{2+}]_i$ to 1 μM caused an additional increase in ChTX- and Ap-sensitive currents so that the currents exhibited theoretical outward rectification. Block of IK_{Ca} and SK_{Ca} channels caused a significant endothelial membrane potential depolarization (≈11 mV) and decrease in $[Ca^{2+}]_i$ in mesenteric arteries in the absence of an agonist. These results indicate that $[Ca^{2+}]_i$ can both activate and block IK_{Ca} and SK_{Ca} channels in endothelial cells, and that these channels regulate the resting membrane potential and intracellular calcium in native endothelium.

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INTRODUCTION

Blood flow is intimately linked to endothelial membrane potential and intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). Endothelial Ca^{2+} influx appears to depend on the electrochemical gradient, and likely occurs through nonvoltage-dependent Ca^{2+} entry pathways, possibly transient receptor potential (TRP) channels. Therefore, hyperpolarization of the endothelium membrane elevates $[Ca^{2+}]_i$ by an increase in Ca^{2+} influx that induces relaxation of the underlying smooth muscle (Luckhoff and Busse, 1990) through endothelium-derived hyperpolarizing factors (EDHFs) and the generation of nitric oxide and prostacyclin. Several types of potassium channels have been proposed to regulate endothelial membrane potential, including large conductance, calcium-sensitive potassium (BK_{Ca}) channels, inward rectifier (K_{ir}) potassium channels, and small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+}-activated potassium channels (Hoger et al., 2002; Shimoda et al., 2002; Fang et al., 2005). SK_{Ca} and IK_{Ca} channels in vascular endothelium appear to have particularly prominent roles, since inhibition of these channels prevents EDHF-mediated vasodilation (Eichler et al., 2003; Weston et al., 2005; Feletou and Vanhoutte, 2006). The current view is that endothelial-dependent vasodilators such as acetylcholine, bradykinin, or substance P elevate intracellular calcium through calcium influx and release; this in turn activates SK_{Ca} and IK_{Ca} channels, which cause membrane potential hyperpolarization and further elevation of intracellular calcium through increases in the calcium electrochemical gradient. The SK_{Ca}- and IK_{Ca}-induced membrane hyperpolarization and elevation of intracellular calcium directly or indirectly induce membrane hyperpolarization and relaxation of the nearby vascular smooth muscle (Feletou and Vanhoutte, 2006). Therefore, SK_{Ca} and IK_{Ca} channels in vascular endothelial cells are thought to act as a positive feedback element, such that their activation causes membrane potential hyperpolarization and thus increased calcium entry (Garland et al., 1995; Marchenko and Sage, 1996; Eichler et al., 2003; Weston et al., 2005; Feletou and Vanhoutte, 2006). However, the role of these channels in the regulation of endothelial membrane potential and intracellular calcium in the absence of endothelial agonists is not known.

Although it is clear that SK_{Ca} and IK_{Ca} channels can affect endothelial membrane potential, it is unclear how membrane potential regulates currents through

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Abbreviations used in this paper: AEC, aortic endothelial cell; Ap, apamin; BK_{Ca}, large conductance Ca^{2+}-activated potassium channel; $[Ca^{2+}]_i$, intracellular Ca^{2+} level; ChTX, charybdotoxin; EC, endothelial cell; EDHF, endothelium-derived hyperpolarizing factor; E_K, equilibrium potential; GHK, Goldman-Hodgkin-Katz; IbTX, iberiotoxin; IK_{Ca}, intermediate conductance Ca^{2+}-activated potassium channel; K_{ir}, inward rectifier potassium channel; K_v, voltage-dependent potassium channel; SK_{Ca}, small conductance Ca^{2+}-activated potassium channel; TRP, transient receptor potential.

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these channels. Expressed SK_{Ca} ($K_{Ca}2.1$-2.3) and IK_{Ca} ($K_{Ca}3.1$) channels lack an intrinsic voltage sensor. Yet, SK_{Ca} and IK_{Ca} currents in native endothelium as well as expressed $K_{Ca}2.2$, $K_{Ca}2.3$, and $K_{Ca}3.1$ channels appear to exhibit inward rectification, i.e., their conductance decreases with membrane potential depolarization (Kohler et al., 1996; Xia et al., 1998; Castle et al., 2003; Eichler et al., 2003; Joiner et al., 2003; Taylor et al., 2003; Si et al., 2006). Although intracellular divalent cations have recently been shown to block the pore of rat $K_{Ca}2.2$ channels exogenously expressed in oocytes (Soh and Park, 2001), evidence for a similar mechanism occurring with IK_{Ca} and SK_{Ca} in native endothelial cells is lacking.

Recent evidence indicates that SK_{Ca} and IK_{Ca} currents in native endothelium are through $K_{Ca}2.3$ and $K_{Ca}3.1$ channels, respectively (Taylor et al., 2003; Si et al., 2006; Kohler and Hoyer, 2007). $K_{Ca}2.1$-2.3 channels are blocked by the bee venom apamin (Ap), whereas $K_{Ca}3.1$ channels are blocked by scorpion toxin, charybdotoxin (ChTX), and by TRAM-34 (Ledoux et al., 2006). ChTX and iberiotoxin (IbTX) block BK_{Ca} channels, which are present in the vascular smooth muscle. Suppression of $K_{Ca}2.3$ channel expression eliminates Ap-sensitive potassium currents in the endothelium, but not IK_{Ca} currents (Taylor et al., 2003). This causes depolarization of the endothelium and vascular smooth muscle, and increases tone and blood pressure (Taylor et al., 2003). Furthermore, molecular evidence supports the idea that functional isoform of SK_{Ca} channels in vascular endothelium is the $K_{Ca}2.3$ (SK3) channel (Kohler et al., 2001; Burnham et al., 2002; Eichler et al., 2003; Taylor et al., 2003; Burnham et al., 2006; Kohler and Hoyer, 2007). Similarly, targeted disruption of gene for $K_{Ca}3.1$ channels eliminates TRAM-34–sensitive, but not Ap-sensitive, potassium currents, and increases vascular tone and blood pressure (Si et al., 2006). Therefore, both channel types appear to be important modulators of vascular function (Kohler and Hoyer, 2007).

One goal of the present study was to determine the properties of SK_{Ca} and IK_{Ca} channels in freshly isolated vascular endothelial cells, in particular their voltage dependence and possible block by intracellular Ca^{2+} and Mg^{2+} ions. Whole cell currents reversed at the potassium equilibrium potential (E_K) and were composed of currents through IK_{Ca} and SK_{Ca} channels, as well as an intracellular Ca^{2+}-insensitive component. However, BK_{Ca} channel and K_{ir} channel blockers did not affect the membrane currents. A pronounced inward rectification was observed for the SK_{Ca} and IK_{Ca} currents positive to the potassium equilibrium potential, E_K. Voltage-dependent block by intracellular Mg^{2+} and Ca^{2+} ions was responsible for this decrease of K^+ efflux through SK_{Ca} and IK_{Ca} channels. The similar voltage dependence of SK_{Ca} and IK_{Ca} current block by Mg^{2+} and Ca^{2+} suggests that their binding sites within the two channel types share common features. Importantly, significant internal Ca^{2+} block occurred at physiological membrane potentials with potentially physiological ion concentrations. These findings suggest a novel negative feedback mechanism in endothelium by which intracellular Ca^{2+} ions would have opposing effect (activation and block) on SK_{Ca} and IK_{Ca} channels, depending on local Ca^{2+} levels. Finally, we found that SK_{Ca} and IK_{Ca} channels regulate the resting membrane potential and $[Ca^{2+}]_i$ in intact endothelium in the absence of stimulation. Preliminary findings have been previously presented (Ledoux, J., and M.T. Nelson. 2005. *FASEB J.* 19:692.2).

MATERIALS AND METHODS

Endothelial Cell isolation and electrophysiology

Aortic endothelial cells were freshly isolated from C57BL6 female mice (3–4 mo old) as previously described (Taylor et al., 2003). Animal procedures used in this study were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use committee of the University of Vermont. In brief, adult female mice were killed by intraperitoneal injection of sodium pentobarbital (150 mg/kg) followed by a thoracotomy. The aorta was removed, cleaned of connective tissue, and cut into small rings. The tissue pieces were then enzymatically digested using dispase (4 mg/ml) in physiological salt solution for 40 min at 35°C. Elastase (0.1 mg/ml) was then added to the enzyme cocktail and the tissue was further incubated for 10 min. After washing the aorta rings in fresh solution, the rings were cut open and gently triturated to dissociate the endothelial cells. Isolated cells were stored at 4°C and used within 6–8 h following isolation.

Electrophysiology

Membrane currents from freshly isolated endothelial cells were recorded using the conventional whole-cell patch clamp technique. Sampled at 2 kHz, endothelial currents were acquired in voltage-clamp mode with an axopatch 200A (Axon Instruments) and analyzed using the pClamp suite (Axon Instruments). The series resistances were ≈3–5 MΩ and the series resistance error was negligible due to the small amplitude of the recorded currents. Therefore, no compensation was applied. The capacitance error was also negligible in our conditions with a time constant (τ) of ≈50 μs. Isolated endothelial cells were identified by their characteristic round and rough shape.

Simultaneous Membrane Potential Recording and Ca^{2+} Imaging In Situ

Mesenteric arteries from mice were cleaned of connective tissue, cut open, and pinned on a sylgard block with the endothelium facing up. The endothelium was preferentially loaded with Fluo-4 (10 μM) for 45 min at 30°C in the presence of pluronic acid (2.5 μg/ml). Ca^{2+} imaging was then performed using a Solamere confocal system (Solamere Technologies) with a CCD camera on an upright Nikon microscope with a 60× water dipping objective (NA 1.0). Images were acquired at 30 frames/s with the QED acquisition software (Media Cybernetics). Ca^{2+} dye was excited using a krypton/argon laser (488 nm) and emission fluorescence was collected above 495 nm. The images were processed using custom designed software (A. Bonev), and the fractional fluorescence was evaluated by dividing the fluorescence of a region of interest (ROI) by an average fluorescence of 50 images without activity from the same ROI. Simultaneous measurement of the membrane potential was performed with the perforated configuration of the

patch clamp technique at a sampling rate of 5 kHz by obtaining a gigaohm seal with a borosilicate micropipette (6–8 MΩ) on the facing endothelium. The membrane potential recordings (perforated patch) were performed without current injection (I = 0 mode). All in situ experiments were performed in the presence of nisoldipine (100 nM) to minimize the contraction of the smooth muscle to KCl and the impact of smooth muscle on endothelial membrane potential.

Solutions

For isolated cell experiments, the following extracellular solution was used (in mM): NaCl 134, KCl 6, glucose 10, HEPES 10 (pH 7.4), $MgCl_2$ 1, and $CaCl_2$ 2. For Fig. 1 B, extracellular KCl concentration was increased to 45 mM with an equivalent reduction of NaCl. The composition of the pipette solution used for the whole-cell experiments was (in mM): KCl 134, HEDTA 5, and HEPES 10 (pH 7.2). The amounts of $MgCl_2$ and $CaCl_2$ added were determined with the software WinMaxC (http://www.stanford.edu/~cpatton/maxc.html) to achieve the indicated free cation concentrations. For 1 mM Mg^{2+} and 3 μM Ca^{2+} (in mM): $MgCl_2$ (5.53) and $CaCl_2$ (0.207); for 1 mM Mg^{2+} and 1 μM Ca^{2+} (mM): $MgCl_2$ (5.51) and $CaCl_2$ (0.0683); for 0 Mg^{2+} and 3 μM Ca^{2+} (mM): $MgCl_2$ (0) and $CaCl_2$ (1.61); for 0 Mg^{2+} and 1 μM Ca^{2+} (mM): $MgCl_2$ (0) and $CaCl_2$ (1.36). For the experiments in the absence of intracellular Ca^{2+}, the $CaCl_2$ was omitted from the solution above (with 5 mM HEDTA) and $MgCl_2$ was added to set the free Mg^{2+} to 1 mM. These experiments were performed at room temperature.

For simultaneous membrane potential and Ca^{2+} imaging experiments in intact endothelium of cut-open mesenteric arteries, a physiological salt solution was used with the following constituents (in mM): NaCl 119, KCl 4.7, $NaHCO_3$ 24, KH_2PO_4 1.2, EDTA 0.0023, $MgCl_2$ 1.2, glucose 11, and $CaCl_2$ 1.6, and the following pipette solution for the simultaneous membrane potential recording (in mM): K-aspartate 110, NaCl 10, KCl 30, $MgCl_2$ 1, HEPES 10 (pH 7.2), and EGTA 0.05. Amphotericin (200 μg/ml) was used to achieve perforated patch. These experiments were performed at 30°C to minimize dye leakage.

Statistics

All data are the mean ± SEM of *n* cells. Data were analyzed using the paired or unpaired Student's *t* test as appropriate and considered significant with P values <0.05.

RESULTS

Electrophysiological Characteristics of Native Endothelial Cells

Freshly isolated aortic endothelial cells (AECs) were dialyzed with 3 μM Ca^{2+} to activate Ca^{2+}-activated K^+ channels using the conventional whole cell configuration of the patch clamp technique. The endothelial cells had a mean capacitance of 10.6 ± 0.8 pF ($n = 31$). Under these conditions, the relationship between membrane current and voltage was examined by applying voltage steps from −100 to + 100 mV from a holding potential of −50 mV. The whole cell currents exhibited inward rectification at membrane potentials positive to the potassium equilibrium potential ($E_K = -83$ mV). This current/voltage (I/V) relationship is strikingly different from that observed in vascular smooth muscle, which exhibits marked increases in voltage-dependent potassium (K_v) channel and BK_{Ca} channel currents at positive voltages (Marijic et al., 2001; Rainbow et al., 2006). The presence of BK_{Ca} currents was probed by applying IbTX (100 nM), a specific inhibitor of BK_{Ca} channels (Fig. 1 A). IbTX did not alter endothelial currents (−100 mV, control −13 ± 8 pA/pF and IbTX −15 ± 11 pA/pF, $n = 4$; +100 mV, control 39 ± 20 pA/pF and IbTX 40 ± 24 pA/pF, $n = 4$) (Fig. 1 A, b). Aortic vascular smooth muscle cells patched in the same conditions showed a large IbTX-sensitive outwardly rectifying current (≈275 pA/pF at +100 mV). These data are consistent with previous reports from native endothelium (Gauthier et al., 2002; Eichler et al., 2003) and suggest that aortic endothelial cells do not have functional BK_{Ca} channels.

Inward rectifier K^+ channels (K_{ir}) have been reported in cultured endothelial cell (Forsyth et al., 1997; Fang et al., 2005), and in some types of native endothelial cell preparations (von Beckerath et al., 1996; Crane et al., 2003; Fang et al., 2006). To evaluate the presence of K_{ir} currents in freshly isolated AECs, the cells were exposed to a high concentration (0.5 mM) of barium, a potent blocker of K_{ir} channels. The K^+ concentration of the superfusate was increased to 45 mM to elevate, if present, K_{ir} inward current at membrane potentials negative to E_K. Application of barium had no effect on the current evoked by a 200-ms voltage ramp from −100 to + 100 mV as illustrated by the mean traces in the absence (black trace) or presence (red trace) of 0.5 mM barium in Fig. 1 B ($n = 5$), suggesting that AECs do not express functional K_{ir} channels.

SK_{Ca} and IK_{Ca} currents have been measured in native endothelial cells (Burnham et al., 2002; Bychkov et al., 2002; Eichler et al., 2003; Taylor et al., 2003; Si et al., 2006). To examine properties of IK_{Ca} channels, the IK_{Ca} (and BK_{Ca}) channel blocker, ChTX (100 nM) was applied to AECs. Currents were evoked by a 200-ms voltage ramp from −100 to + 100 mV (Fig. 2 A). The current in cells dialyzed with 3 μM Ca^{2+} and 1 mM Mg^{2+} exhibited inward rectification and reversed near E_K ($E_{Rev} = -79 \pm 2$ mV; $E_K = -83$ mV), indicating that the membrane currents are largely carried by potassium ions. In contrast to IbTX, ChTX significantly reduced the currents (−25 ± 4% at + 80 mV, $n = 6$). The ChTX-sensitive current also exhibited marked inward rectification and reversed near E_K (Fig. 2 C; −79 ± 4 mV). Addition of the selective blocker of SK_{Ca} ($K_{Ca}2.1$-2.3) channels, Ap (300 nM), further reduced the current (−17 ± 3% of the current in the presence of ChTX at + 80 mV, $n = 5$). The Ap-sensitive current also exhibited inward rectification and reversed near E_K (Fig. 2 D; −83 ± 3 mV). The contribution of the ChTX-sensitive current, IK_{Ca}, to the total current in the presence of 3 μM Ca^{2+} and 1 mM Mg^{2+} was larger than the Ap-sensitive current, SK_{Ca} (4.2 ± 1.4 pA/pF and 2.4 ± 0.5 pA/pF at +80 mV for IK_{Ca} and SK_{Ca} currents, respectively; $P < 0.05$, $n = 6$ and 5).

SK_{Ca} and IK_{Ca} channel activation require intracellular calcium ($EC_{50} \approx 500$ nM; Kohler et al., 1996; Hirschberg

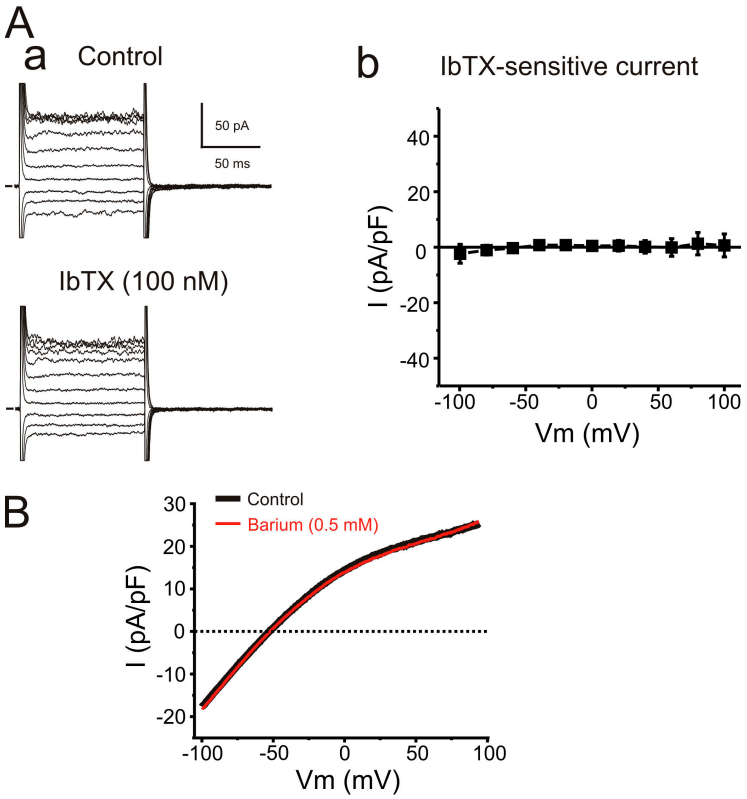

Figure 1. Functional BK_{Ca} or K_{ir} channels are not present in freshly isolated aortic endothelial cells. (Aa) Typical family of traces recorded from freshly isolated aortic endothelial cells dialyzed with a pipette solution containing 1 mM Mg^{2+} and 3 μM Ca^{2+} before (Control) or following application of 100 nM IbTX. From a holding potential of −50 mV, currents were evoked by 100-ms voltage steps from −100 to +100 mV in a 10-mV increment followed by a 100-ms repolarizing step to −50 mV. (Ab) Mean IbTX-sensitive current recorded from four experiments similar to the one shown in Aa. (B) Mean ramp currents in the absence (Control, black trace) and presence (Barium, red trace) of 0.5 mM barium ($n = 5$). These currents were elicited using 45 mM extracellular K^+ to emphasize potential K_{ir} currents. Currents were induced by a 200-ms voltage ramp protocol from −100 mV to +100 mV (HP = −60 mV).

et al., 1998). Therefore, the removal of intracellular calcium on endothelial currents was examined by dialyzing the cells with a Ca^{2+}-free intracellular solution containing 5 mM HEDTA (Mg^{2+} was maintained at 1 mM) (Fig. 2, A and B). Removing intracellular calcium significantly reduced the membrane current, and this remaining current was insensitive to ChTX and Ap (9.8 ± 0.9 pA/pF and 9.6 ± 1.3 pA/pF at +80 mV in the absence and presence of ChTX and Ap, respectively; $n = 4$). The membrane current in cells dialyzed with the 0 Ca^{2+} solution was similar in amplitude to the currents in cells dialyzed with 3 μM Ca^{2+} in the presence of ChTX and Ap (10 ± 1 pA/pF at +80 mV, $n = 5$). Interestingly, in all cases, the currents reversed near E_K, suggesting that a $[Ca^{2+}]_i$-insensitive potassium current contributes to the membrane current (Fig. 2, A and B). These results

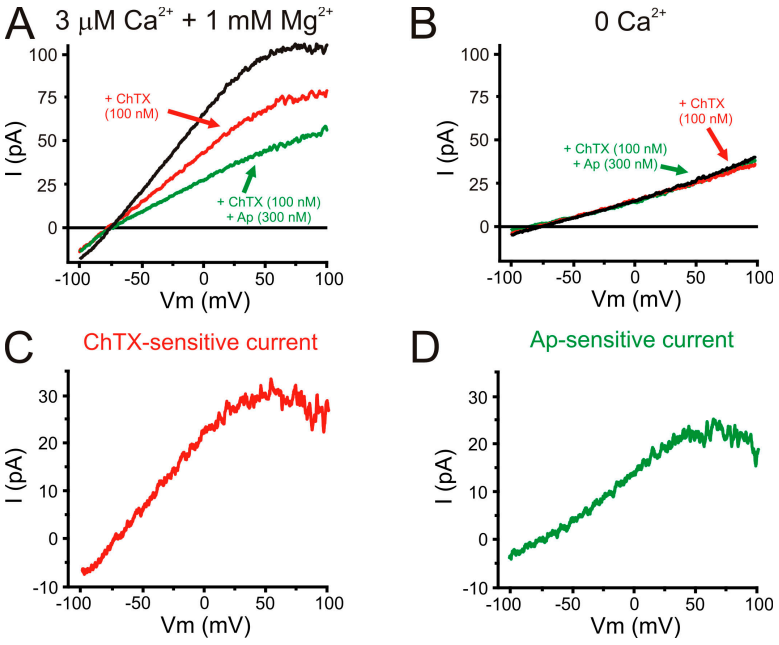

Figure 2. Contribution of Ca^{2+}-dependent currents, SK_{Ca} and IK_{Ca}, to membrane currents in AECs. (A) Examples of ramp currents in the absence (black trace; E_{rev} = −78.4 mV) or in presence of 100 nM ChTX, an IK_{Ca} inhibitor, alone (ChTX, red trace, E_{rev} = −74.5 mV) or in combination with 300 nM Ap, a SK_{Ca} inhibitor (Ap, green trace, E_{rev} = −75.6 mV). The AEC was dialyzed with a pipette solution containing 5 mM HEDTA with 1 mM free Mg^{2+} and 3 μM free Ca^{2+}. (B) Recording of currents as in A except that the cell was dialyzed without addition of Ca^{2+}. IK_{Ca} and SK_{Ca} currents from the experiment presented in A were obtained as the ChTX- (C, E_{rev} = −75.4 mV) and Ap-sensitive (D, E_{rev} = −76.2 mV) currents, respectively. Currents were evoked by a 200-ms voltage ramp protocol from −100 to + 100 mV (HP = −60 mV).

indicate that SK_{Ca}, IK_{Ca}, and Ca^{2+}-insensitive K^+ conductances contribute significantly to the overall membrane conductance of isolated AECs.

The Basis of the Apparent Voltage Dependence of IK_{Ca} and SK_{Ca}: Block by Intracellular Mg^{2+} and Ca^{2+}

SK_{Ca} and IK_{Ca} channels do not exhibit intrinsic voltage dependence, and therefore, in a physiological potassium gradient, their current–voltage relationship should exhibit theoretical outward rectification. The decrease in current and increase in current noise at positive potentials suggest that intracellular cations are blocking IK_{Ca} and SK_{Ca} channels in a voltage-dependent manner (Fig. 2, C and D). To investigate the involvement of intracellular divalent cations in the apparent voltage dependence of IK_{Ca} and SK_{Ca} currents, AECs were dialyzed with pipette solutions containing different concentrations of free Mg^{2+} and Ca^{2+}, and ChTX- and Ap-sensitive currents were analyzed. As the IK_{Ca} and SK_{Ca} currents are relatively small and intracellular calcium is required for activation, it was not possible to evaluate intracellular calcium block <1 μM. Therefore, SK_{Ca} and IK_{Ca} currents were compared with the predictions from the Goldman-Hodgkin-Katz (GHK) constant field equation. The relationship between any type of current, in this case a macroscopic K^+ current, and the membrane potential can be described by the appropriately modified GHK constant field equation as follows:

$$I_K = N_K P_K \bullet \frac{EF^2}{RT} \bullet \frac{[K]_o - [K]_i \bullet e^{(EF/(RT))}}{1 - e^{(EF/(RT))}}, \quad (1)$$

where I_K is the K^+ current density in pA/pF, N_K is the channel density (channels/pF), P_K is the permeability of a channel to K^+ ions in cm/s, $[K]_{o/i}$ is the concentration of K^+ in the extracellular or intracellular compartments, E is the membrane potential in V, F is the Faraday's constant, R is the gas constant, and T the temperature in Kelvin. The permeability constants were calculated using a simplified GHK equation (Benham et al., 1986) and using a single channel conductance of 40 and 10 pS for IK_{Ca} and SK_{Ca}, respectively (Kohler et al., 1996; Ishii et al., 1997), in the presence of symmetrical K^+ (120 mM). The permeability constants of IK_{Ca} and SK_{Ca} channels obtained were 8.7872 E − 14 and 2.1968 E − 14 cm/s, respectively. Based on the results presented below, the inward rectification of IK_{Ca} and SK_{Ca} is due to intracellular block of the pore by Ca^{2+} and Mg^{2+}, and membrane potential depolarization drives the blocking ion into the intracellular side of the pore. Therefore, the inward component of the ramp-evoked current (i.e., negative to E_K) is assumed not to experience much block. This region was fit with the Eq. 1 and then extrapolated up to + 100 mV to define the current–voltage relationship in the absence of blocking ions.

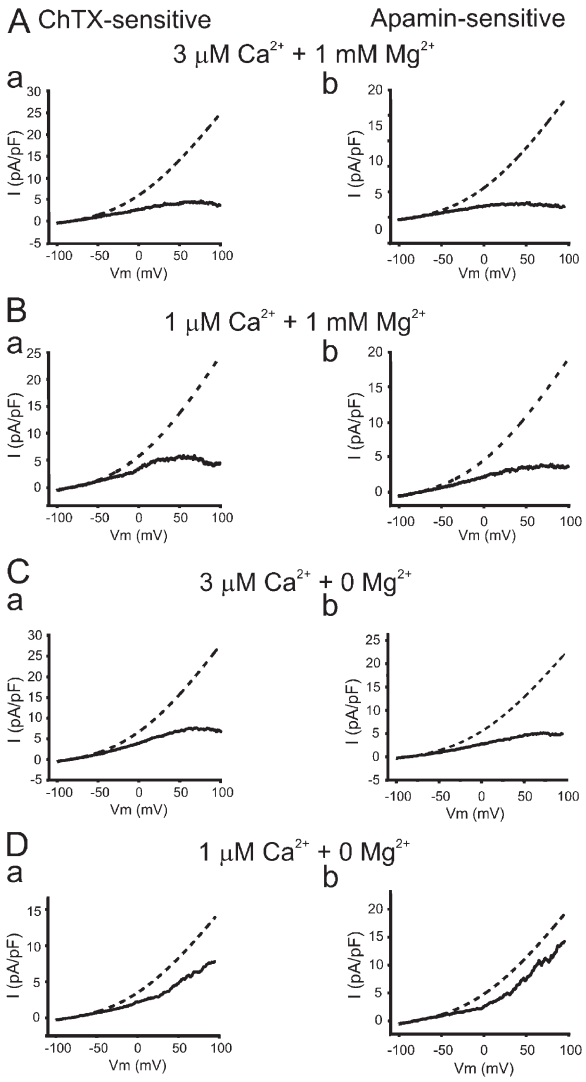

Figure 3. The inward rectification of IK_{Ca} and SK_{Ca} is caused by intracellular Mg^{2+} and Ca^{2+}. (A and B) Mean IK_{Ca} (a) and SK_{Ca} (b) currents (filled lines) elicited by voltage ramp protocol in AECs dialyzed with 1 mM free Mg^{2+} and (A) 3 μM free Ca^{2+} (n = 6 and 5, respectively) or (B) 1 μM free Ca^{2+} (n = 5 and 5). Predictions from the GHK constant field equation (dashed lines) were obtained by fitting the mean inward currents of IK_{Ca} (a) and SK_{Ca} (b) of AECs (filled lines) in each condition. (C and D) Similar to A and B except that endothelial cells were dialyzed with a Mg^{2+}-free ($[Mg^{2+}]$ = 0) pipette solution containing either (C) 3 μM free Ca^{2+} (n = 4 and 4) or (D) 1 μM Mg^{2+} (n = 4 and 5 for IK_{Ca} and SK_{Ca}, respectively). Predictions from the GHK constant field equation (dashed lines) were obtained by fitting the mean inward currents of IK_{Ca} (a) and SK_{Ca} (b) of AECs (filled lines) in each condition. Currents were evoked by a 200-ms voltage ramp protocol from −100 to + 100 mV (HP = −60 mV). $[K]_o$, 6 mM; $[K]_i$, 150 mM; and P_K, 8.7872 E − 14 and 2.1968 E − 14 for IK_{Ca} and SK_{Ca}, respectively.

Removal of internal Mg^{2+} in the presence of 3 μM intracellular Ca^{2+} increased SK_{Ca} and IK_{Ca} currents but did not eliminate inward rectification (Fig. 3, A and C, solid lines). The absence of intracellular Mg^{2+} resulted in an approximate twofold increase in the outward current amplitude at

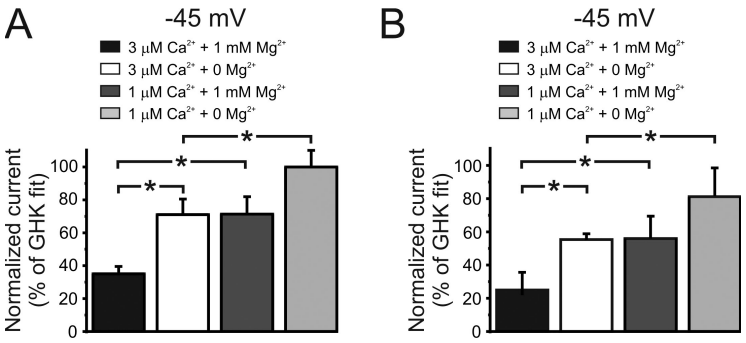

Figure 4. Comparison between native and theoretical IK_{Ca} and SK_{Ca} currents. IK_{Ca} (A) and SK_{Ca} (B) currents at −45 mV in cells dialyzed with different pipette solutions normalized to the GHK predictions depicted in Fig. 3. For 1 mM Mg^{2+} and 3 μM Ca^{2+} (% of GHK fit): IK_{Ca} (35 ± 4) and SK_{Ca} (25 ± 10); for 0 Mg^{2+} and 1 μM Ca^{2+} (mM): IK_{Ca} (71 ± 9) and SK_{Ca} (55 ± 5); for 1 mM Mg^{2+} and 3 μM Ca^{2+} (mM): IK_{Ca} (71 ± 11) and SK_{Ca} (56 ± 13); for 0 Mg^{2+} and 1 μM Ca^{2+} (mM): IK_{Ca} (99 ± 10) and SK_{Ca} (80 ± 17). *, $P < 0.05$.

+80 mV for both IK_{Ca} (4.4 ± 1.8 pA/pF with 1 mM Mg^{2+} and 7.4 ± 0.3 pA/pF without Mg^{2+}, respectively; $P < 0.05$, n = 6 and 5) and SK_{Ca} (2.8 ± 0.5 pA/pF with 1 mM Mg^{2+} and 4.9 ± 0.8 pA/pF without Mg^{2+}, $P < 0.05$, n = 5 and 5). These data support the concept that voltage-dependent block of SK_{Ca} and IK_{Ca} channels by intracellular Mg^{2+} is a mechanism contributing to the voltage-dependent decrease in potassium efflux through these channels.

To examine the effect of intracellular Ca^{2+} on the rectification properties of IK_{Ca} and SK_{Ca}, pipette Ca^{2+} was lowered from 3 to 1 μM, which should still cause near-maximal activation of SK_{Ca} and IK_{Ca} channels. As depicted in Fig. 3 B, lowering intracellular Ca^{2+} from 3 to 1 μM, in the presence of 1 mM Mg^{2+}, increased both ChTX- and Ap-sensitive currents, IK_{Ca} and SK_{Ca}, respectively. Similarly, lowering intracellular Ca^{2+} from 3 to 1 μM in the absence of internal Mg^{2+} dramatically increased SK_{Ca} and IK_{Ca} currents (Fig. 3 D, solid lines). The amplitude of IK_{Ca} and SK_{Ca} currents recorded at +80 mV in the presence of 1 μM Ca^{2+} are 14 ± 3 and 9 ± 2 pA/pF, respectively (n = 5 and 6). Under these conditions, both IK_{Ca} and SK_{Ca} currents now exhibited theoretical outward rectification. These results indicate that intracellular calcium between 1 and 3 μM caused substantial voltage-dependent block of IK_{Ca} and SK_{Ca} channels. Thus, it appears that intracellular calcium both activates, via calmodulin, and blocks IK_{Ca} and SK_{Ca} channels.

The theoretical currents (dashed) and measured currents (solid) for IK_{Ca} and SK_{Ca} are shown on Fig. 3 in the presence (A and B, with 3 and 1 μM Ca^{2+}, respectively) and absence of Mg^{2+} (C and D, with 3 and 1 μM Ca^{2+}, respectively). With free intracellular Ca^{2+} set to 3 μM, the current–voltage relationships of IK_{Ca} and SK_{Ca} were significantly smaller than that predicted from the GHK equation (Fig. 3 C, a and b). Since the physiological membrane potentials of endothelium spans from −55 to −35 mV (Chen and Cheung, 1992; Burnham et al., 2002; Taylor et al., 2003; Eichler et al., 2003; Weston et al., 2005), the impact of intracellular cations on SK_{Ca} and IK_{Ca} was further analyzed at −45 mV. Fig. 4 shows that in the presence of Mg^{2+} (1 mM) and elevated Ca^{2+} (3 μM), the currents were dramatically smaller compared with the predicted GHK at −45 mV (35 ± 4% and 25 ± 10% of the predicted GHK current for IK_{Ca} and SK_{Ca}, respectively; n = 6 and 5). Lowering intracellular calcium to 1 μM in the presence of Mg^{2+} (1 mM) increased IK_{Ca} and SK_{Ca} currents closer to the currents predicted by the GHK equation (Fig. 4). In 1 μM Ca^{2+} and in the absence of Mg^{2+}, the currents exhibited almost theoretical outward rectification (Fig. 3 D). Under these conditions, IK_{Ca} and SK_{Ca} currents were 99 ± 10% and 80 ± 17%, respectively (n = 4 and 5) of the predicted GHK current at −45 mV (Fig. 4).

The numbers of IK_{Ca} and SK_{Ca} channels in a single endothelial cell are not known. The IK_{Ca} and SK_{Ca} channel density (N_K) can be estimated by the fit of Eq. 1 to the macroscopic currents shown in Fig. 3. However, since the macroscopic current is a function of the open state probability (P_o), the estimated N_K was corrected for the P_o of the channels. A maximal open state probability ($P_{o(max)}$) of 0.6 was used, based on the measurements of Hirschberg et al. (1998). Using this value of P_{omax}, the density of IK_{Ca} and SK_{Ca} channels in a single endothelial cell was then estimated to be 9.4 ± 0.1 and 29.0 ± 0.3 channels/100 μm^2, respectively. A single endothelial cell would then have ≈99 IK_{Ca} and ≈307 SK_{Ca} functional channels in its cell membrane.

Intracellular Block of Channel Pore

The inward rectification of IK_{Ca} and SK_{Ca} by Mg^{2+} and Ca^{2+} ions could be interpreted as divalent cations binding to a site within the pore, thus preventing passage of K^+ ions. Using the simplest model, the binding site would be inside the pore and accessible from the cytoplasm. According to the Woodhull model (Woodhull, 1973), the blocking cation penetrates partway through the electrical field of the membrane. Therefore, depolarization will favor penetration of the blocking cations into the permeation pathway and thereby decrease K^+ efflux. The blocking process is analogous to the well-described block of K_{ir} channels by extracellular barium in smooth muscle (Quayle et al., 1993). The apparent K_d of the blocking intracellular divalent cation can be estimated using the following relation:

$$\left(\frac{I_{blocked}}{I_{max}}\right) = \frac{1}{1+\frac{[divalent]}{K_d}}, \quad (2)$$

where K_d is the apparent dissociation constant, [divalent] is the intracellular concentration of the blocking divalent cation, and I_{max} and $I_{blocked}$ are the currents recorded with nominally zero and the highest intracellular concentration of the blocking cation, respectively. Since intracellular Ca^{2+} cannot be nominally zero, the current value from the GHK equation was used as I_{max} for Ca^{2+}. The values obtained were then plotted as a function of the membrane potential as depicted in Fig. 5 for the SK_{Ca} channels. The apparent K_d is an exponential function of the membrane potential as expected for an ion binding site within the membrane voltage field. The relationship between the apparent K_d and the membrane potential between +40 and +90 mV was fit with the following relation:

$$K_d(V) = \frac{K_d(0)}{e^{\left(\frac{zF(1-\theta)E}{RT}\right)}}, \quad (3)$$

where $K_d(0)$ is the K_d at 0 mV, z is the valence of the ion, θ is the slope factor representing the sensitivity of the K_d to the applied voltage from the inside of the membrane, and F, E, R, and T have their usual meanings. The $K_d(0)$ for Mg^{2+} obtained from the fitted lines are 2 and 3 mM for IK_{Ca} and SK_{Ca} with a slope factor of -0.10 and -0.20, respectively. For Ca^{2+}, a lower $K_d(0)$ was isolated from the fitted lines, 5 and 3 μM ($\theta = -0.17$ and -0.20) for IK_{Ca} and SK_{Ca}, respectively. These results are consistent with our findings suggesting that Ca^{2+} is more effective at blocking the pore than Mg^{2+}. Also, the slope factor values suggest that IK_{Ca} and SK_{Ca} channels have similar Mg^{2+} and Ca^{2+} binding sites within their channel pores.

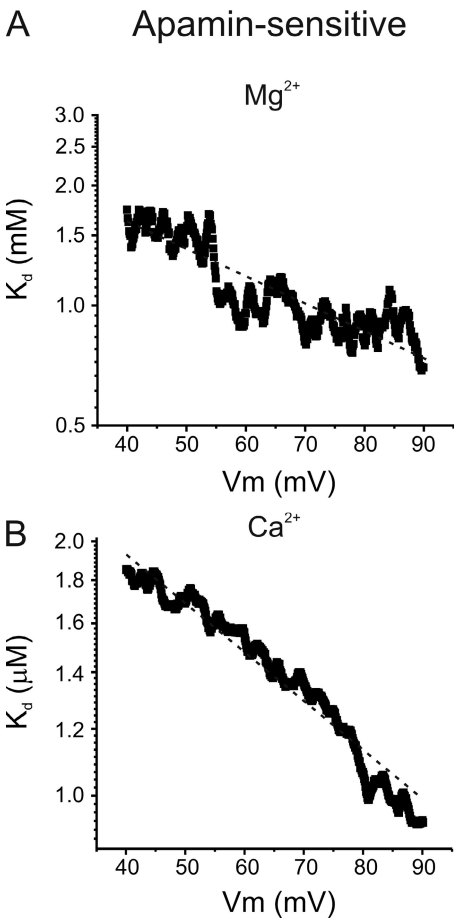

Figure 5. Voltage dependence of Mg^{2+} and Ca^{2+} block of SK_{Ca}. (A) Voltage dependence of the apparent K_d of Mg^{2+} calculated with Eq. 2. Dashed line was fitted to data with Eq. 3, with a K_d at 0 mV of 3 mM and θ (slope factor indicating voltage sensitivity of K_d) = −0.20. (B) Voltage dependence of the apparent K_d of Ca^{2+} calculated with Eq. 2. Dashed line was fitted to data with Eq. 3, with a K_d at 0 mV of 3 μM and θ = −0.20.

Ca^{2+}-activated K^+ Channels and their Impact on Resting Membrane Potential and Intracellular Ca^{2+} Levels

Membrane potential regulates Ca^{2+} entry, and hence intracellular Ca^{2+}, in endothelial cells through changes in the Ca^{2+} electrochemical gradient (Luckhoff and Busse, 1990). Although previous studies have demonstrated the role of IK_{Ca} and SK_{Ca} channels in the regulation of endothelial $[Ca^{2+}]_i$ following stimulation by ACh, bradykinin, or substance P (Eichler et al., 2003; Weston et al., 2005), the impact of these channels on membrane potential and $[Ca^{2+}]_i$ under resting conditions remains unclear. To investigate the impact of IK_{Ca} and SK_{Ca} channels on the basal endothelial $[Ca^{2+}]_i$ and membrane potential, intact endothelium preferentially loaded with the Ca^{2+} indicator Fluo-4 was used to simultaneously monitor the intracellular Ca^{2+} levels and membrane potential in cut-open mesenteric arteries (Fig. 6). The resting membrane potential of endothelium measured in current clamp mode of the perforated patch clamp technique was found to be -52 ± 2 mV ($n = 6$ vessels). Exposure to ChTX (100 nM) depolarized the endothelial membrane potential by 8 ± 1 mV ($n = 6$). Addition of Ap (300 nM) to the ChTX superfusate further depolarized the membrane potential by 3.1 ± 0.4 mV ($n = 6$). ChTX, alone, and ChTX + Ap significantly reduced Ca^{2+} fluorescence $22 \pm 5\%$ and $43 \pm 8\%$ ($n = 6$ and 6). The exposure of vessels to an extracellular solution containing 60 mM K^+ depolarized the endothelium to a membrane potential of -17 ± 1 mV, close to E_K (-20 mV), and was associated with a $78 \pm 4\%$ decrease in Ca^{2+} fluorescence ($n = 5$).

The fluorescent Ca^{2+} indicator, Fluo-4, provides fractional changes in intracellular Ca^{2+}. To provide a sense of changes in intracellular Ca^{2+}, a baseline (F_o) value of intracellular Ca^{2+} previously measured with ratiometric Ca^{2+} indicator Fura-2 was used (Knot et al., 1999) in conjunction with the following equation (Jaggar et al., 1998):

$$[Ca^{2+}] = \frac{KR}{\frac{K}{[Ca^{2+}]_{Fo}} + 1 + R}, \quad (4)$$

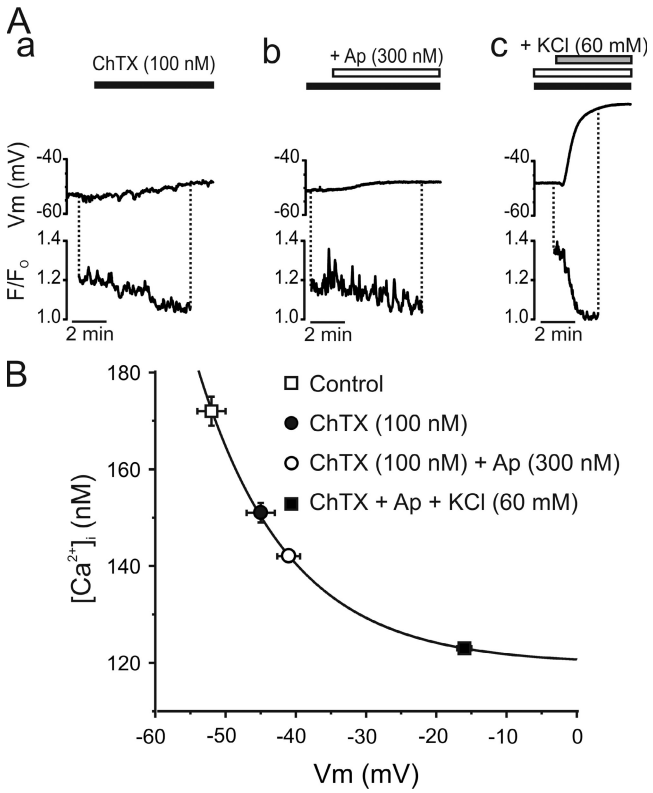

Figure 6. IK_{Ca} and SK_{Ca} regulate intracellular Ca^{2+} of resting endothelium in mesenteric arteries. (A) Perforated patch clamp was used to record membrane potential of Fluo-4–loaded endothelium to simultaneously measure changes in intracellular Ca^{2+}. Time course graph illustrating the membrane potential and relative fluorescence of resting endothelium exposed to ChTX alone (black bars), with the addition of Ap (+Ap; white bars), and following the addition of 60 mM KCl (KCl; gray bars). (B) Voltage dependence of intracellular Ca^{2+} concentration from nonstimulated endothelium. Intracellular Ca^{2+} concentrations in each condition were estimated with Eq. 4 using $[Ca^{2+}]_{Fo}$ = 123 nM in the presence of 60 mM KCl (Knot et al., 1999) and associated with the corresponding membrane potential recorded. The value used for the K_d of Fluo-4 at 30°C was 370 nM (Woodruff et al., 2002). Solid line was fitted to data with the function Y = A*exp(−x/t) + B, where B = 120 ± 1 nM, A = 0.9 ± 0.4, and t = 13 ± 2.

where K is the K_d of Fluo-4 for Ca^{2+}, R is the fractional change in fluorescence (F/F_o), $[Ca^{2+}]_{Fo}$ is the Ca^{2+} concentration (measured with Fura-2; Knot et al., 1999) at F_o and $[Ca^{2+}]$ is the endothelial intracellular Ca^{2+}. The $[Ca^{2+}]_i$ previously measured in the presence of 60 mM extracellular K^+ in rat endothelium with the ratiometric Ca^{2+} indicator Fura-2 was 123 nM (Knot et al., 1999). The same approach was used to estimate $[Ca^{2+}]$ in the presence of ChTX and Ap. This approach yielded a $[Ca^{2+}]_i$ in physiological external potassium of 176 nM, which was similar to the value previously obtained with Fura-2 (174 nM) in rat coronary artery endothelium (Knot et al., 1999). Membrane depolarization induced by blocking IK_{Ca} and SK_{Ca} channels reduced $[Ca^{2+}]_i$ (Fig. 6 B) to a similar level as obtained previously with elevations of external potassium (Knot et al., 1999). Thus, blocking IK_{Ca} and SK_{Ca} channels causes a membrane potential depolarization and a decrease in endothelial calcium in the absence of an agonist.

DISCUSSION

Endogenous Currents in Native Endothelium

Most studies on endothelial ion channels have been performed on cultured cells in which phenotypical changes might be induced by culture media (Kestler et al., 1998; Jow et al., 1999). Although the presence of K_{ir} currents has been reported in cultured endothelium (Shimoda et al., 2002; Fang et al., 2005) and in endothelium isolated from some species (von Beckerath et al., 1996; Fang et al., 2006; Crane et al., 2003), we did not detect K_{ir} currents in endothelial cells freshly isolated from mouse aorta. In agreement with Gauthier et al. (2002), we found that freshly isolated endothelial cells do not exhibit BK_{Ca} currents. Interestingly, BK_{Ca} channel expression was shown to be induced in human endothelial cells by culture passage (Kestler et al., 1998; Jow et al., 1999). However, BK_{Ca} current and channel expression have been reported in porcine and rabbit endothelium (Rusko et al., 1992; Papassotiriou et al., 2000), suggesting that the expression of BK_{Ca} channels may be species or vascular bed dependent.

The current–voltage relationship of AECs suggested that the majority of membrane current was carried by potassium channels. When cells were dialyzed with 3 μM Ca^{2+}, ChTX and Ap, but not IbTX, inhibited a substantial fraction (44%) of the K^+ current at +80 mV, indicating that a significant portion of the potassium conductance is carried by SK_{Ca} and IK_{Ca} channels. In the absence of intracellular Ca^{2+}, the K^+ currents were significantly reduced, and ChTX and Ap had no effect. Therefore, local Ca^{2+} influx in the absence of intracellular Ca^{2+} does not seem to be sufficient to activate a substantial number of ChTX- and Ap-sensitive channels. However, the remaining current reversed at the potassium equilibrium potential, indicating that another type of potassium conductance is also present in AECs, and that this current would be more prominent at low intracellular calcium levels. K_{ATP} and two-pore-domain K^+ channels have been reported in endothelial cells (Janigro et al., 1993; Chatterjee et al., 2003; Garry et al., 2007) and are possible candidates for the Ca^{2+}-independent potassium channel. In contrast, vascular myocytes have prominent BK_{Ca} currents, K_V and voltage-dependent Ca^{2+} channels, and do not exhibit SK_{Ca} and IK_{Ca} currents (Thorneloe and Nelson, 2005; Ledoux et al., 2006).

Ca^{2+}-activated K^+ Currents in Aortic Endothelial Cells

It has been well established that the ChTX- and Ap-sensitive Ca^{2+}-dependent K^+ currents recorded in native

endothelial cells (IK_{Ca} and SK_{Ca} currents) are carried by $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, respectively (Kohler and Hoyer, 2007). Although the concentration of Ap used (300 nM) is sufficient to inhibit all three $K_{Ca}2.x$ isoforms (Ledoux et al., 2006), the endothelial $K_{Ca}2.x$ current has previously been shown using molecular biology, immunohistological, and electrophysiological approaches to result from the functional expression of $K_{Ca}2.3$ channels (Kohler et al., 2001; Burnham et al., 2002; Bychkov et al., 2002; Eichler et al., 2003; Taylor et al., 2003; Burnham et al., 2006; McNeish et al., 2006; Si et al., 2006; Sandow and Tare, 2007). Although well characterized in expression system, little information is available on endogenous endothelial $K_{Ca}2.3$ and $K_{Ca}3.1$ currents. The channel density of $K_{Ca}3.1$ and $K_{Ca}2.3$ in AECs was estimated to be ≈10 and ≈29 channels/100 μm^2 or ≈99 and ≈307 channels/cell, respectively. Even with a lower channel density, $K_{Ca}3.1$ current amplitude appears to be ≈1.75-fold larger than the $K_{Ca}2.3$ current, which reflects the higher single channel conductance of $K_{Ca}3.1$ channels. In contrast with our findings, Si et al. (2006) reported that $K_{Ca}2.3$ and $K_{Ca}3.1$ currents from mouse AECs had similar amplitude. The inward-rectifying current–voltage relationship of the Ca^{2+}-dependent currents in AECs is similar to what has been reported for heterologous expression of $K_{Ca}3.1$ and $K_{Ca}2.3$ (Kohler et al., 1996; Ishii et al., 1997; Xia et al., 1998; Castle et al., 2003) and in endothelium (Eichler et al., 2003; Taylor et al., 2003; Si et al., 2006). Inward rectification was also observed in AECs perforated patch cells (unpublished data), suggesting that $K_{Ca}3.1$ and $K_{Ca}2.3$ channels present the same characteristic I/V relationship with unaltered cytoplasm content.

Inward Rectification and Intracellular Divalent Cations

One aim of our study was to investigate the role of physiological intracellular divalent cations (Mg^{2+} and Ca^{2+}) in the regulation of endothelial $K_{Ca}3.1$ and $K_{Ca}2.3$ channels in native endothelial cells, using physiological levels of potassium. Intracellular divalent cations (Ba^{2+}, Sr^{2+}, Mg^{2+}, Ca^{2+}) have been shown to block rat $K_{Ca}2.2$ channels exogenously expressed in *Xenopus* oocytes (Soh and Park, 2001, 2002). The authors found that the apparent dissociation constant (K_d) for intracellular Ca^{2+} and Mg^{2+} at +90 mV for $K_{Ca}2.2$ channels was 19.3 and 180 µM, respectively (Soh and Park, 2002). In contrast, our data would indicate an apparent K_d for Mg^{2+} at +90 mV of 1.2 and 0.7 mM for $K_{Ca}3.1$ and $K_{Ca}2.3$, respectively. Soh and Park (2001) reported that Ca^{2+} slightly reduces the apparent affinity for Mg^{2+} between 0.4 and 2 µM $[Ca^{2+}]_i$ (K_d for Mg^{2+} from 130 to 180 µM in the presence of 0.4 and 2 µM Ca^{2+}, respectively). To minimize the impact of Ca^{2+} on the apparent K_d for Mg^{2+}, the experimental data used in the present study to calculate the apparent K_d for Mg^{2+} were obtained with solutions containing identical Ca^{2+} concentration (3 µM). Also at +90 mV, our results indicate an apparent K_d for Ca^{2+} (in the absence of Mg^{2+}) of 1.3 and 0.99 µM for $K_{Ca}3.1$ and $K_{Ca}2.3$, respectively. The inhibitory binding site for Mg^{2+} and Ca^{2+} is within 10–20% of the electrical field sensed by the ion from the cytoplasmic side of the plasma membrane, a value similar to the reported δ for rat $K_{Ca}2.2$ (17%) (Soh and Park, 2001, 2002). Our findings are in agreement with the idea that the divalent ion binding site resides inside the channel pore closer to the cytoplasmic side (Soh and Park, 2002). Soh and Park (2002), based on functional analysis of mutations, identified residue Ser-359 in the pore region of $K_{Ca}2.2$ as being critical for rectification. Interestingly, Ser-359 appears to be conserved in $K_{Ca}2.3$ channels, but not $K_{Ca}3.1$ (Soh and Park, 2002).

Our results suggest that elevation of intracellular Ca^{2+} levels can considerably decrease the current at −45 mV, a physiological membrane potential (Chen and Cheung, 1992; Marchenko and Sage, 1993; Taylor et al., 2003). Indeed, decreasing $[Ca^{2+}]_i$ from 3 to 1 µM significantly increased $K_{Ca}3.1$ and $K_{Ca}2.3$ currents, even though the channels should be maximally activated at 1 µM $[Ca^{2+}]_i$ (EC_{50} ≈ 500 nM) (Kohler et al., 1996; Hirschberg et al., 1998). It is clear then that depending on their concentration, intracellular Ca^{2+} ions could potentially have opposing effects in the regulation of membrane potential by $K_{Ca}3.1$ and $K_{Ca}2.3$ channels. Furthermore, voltage-dependent block by intracellular Ca^{2+} and Mg^{2+} would modulate $K_{Ca}3.1$ and $K_{Ca}2.3$ currents, and thus their contributions to membrane conductance.

Following endothelial stimulation, $K_{Ca}3.1$ and $K_{Ca}2.3$ channel activation would increase Ca^{2+} influx through membrane potential hyperpolarization. This $[Ca^{2+}]_i$ rise would further increase the activity of $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, generating a positive feedback loop (Garland et al., 1995; Feletou and Vanhoutte, 2006; Ledoux et al., 2006). Membrane potential hyperpolarization would relieve intracellular Mg^{2+} and Ca^{2+} block, and thus increase contribution of $K_{Ca}3.1$ and $K_{Ca}2.3$ channels to the membrane conductance. However, the rise in intracellular calcium would block $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, and thus decrease channel conductance. The degree of intracellular calcium block would therefore depend on the membrane potential and the level of calcium sensed by the channels. Indeed, recent evidence indicates that the endothelium has localized submembrane microdomains where the local Ca^{2+} level is higher at the cell membrane (Isshiki et al., 2004; Ledoux, J., A.D. Bonev, and M.T. Nelson. 2007. *FASEB J.* 21:745.18). Recent evidence also suggests a differential localization of $K_{Ca}3.1$ and $K_{Ca}2.3$ that might increase the degree of complexity in the regulation of the channels (Absi et al., 2007).

Regulation of Basal Membrane Potential and Intracellular Ca^{2+} by $K_{Ca}3.1$ and $K_{Ca}2.3$ Channels

In intact endothelium, the resting membrane potential, measured with microelectrodes (Chen and Cheung, 1992;

Burnham et al., 2002; Taylor et al., 2003) or the patch clamp technique (this study; Burnham et al., 2002; Eichler et al., 2003; Weston et al., 2005), is −55 to −35 mV, and can be hyperpolarized to ≈−75 mV by $K_{Ca}3.1$ and $K_{Ca}2.3$ stimulators (Quignard et al., 2000; Burnham et al., 2002) or endothelial agonists (e.g., ACh, substance P, bradykinin) (Chen and Cheung, 1992; Marchenko and Sage, 1993; Quignard et al., 2000; Burnham et al., 2002; Eichler et al., 2003; Weston et al., 2005). The induced hyperpolarizations appear to be mediated by activation of $K_{Ca}3.1$ and $K_{Ca}2.3$ channels, since they are inhibited by blockers of these channels or targeted gene disruption (Kohler and Hoyer, 2007).

The resting membrane potential in intact endothelium is significantly positive to E_K, indicating that sodium- or chloride-permeable channels also contribute to the membrane potential in the intact endothelium. This depolarizing current may be mediated by TRP (transient receptor potential) channels expressed in endothelial cells (Nilius et al., 2003; Yao and Garland, 2005). However, only K^+ currents were detected in isolated AECs, suggesting that cell isolation or lower temperature affected the functionality of channels that mediate the depolarizing current.

Little is known about the role of $K_{Ca}3.1$ and $K_{Ca}2.3$ channels in the regulation of endothelium membrane potential and intracellular Ca^{2+} in the absence of agonists. Block of $K_{Ca}2.3$ channels by Ap or by silencing its gene caused a 15-mV depolarization of the membrane potential of mesenteric artery endothelial cells in a mouse model that conditionally overexpresses $K_{Ca}2.3$ channels (Taylor et al., 2003). Here, we show that blocking $K_{Ca}3.1$ channels with ChTX depolarizes the endothelium membrane by ≈8 mV, and blocking $K_{Ca}2.3$ channels causes an additional ≈3 mV depolarization (Fig. 6). Therefore, $K_{Ca}3.1$ and $K_{Ca}2.3$ channels likely regulate endothelial function in the absence and presence of agonists.

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