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Inward Rectifier K⁺ Currents and Kir2.1 Expression in Renal Afferent and Efferent Arterioles

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Hyposmotic challenge inhibits inward rectifying K⁺ channels in cerebral arterial smooth muscle cells

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Wu BN, Luykenaar KD, Brayden JE, Giles WR, Corteling RL, Wiehler WB, Welsh DG. Hyposmotic challenge inhibits inward rectifying K⁺ channels in cerebral arterial smooth muscle cells. *Am J Physiol Heart Circ Physiol* 292: H1085–H1094, 2007. First published October 20, 2006; doi:10.1152/ajpheart.00926.2006.—This study sought to define whether inward rectifying K⁺ (K_{IR}) channels were modulated by vasoactive stimuli known to depolarize and constrict intact cerebral arteries. Using pressure myography and patch-clamp electrophysiology, initial experiments revealed a Ba²⁺-sensitive K_{IR} current in cerebral arterial smooth muscle cells that was active over a physiological range of membrane potentials and whose inhibition led to arterial depolarization and constriction. Real-time PCR, Western blot, and immunohistochemical analyses established the expression of both K_{IR2.1} and K_{IR2.2} in cerebral arterial smooth muscle cells. Vasoconstrictor agonists known to depolarize and constrict rat cerebral arteries, including uridine triphosphate, U46619, and 5-HT, had no discernable effect on whole cell K_{IR} activity. Control experiments confirmed that vasoconstrictor agonists could inhibit the voltage-dependent delayed rectifier K⁺ (K_{DR}) current. In contrast to these observations, a hyposmotic challenge that activates mechanosensitive ion channels elicited a rapid and sustained inhibition of the K_{IR} but not the K_{DR} current. The hyposmotic-induced inhibition of K_{IR} was 1) mimicked by phorbol-12-myristate-13-acetate, a PKC agonist; and 2) inhibited by calphostin C, a PKC inhibitor. These findings suggest that, by modulating PKC, mechanical stimuli can regulate K_{IR} activity and consequently the electrical and mechanical state of intact cerebral arteries. We propose that the mechanoregulation of K_{IR} channels plays a role in the development of myogenic tone.

cerebral arteries; potassium channels; vasoconstrictor stimuli

THE MAGNITUDE AND DISTRIBUTION of tissue blood flow is controlled by an integrated network of resistance arteries. Under dynamic conditions, tone within an arterial network is regulated by multiple stimuli initiated by changes in intraluminal pressure (15, 22, 43), blood flow (12), neuronal activity (37), and tissue metabolism (14). These vasoactive stimuli elicit diameter responses by activating transduction pathways that alter the Ca²⁺ sensitivity of the myofilaments (39), Ca²⁺ release from internal stores (31), and/or Ca²⁺ influx through voltage-operated Ca²⁺ channels (17, 25, 41). The activity of voltage-operated Ca²⁺ channels is in turn coupled to resting membrane potential (V_M).

In vascular smooth muscle, V_M is determined by a dynamic interplay between depolarizing inward and hyperpolarizing

outward currents. While inward current is governed by chloride and sodium conductances, outward current is principally driven by voltage-gated (K_V), Ca²⁺-activated (BK_{Ca}), ATP-sensitive (K_{ATP}), and inward rectifying (K_{IR}) K⁺ channels (27, 34). Past studies have carefully characterized the regulatory and compositional properties of several smooth muscle conductances. Despite this work, a number of conductances including K_{IR} have received limited experimental analysis. K_{IR} channels are aptly named for their ability to pass current more readily in the inward direction. To date, molecular approaches have identified seven subfamilies, including those that encode for weakly rectifying (K_{IR1.x}), strongly rectifying (K_{IR2.x}), G protein-coupled (K_{IR3.x}), and K_{ATP} [K_{IR6.x} + sulfonylurea receptor (SUR) subunit] K⁺ channels (2, 5). In the cerebral circulation, smooth muscle K_{IR} channels are potentially blocked by micromolar Ba²⁺, activated by extracellular K⁺, and thought to be principally composed of K_{IR2.1} subunits (3, 33). Since their initial identification (7), cerebral arterial K_{IR} channels generally have been regarded as a tonic background conductance (27, 40). As such, they are presumed to be unresponsive to a range of agonists and mechanical perturbations that constrict arteries through mechanisms that partly depend on electromechanical coupling. While recent studies have started to challenge this perception (29, 30), experimental observations supporting the concept of smooth muscle K_{IR} channel regulation by vasoactive stimuli remain limited.

The present study examined whether vasoactive stimuli known to depolarize and constrict cerebral arteries suppress K_{IR} channel activity. Using functional, electrical, and molecular approaches, we confirmed that K_{IR} channels are 1) present in cerebral arterial smooth muscle cells, 2) active over a physiological V_M range, and 3) likely composed of both K_{IR2.1} and K_{IR2.2} subunits. Subsequent experiments demonstrated that whole cell K_{IR} activity was unaffected by several vasoconstrictor agonists including uridine triphosphate (UTP), U46619, and 5-HT. In contrast, K_{IR} currents were potently suppressed by hyposmotic challenge, a stimulus known to activate mechanosensitive ion channels in the resistance vasculature (11, 43, 44). Further investigation revealed that this hyposmotic-induced suppression was dependent on protein kinase C (PKC) signaling. On the basis of the observations, we propose that smooth muscle K_{IR} channels are mechanically sensitive and that this property facilitates the participation of this conductance in myogenic tone development.

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MATERIALS AND METHODS

Animal and dissection procedures. Animal procedures were approved by the Animal Care and Use Committee at the University of Calgary or the University of Vermont. Briefly, female Sprague-Dawley rats (10–12 wk of age) were euthanized via carbon dioxide asphyxiation. The brain was carefully removed and placed in cold phosphate-buffered (pH 7.4) saline solution containing (in mM) 138 NaCl, 3 KCl, 10 Na_2HPO_4 , 2 NaH_2PO_4 , 5 glucose, 0.1 CaCl_2 , and 0.1 MgSO_4 . Cerebral and basilar arteries were carefully dissected out of surrounding tissue and cut into 2-mm segments.

Intact cerebral arteries. Cerebral arterial segments were mounted in a customized arteriograph and superfused with warm (37°C) physiological salt solution (PSS; pH 7.4) containing (in mM) 119 NaCl, 4.7 KCl, 20 NaHCO_3 , 1.7 KH_2PO_4 , 1.2 MgSO_4 , 1.6 CaCl_2 , and 10 glucose. Endothelial cells were removed by passing air bubbles through the vessel lumen (2 min); successful removal was confirmed by the loss of acetylcholine-induced dilations. Cerebral arteries were equilibrated for 60 min, and contractile responsiveness was then assessed by briefly (~10 s) exposing the tissue to a 60 mM KCl challenge. Following equilibration, cerebral arteries were maintained at low (15 mmHg) or high (80 mmHg) intravascular pressure in the absence or presence of 30 μM Ba^{2+} (K_{IR} channel inhibitor). Arterial diameter was monitored using an automated edge detection system (IonOptix, Milton, MA). Smooth muscle V_M was assessed by inserting a glass microelectrode backfilled with 1 M KCl (tip resistance = 120–150 M Ω) into the vessel wall. The criteria for successful cell impalement included 1) a sharp negative V_M deflection on entry, 2) a stable recording for at least 1 min following entry, and 3) a sharp return to baseline on electrode removal.

Isolation of arterial smooth muscle cells. Smooth muscle cells from rat basilar arteries were enzymatically isolated as previously described (43). Briefly, arterial segments were placed in an isolation medium (37°C, 10 min) containing (in mM) 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl_2 , 10 glucose, and 10 HEPES with 1 mg/ml albumin (pH 7.4). Vessels were then exposed to a two-step digestion process that involved 1) a 15-min incubation in isolation media (37°C) containing 0.6 mg/ml papain and 1.8 mg/ml dithioerythritol, and 2) a 10-min incubation in isolation medium containing 100 μM Ca^{2+} , 0.7 mg/ml type F collagenase, and 0.4 mg/ml type H collagenase. Following treatment, tissues were washed repeatedly with ice-cold isolation medium and triturated with a fire-polished pipette. Liberated smooth muscle cells were stored in ice-cold isolation medium for use the same day.

Electrophysiology. Conventional patch-clamp electrophysiology was used to measure whole cell currents in isolated smooth muscle cells. Briefly, recording electrodes (resistance of 4–7 M Ω) were fashioned from borosilicate glass, covered in sticky wax to reduce capacitance, and backfilled with solution containing (in mM) 5 NaCl, 35 KCl, 100 K-gluconate, 1 CaCl_2 , 0.5 MgCl_2 , 10 HEPES, 10 EGTA, 2.5 $\text{Na}_3\text{-ATP}$, and 0.2 GTP (pH 7.2). This pipette was then gently lowered onto an isolated cell and negative pressure applied to rupture the membrane. Cells with a seal resistance >10 G Ω were then voltage clamped (–60 mV) and equilibrated for 15 min in a bath solution containing (in mM) 90 NaCl, 5 KCl, 0.5 MgCl_2 , 10 HEPES, 10 glucose, 0.1 CaCl_2 , and 100 D-mannitol (pH 7.4). K_{IR} activity was assessed by ramping cells between –120 and 20 mV (0.047 mV/ms) and quantifying the component of the whole cell current that was sensitive to 30 μM Ba^{2+} . In contrast, voltage-dependent delayed rectifier K^+ (K_{DR}) current activity was assessed by stepping cells to voltages between –70 and +40 mV (300 ms) and monitoring peak outward current. Following this voltage pulse, cells were stepped back to –40 mV (500 ms) to determine whether tail currents were present. Control experiments confirmed that the pipette solution did minimize K_{ATP} and K_{Ca} channel activity. Whole cell currents were recorded on an Axopatch 200B amplifier (Axon Instruments, Union City, CA), filtered at 1 kHz, digitized at 5 kHz, and stored on a computer for subsequent analysis with Clampfit 8.1 software. Cell capacitance

ranged between 14 and 18 pF and was measured with the cancellation circuitry in the voltage-clamp amplifier. Cells that displayed a noticeable shift in capacitance (>0.3 pF) during experiments were excluded from analysis. A 1 M NaCl-agar salt bridge between the reference electrode and the bath solution was used to minimize offset potentials (<2 mV). All experiments were performed at room temperature (20–22°C).

Ba^{2+} -sensitive K_{IR} currents were first assessed in bath solutions containing 5, 20, and 60 mM K^+ . To elevate the bath concentration of K^+ , KCl was substituted for NaCl; osmolarity was maintained at ~305 mosM, as confirmed with a bioosmometer. In all subsequent K_{IR} experiments, extracellular K^+ was maintained at 20 mM, and the magnitude of the Ba^{2+} -sensitive current was monitored in response to UTP (30 μM), U46619 (0.1 μM), 5-HT (0.1 μM), or phorbol-12-myristate-13-acetate (PMA; 50 nM) or to hyposmotic challenge \pm calphostin C (300 nM). To render bath solutions hyposmotic (205 mosM), D-mannitol was removed. K_{DR} activity was also assessed in the absence and presence of UTP, U46619, and hyposmotic challenge.

Real-time PCR analysis of K_{IR} subtypes. Whole basilar arteries or ~300 isolated smooth muscle cells were placed in RNase- and DNase-free collection tubes. Total RNA was extracted (RNeasy Mini kit with DNase treatment; Qiagen, Valencia, CA), and first-strand cDNA was synthesized using the Sensi-script RT kit (Qiagen) with oligo d(T) primer. To optimize reaction specificity, real-time PCR was initially performed on each primer set using rat brain cDNA, SYBR green (Bio-Rad, Hercules, CA), and a range of annealing temperatures (52–62°C). On the basis of melt curve analysis, 1 μl of each reaction product was placed on a DNA 500 lab chip and examined using a bioanalyzer (model no. 2100, Agilent Technologies). A second aliquot of product was electrophoresed on a 1.5% (wt/vol) agarose gel, extracted using a gel extraction kit (Qiagen), and sequenced at the University of Calgary Core DNA facility. Having ascertained an ideal annealing temperature, real-time PCR efficiency was determined for all primer sets, with serial dilutions of brain cDNA used as template. Reaction efficiencies were as follows: $K_{IR2.1}$, 94.4%; $K_{IR2.2}$, 94.9%; $K_{IR2.3}$, 98.8%; $K_{IR2.4}$, 98.0%. The optimal real-time PCR reaction consisted of a hot start (95°C for 3 min) followed by 40 cycles of 95°C for 15 s, 55.1°C for 30 s, and 72°C for 30 s. A melt curve analysis was performed on each reaction, and the threshold cycle was determined using software provided with the Bio-Rad iCycler. Forward (F) and reverse (R) primers specific to rat $K_{IR2.1}$ –2.4 were as follows: $K_{IR2.1}$ (accession no. NM_017296) (F) 5'-AGAGGAAGAGGACAGTGAGAAC-3', $K_{IR2.1}$ (R) 5'-TCGCCTGGTTGTGGAGATC-3'; $K_{IR2.2}$ (accession no. NM_053981) (F) 5'-GCAGCCTTCTCTCTCCATTGA-3', $K_{IR2.2}$ (R) 5'-GACTGAGC-CACCACCATGAAG-3'; $K_{IR2.3}$ (accession no. NM_053870) (F) 5'-CC-TGGACCGCATCTTCTTGG-3', $K_{IR2.3}$ (R) 5'-CAGGATGACCA-C AATCTCAAAGTC-3'; $K_{IR2.4}$ (accession no. NM_170718) (F) 5'-ATGAGTTGACTATCGACTTCC-3', $K_{IR2.4}$ (R) 5'-GGGA-GCCAGGAAAACCTGA CTTA-3'. K_{IR} mRNA levels were expressed relative to $K_{IR2.1}$.

All smooth muscle cell samples were screened a priori for template and endothelial cell contamination using conventional PCR techniques. In these control experiments, 2 μl of first-strand cDNA were added to a PCR reaction containing 1.5 mM MgCl_2 , 0.25 μM forward and reverse primers (University of Calgary, Core DNA facility), 0.2 mM deoxynucleotide triphosphates, and 2.5 units of recombinant *Taq* DNA polymerase (Qiagen). PCR reactions were hot started (94°C for 3 min) and underwent 35 cycles of 94°C for 1.0 min, 60°C for 0.5 min, and 72°C for 0.75 min. PCR samples were then exposed to a final extension period at 72°C for 10 min. Forward and reverse primers specific to RhoA (accession no. NM_057132) were as follows: (F) 5'-CGGGATCCCGATGGCTGCCATCCAGGAAG-3'; (R) 5'-GGAATTCCTCACAA-GATCGAAGGCAAC-3'. Nested primers (F1/R1, F2/R2) for endothelin-1 (accession no. NM_012548) were as follows: (F1) 5'-GAGCTGAGAAGGAAGTGCAGAG-3'; (R1) 5'-GGTCTT-GATGCTGTTGCTGATG-3'; (F2) 5'-TGTGTCTACTTCTGC-

CACCTG-3'; (R2) 5'-GCCTCCAACCTTCTTAGTTTTCTT-3'. The expected amplicon sizes for RhoA and endothelin-1 were 550 and 350, respectively. DNA sequencing identified each reaction product, and control experiments confirmed the absence of genomic DNA contamination.

Western blotting. Whole basilar arteries were placed in 100 μ l of lysis buffer (pH 7.4) containing (in mM) 150 NaCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES with 0.5% Tween and 10% mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Samples were mechanically disrupted, exposed to three liquid nitrogen freeze-thaw cycles, and then centrifuged (10 min, 13,000 rpm). Supernatant was placed in a clean tube, assayed for total protein, and stored at -20°C for up to 1 wk. Samples were prepared for electrophoresis by placing 15 μ l of supernatant in 5 μ l of 4 \times sample buffer and 2 μ l of DTT. After heating (10 min, 90°C), 20 μ g of protein were loaded to run on a 10% polyacrylamide gel. Protein was transferred to a polyvinylidene difluoride (PVDF) membrane, blocked for 2 h (5% nonfat milk in Tris-buffered saline), and incubated overnight (4°C) with a primary antibody (K_{IR} 2.1 or K_{IR} 2.2, 1:200 dilution; Sigma-Aldrich) diluted in milk-Tris buffer. The PVDF membrane was subsequently washed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:1,000; Jackson Laboratories, West Grove, PA) diluted in milk-Tris buffer (1 h, 20–22°C). Washing was repeated, and the blot was developed using chemiluminescent substrate (Pierce Biochemicals, Rockford, IL).

Immunohistochemistry. K_{IR} 2.1 and K_{IR} 2.2 protein expression was detected in smooth muscle cells isolated from basilar myocytes.

Isolated cells were air dried onto poly-L-lysine-coated slides and washed with Tris-buffered saline (pH 7.4) containing 50 mM Tris, 0.9% NaCl, 0.1% BSA, and 0.01% Triton X. Sections were lightly fixed in 2% paraformaldehyde-Tris-buffered saline (20 min) and incubated (4 h) with a quench solution containing 100 mM Tris, 1.8% NaCl, 5% goat serum, 2% BSA, and 0.2% Triton X. Sections were subsequently incubated overnight (4°C) with a polyclonal (K_{IR} 2.1 or K_{IR} 2.2; 1:200) and a monoclonal (endothelial nitric oxide synthase, 1:2,500; Chemicon, Temecula, CA) antibody diluted in quench solution. Slides were then washed with Tris-buffered saline and incubated for 4 h (20–22°C) with an anti-rabbit-Cy3 and an anti-mouse-Cy5 IgG-specific secondary antibody (Molecular Probes, Eugene, OR). After further washing, slides were air dried and mounted in anti-fade media (Molecular Probes). Smooth muscle cells were viewed and photographed using a Zeiss fluorescent microscope coupled to a \times 63 oil immersion lens.

Chemicals, drugs, and enzymes. Buffer reagents, collagenases (types F and H), UTP, and 5-HT were obtained from Sigma-Aldrich. Papain was acquired from Worthington (Lakewood, NJ). PMA, calphostin C, and U46619 were purchased from Calbiochem (La Jolla, CA). PMA was dissolved in DMSO, with a final solvent concentration \leq 0.05%.

Statistical analysis. Data are expressed as means \pm SE, and *n* indicates the number of vessels or cells. Paired *t*-tests were performed to statistically compare the effects of a given condition/treatment on arterial diameter, V_M , or whole cell current. *P* values \leq 0.05 were considered statistically significant.

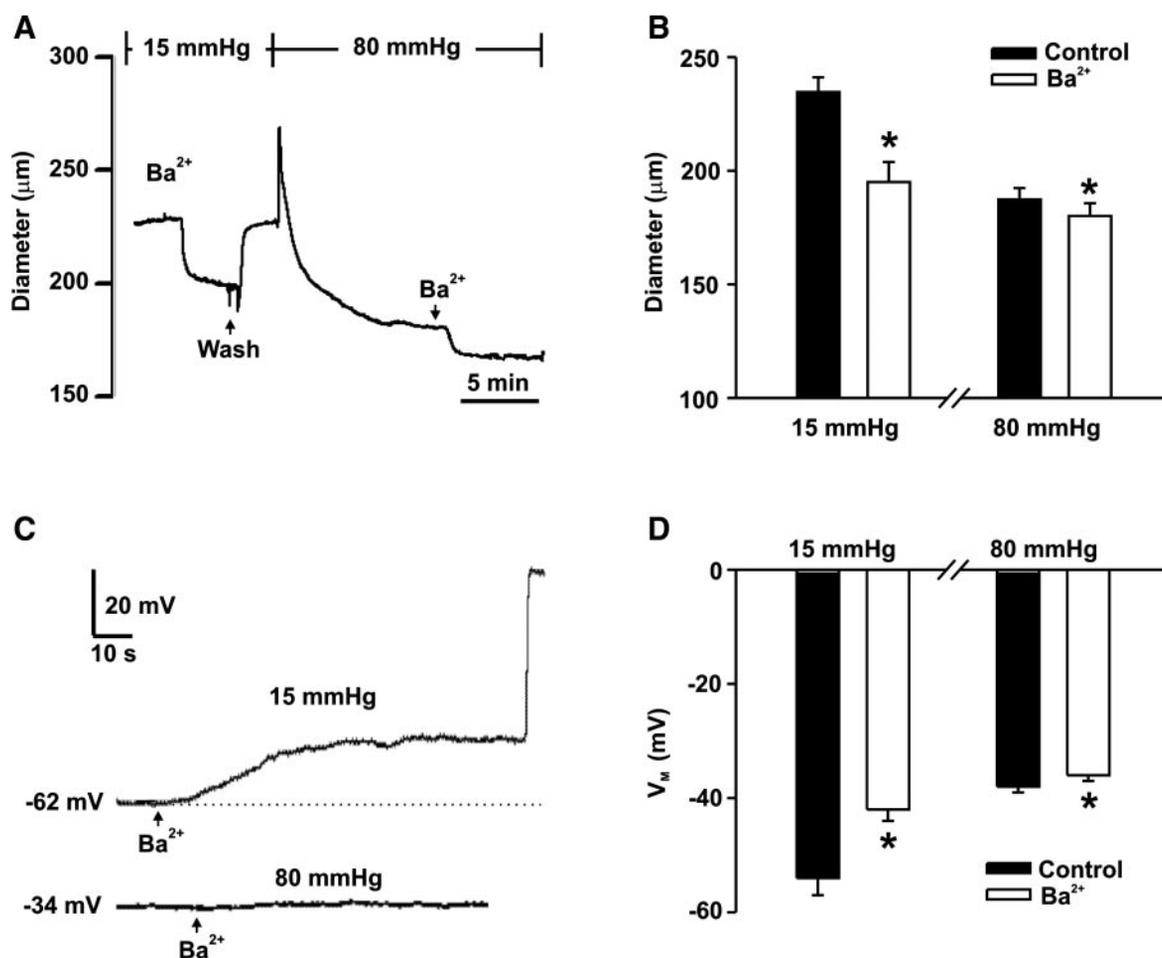


Fig. 1. Inward rectifying K⁺ (K_{IR}) channels regulate cerebral arterial diameter and membrane potential (V_M). A and C: representative traces demonstrating the effects of Ba²⁺ (K_{IR} channel inhibitor; 30 μ M) on arterial diameter and V_M at an intravascular pressure of 15 or 80 mmHg. B and D: summary data (*n* = 6/group) illustrating the effects of Ba²⁺ on arterial diameter and V_M at 15 and 80 mmHg. Data are means \pm SE. *Significant difference from control.

RESULTS

K_{IR} in intact cerebral arteries and isolated smooth muscle cells. To examine whether K_{IR} channels were functionally active over a physiological V_M range, initial experiments examined the Ba^{2+} responsiveness of denuded cerebral arteries at low (15 mmHg) and high (80 mmHg) intravascular pressure (Fig. 1). At low intravascular pressure, where myogenic tone was limited and V_M negative, 30 μM Ba^{2+} elicited a profound constriction (235 ± 7 to 195 ± 9 μm) and arterial depolarization (-54 ± 3 to -42 ± 2 mV). Elevating intravascular pressure to 80 mmHg depolarized and constricted cerebral arteries. In this myogenically active state, Ba^{2+} superfusion continued to constrict (187 ± 5 to 180 ± 6 μm) and depolarize (-38 ± 1 to -36 ± 1 mV) arteries, albeit in a more modest manner.

Following the preceding experiments, smooth muscle cells were enzymatically isolated, and K_{IR} currents were monitored using pipette solutions that minimize K_{ATP} and K_{Ca} channel activity. As noted in Fig. 2A, the whole cell current consisted of Ba^{2+} -sensitive and -insensitive components. The Ba^{2+} -sensitive component was inward at voltages negative to the apparent K^+ equilibrium potential (E_K). Characteristic of K_{IR} (33, 35), increases in extracellular K^+ augmented the Ba^{2+} -sensitive component and elicited a rightward shift in the

reversal potential (Fig. 2, B and C). K_{IR} activity was stable over time (Fig. 2D), and in two of six cells, a small Ba^{2+} -sensitive outward current was observable over the physiological V_M range and in a bath solution containing 5 mM K^+ (Fig. 2B, inset). The Ba^{2+} -insensitive component of the whole cell current was outward and predominated at voltages positive to -30 mV. This component can be primarily attributed to K_{DR} channel activity (25).

To address the potential molecular identity of smooth muscle K_{IR} channels, mRNA and protein analyses were performed on whole arteries and isolated smooth muscle cells. Samples were initially screened for template (i.e., RhoA) and for endothelial cell contamination (i.e., endothelin-1; ET-1). Figure 3A highlights that RhoA mRNA was consistently found in both tissue preparations, whereas ET-1 mRNA was only observed in whole artery samples. Real-time PCR analysis revealed the presence of $K_{IR2.1}$, $K_{IR2.2}$, and $K_{IR2.4}$ mRNA in whole arteries (Fig. 3, B and C). In comparison, only $K_{IR2.1}$ and $K_{IR2.2}$ mRNA were observed in aliquots of isolated smooth muscle cells (~ 300), with the latter found at levels approximately threefold higher than the former. Western blot analysis confirmed the expression of $K_{IR2.1}$ and $K_{IR2.2}$ protein in whole basilar artery (Fig. 4A). Likewise, immunocytochemical approaches revealed the presence of $K_{IR2.1}$ and $K_{IR2.2}$ protein in cerebral arterial smooth muscle cells (Fig. 4B).

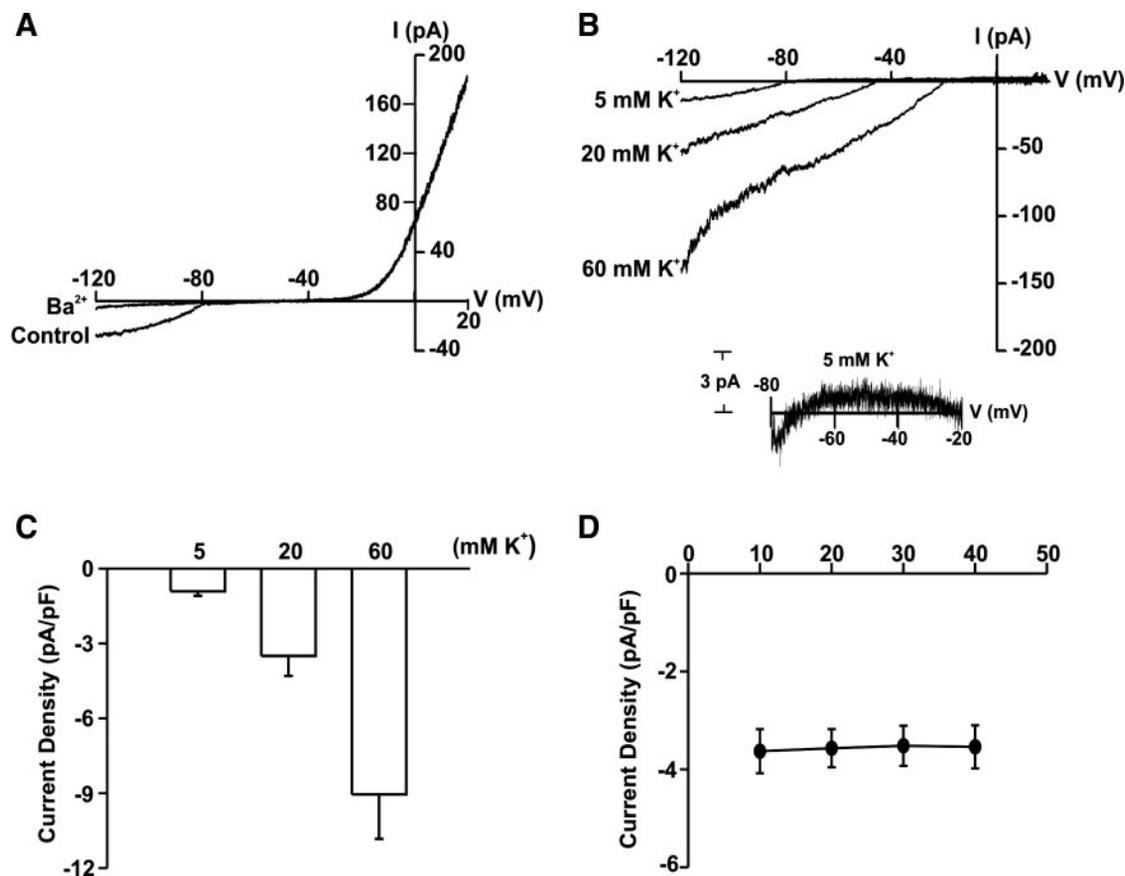


Fig. 2. K_{IR} current in cerebral arterial smooth muscle cells. A: representative traces demonstrating the effect of Ba^{2+} (30 μM) on whole cell current. B: representative traces demonstrating the effects of extracellular K^+ concentration ($[K^+]$) on the Ba^{2+} -sensitive K_{IR} current. Inset: outward K_{IR} current in a smooth muscle cell bathed in the 5 mM K^+ solution. C: summary data illustrating the effects of extracellular $[K^+]$ on the Ba^{2+} -sensitive K_{IR} current at -120 mV ($n = 6$). D: effects of time on the Ba^{2+} -sensitive K_{IR} current at -120 mV ($n = 6$). Data are means \pm SE.

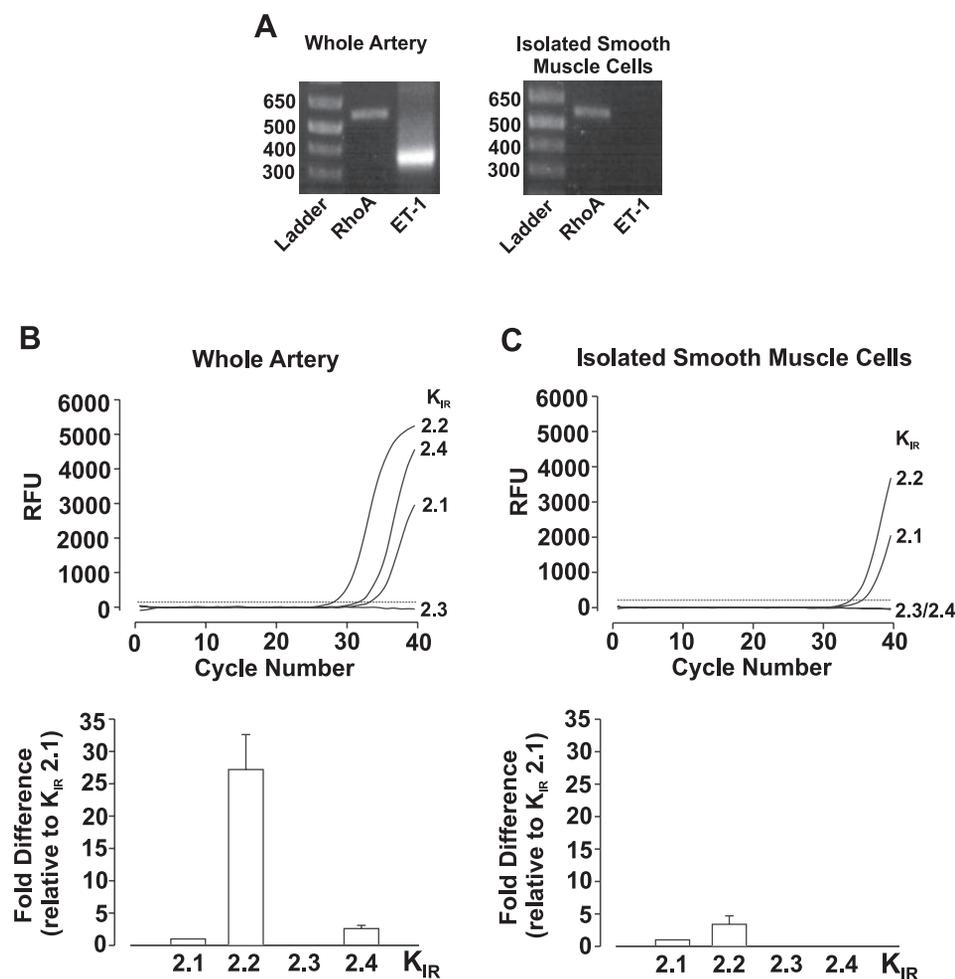


Fig. 3. PCR analysis of $K_{IR}2.1-2.4$. *A*: conventional RT-PCR analysis of whole basilar arteries and isolated smooth muscle cells for RhoA (product marker) and endothelin-1 (ET-1; endothelial cell-specific marker) mRNA. Ladder markers are measured in bp. *B*: real-time PCR reaction plot and mRNA expression of $K_{IR}2.1-2.4$ in whole arteries ($n = 4$). *C*: real-time PCR reaction plot and mRNA expression of $K_{IR}2.1-2.4$ in isolated smooth muscle cells ($n = 4$). Data are expressed relative to $K_{IR}2.1$. RFU, relative fluorescence units.

Regulation of K_{IR} current in isolated myocytes. To test whether K_{IR} channels are actively regulated, patch-clamp electrophysiology was used to monitor the Ba^{2+} -sensitive currents in the absence or presence of various vasoconstrictor stimuli.

As noted in Fig. 5, the 20-min application of UTP, U46619, or 5-HT at concentrations that depolarize and/or constrict intact cerebral arteries (20, 25) had no effect on the Ba^{2+} -sensitive K_{IR} current. While ineffective at modulating K_{IR} , both UTP

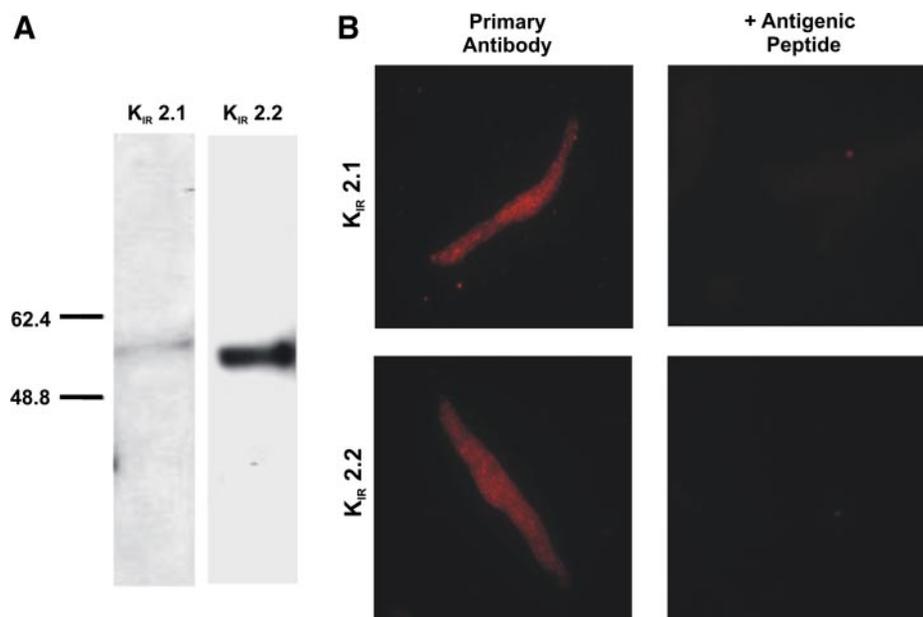


Fig. 4. $K_{IR}2.1$ and $K_{IR}2.2$ protein expression. *A*: Western blot analysis of whole basilar artery probed with an anti- $K_{IR}2.1$ (left) or anti- $K_{IR}2.2$ (right) polyclonal antibody. *B*: smooth muscle cells isolated from the basilar artery were treated with a $K_{IR}2.1$ or $K_{IR}2.2$ polyclonal antibody and IgG secondary antibody conjugated to Cy3 (left). Staining was absent when the antigenic peptide was incubated with the primary antibody (right).

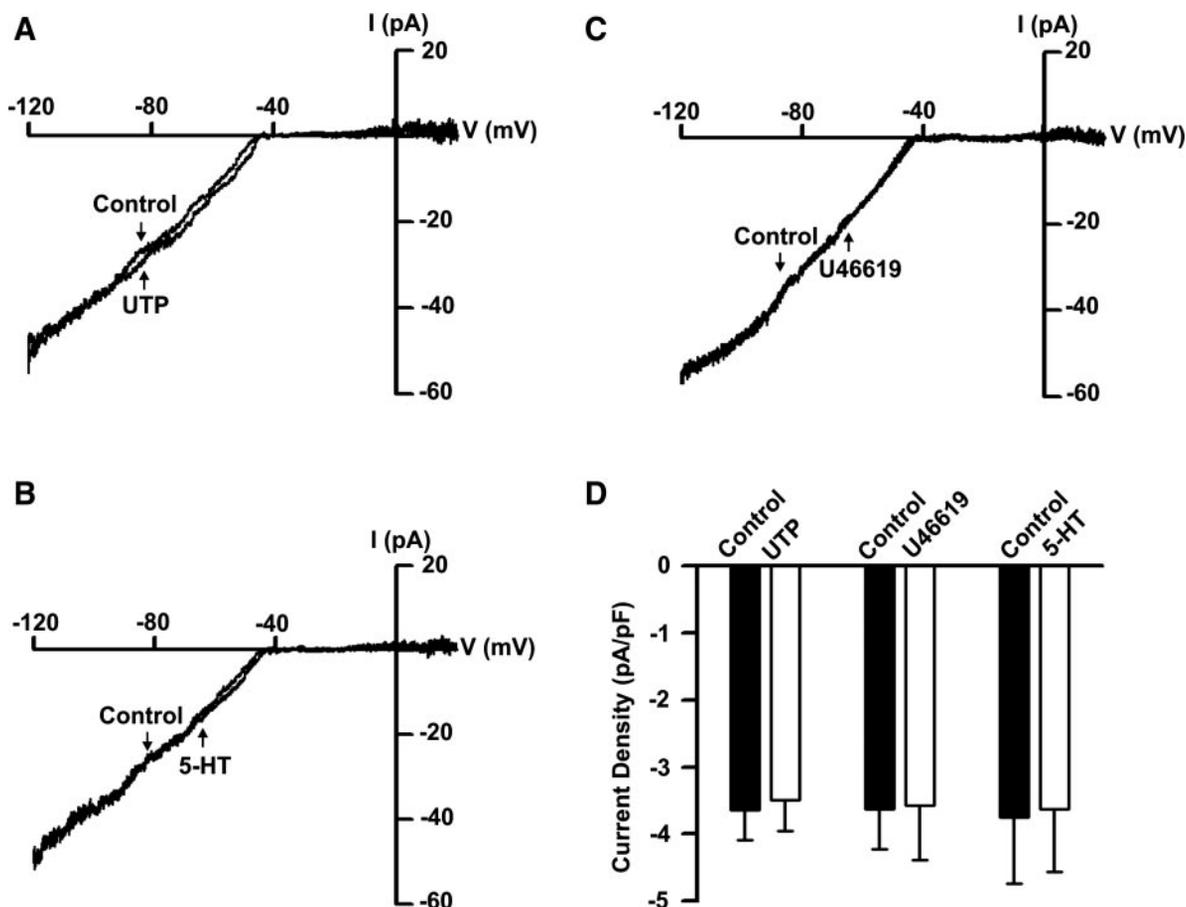


Fig. 5. Vasoconstrictor agonists do not affect the K_{IR} current. A–C: representative traces of the Ba^{2+} -sensitive K_{IR} current before and after a 20-min exposure to uridine triphosphate (UTP; 30 μ M), U46619 (0.1 μ M), and 5-HT (0.1 μ M). D: summary data illustrating the effects of UTP ($n = 6$), U46619 ($n = 6$), and 5-HT ($n = 6$) on the Ba^{2+} -sensitive K_{IR} current at -120 mV. Data are means \pm SE.

and U46619 suppressed outward K_{DR} activity in control experiments (Fig. 6). In contrast, a 100 mosM reduction in bath osmolarity rapidly and sustainably inhibited the Ba^{2+} -sensitive K_{IR} current in cerebral arterial smooth muscle cells (Fig. 7). This stimulus, which has been used previously in smooth muscle to activate mechanically sensitive inward currents (11, 43, 44), did not alter whole cell K_{DR} activity (Fig. 8). In theory, one might expect outward current to rise with hypotonic challenge because of the activation of a background swelling-activated cation current. Previous work has, however, shown that this current is small even at positive voltages (~ 5 – 10 pA at $+40$ mV). Thus it is unlikely that such a current could be accurately discerned from the recordings in Fig. 8.

Previous studies have shown that hypotonic stimuli modulate smooth muscle ion channels through the activation of PKC (8, 38, 45). In light of these observations, we sought to define the involvement of this kinase in the regulation of K_{IR} . As noted in Fig. 9, A and C, the PKC activator PMA rapidly and sustainably suppressed the K_{IR} current in cerebral arterial smooth muscle cells. This PMA-induced suppression was blocked by calphostin C, a PKC inhibitor ($n = 2$; data not shown). Calphostin C also abolished the hypotonic-induced suppression of the K_{IR} current (Fig. 9, B and C).

DISCUSSION

This study examined whether stimuli that depolarize and constrict cerebral arteries suppress K_{IR} channel activity. Initial observations confirmed that K_{IR} channels were present in cerebral smooth muscle cells, active over a physiological V_M range, and likely comprised of $K_{IR2.1}$ and $K_{IR2.2}$. Using whole cell patch-clamp electrophysiology, we found that vasoconstrictor agonists including UTP, U46619, and 5-HT did not alter K_{IR} activity. In contrast, K_{IR} activity was suppressed by a reduction in bath osmolarity, a swelling stimulus that activates mechanosensitive ion channels and induces myogenic-like responses in resistance arteries (11, 43, 44). Experiments further demonstrated that the hypotonic-induced inhibition of K_{IR} depended on the activation of PKC. We propose that mechanically sensitive K_{IR} channels contribute to the initiation of myogenic tone in the cerebral circulation.

K_{IR} channels in intact cerebral arteries. Cerebral arteries are coupled in series and parallel to one another, forming an arterial network that controls the magnitude and distribution of tissue blood flow. Under dynamic conditions, tone within this network is regulated by changes in tissue metabolism (14), neuronal activity (37), blood flow (12), and intraluminal pressure (15, 22, 43). Many of these stimuli initiate vasomotor

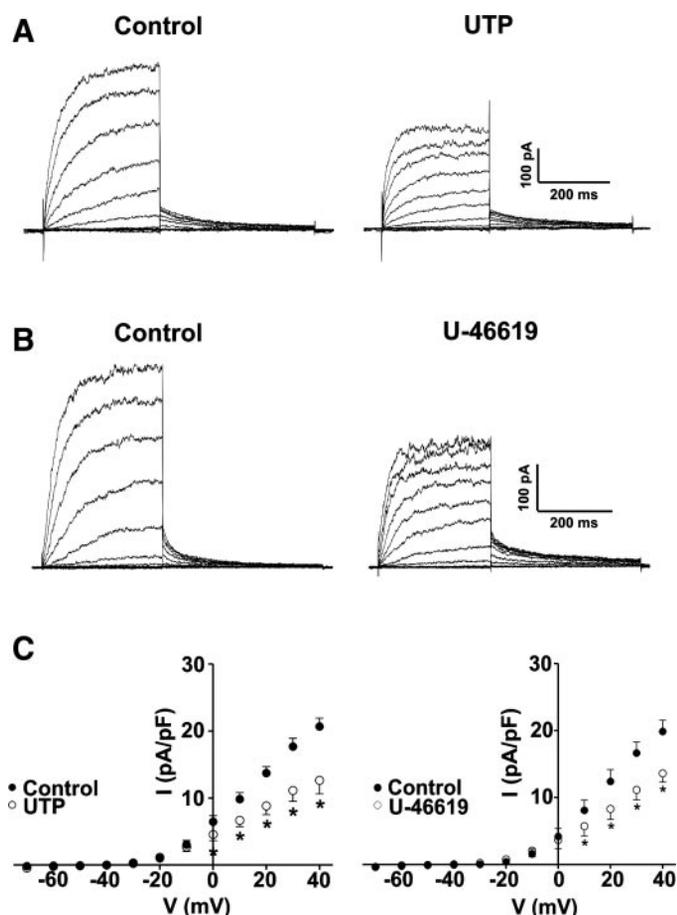


Fig. 6. Vasoconstrictor agonists inhibit the voltage-dependent delayed rectifier K^+ (K_{DR}) current. *A* and *B*: representative traces of the K_{DR} current before and after a 20-min exposure to UTP (30 μ M) and U46619 (0.1 μ M). *C*: summary data illustrating the effects of UTP ($n = 6$) and U46619 ($n = 6$) on the K_{DR} current. Data are means \pm SE. *Significant difference from control.

responses by activating transduction pathways that alter resting V_M and the influx of Ca^{2+} through voltage-operated Ca^{2+} channels (17, 25, 41). In vascular smooth muscle, steady-state V_M is set by an array of depolarizing inward and hyperpolarizing outward currents including K_{IR} (23, 27, 34). K_{IR} channels are aptly named for their distinctive ability to pass current more

readily in the inward direction. Structurally, these channels consist of four α -subunits, each of which is comprised of two transmembrane domains and a GYG-containing "P-loop" that confers K^+ selectivity (2). Since their initial isolation in arterial smooth muscle cells (10), vascular studies have consistently asserted that K_{IR} channels principally function as a background conductance (27, 40). To operate in this manner, K_{IR} channels would have to display activity over a range of resting membrane potentials. This functional activity is evident in Fig. 1, where micromolar Ba^{2+} elicited electrical and mechanical responses in arteries maintained in a depolarized or hyperpolarized state through changes in intravascular pressure. This broad range of activity ensures that K_{IR} can function in a housekeeping manner. However, it also invites the suggestion that these channels may serve as a target for vasoactive stimuli to initiate arterial depolarization and constriction.

K_{IR} regulation in cerebral arterial smooth muscle cells. To address whether K_{IR} channels are actively regulated by vasoconstrictor stimuli, it is important to first establish the basic electrical and molecular properties of this current in cerebral arterial smooth muscle cells. Consistent with past vascular investigations (32, 33, 35), this study observed a prominent K_{IR} current that was blocked by micromolar Ba^{2+} , potentiated by extracellular K^+ , and displayed sustained activity over a 40-min recording period (Fig. 2). While inward rectification is the most distinctive feature of this current, it is the outward current between -20 to -60 mV that is physiologically important. In this study, a small outward component was observed in a limited number of cells, and it was generally no greater than 2 pA. Although subtle, modeling studies have shown that such currents can clearly alter V_M as long as the input resistance of a smooth muscle cell remains sufficiently high (7, 19). An analysis of mRNA and protein (Figs. 3 and 4) revealed the expression of both $K_{IR2.1}$ and $K_{IR2.2}$ in cerebral arterial smooth muscle cells, raising the possibility that arterial K_{IR} channels are perhaps heteromultimeric in nature. As such, it is worthwhile to note that $K_{IR2.1}$ and $K_{IR2.2}$ can coassociate and that the resulting heteromultimer displays electrophysiological properties including a Ba^{2+} sensitivity that corresponds well with the native cerebral arterial K_{IR} current (24, 36). These findings are in contrast to a previous study (3) that found little evidence for $K_{IR2.2}$ expression in vascular smooth muscle.

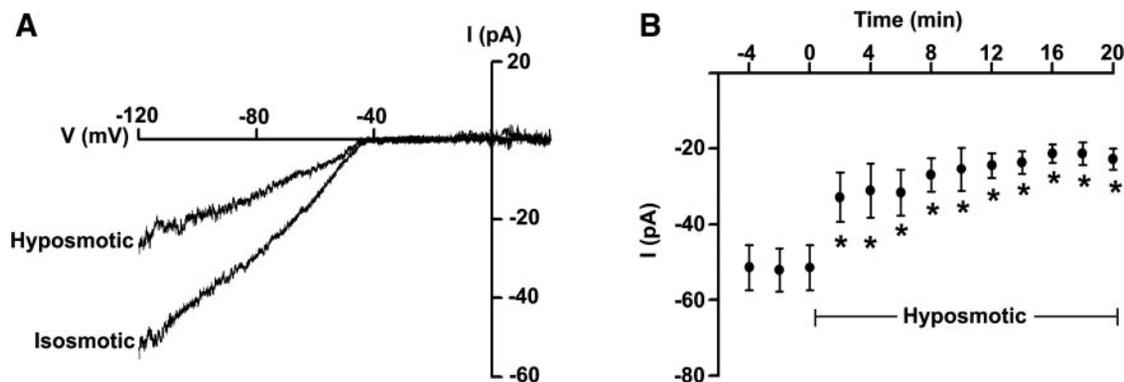


Fig. 7. Hypotonic challenge inhibits the K_{IR} current. *A*: representative traces of the Ba^{2+} -sensitive K_{IR} current before and after 6 min of hypotonic challenge. Bath osmolarity was reduced from 305 (isosmotic) to 205 (hypotonic) mosM. *B*: time-course data documenting the effect of hypotonicity on the Ba^{2+} -sensitive K_{IR} current at -120 mV ($n = 6$). Data are means \pm SE. *Significant difference from isosmotic conditions.

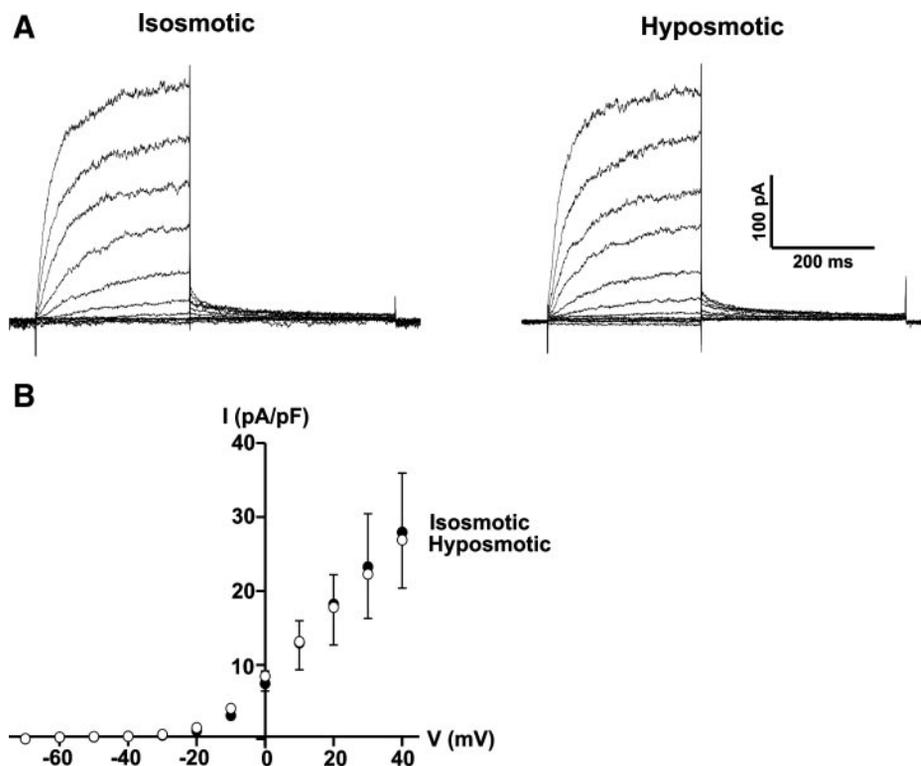


Fig. 8. Hypotonic challenge does not inhibit the K_{DR} current. *A*: representative traces of the K_{DR} current before and after 20 min of hypotonic challenge. Bath osmolarity was reduced from 305 (isosmotic) to 205 (hypotonic) mosM. *B*: summary data illustrating the effects of hypotonic stimuli ($n = 6$) on the K_{DR} current. Data are means \pm SE.

Clarification of this issue awaits further detailed molecular characterization studies.

With K_{IR} activity prominent at negative potentials and in elevated extracellular K^+ , the influence of vasoactive stimuli on cerebral arterial K_{IR} currents was quantified at -120 mV and in bath solutions containing 20 mM K^+ . Initial trials focused on vasoconstrictor agonists including UTP and U46619, agents previously shown to strongly depolarize and constrict intact cerebral arteries (25). As illustrated in Fig. 5, K_{IR} activity was not substantively altered by these vasoconstrictors; 5-HT applied at concentrations known to constrict cerebral arteries was also ineffective (20). This absence of modulation could not be ascribed to enzymatic digestion or to the whole cell recording conditions, since control experiments confirmed that UTP and U46619 suppressed whole cell K_{DR} activity (Fig. 6). Agonist modulation of K_{IR} has, in general, been poorly examined in vascular tissue. The recent work of Park and colleagues (29, 30) is a notable exception, with these limited studies demonstrating that smooth muscle K_{IR} activity can be suppressed if agonists mobilize PKC. While UTP and U46619 are often presumed to mobilize PKC in all resistance arteries, the cerebral circulation appears to be an exception (25, 41). This view is based on recent findings showing that PKC blockers do not prevent these agonists from depolarizing cerebral arteries or from suppressing K_{DR} activity (25). Indeed, in the cerebral circulation, vasoconstrictors like UTP and U46619 appear to elicit electrical and mechanical responses by mobilizing RhoA and Rho kinase (25).

The preceding results should not be interpreted in a manner that suggests all vasoactive stimuli initiate cerebral vascular responses independent of PKC. Indeed, several studies have reported an important role for this kinase in enabling intravas-

cular pressure to depolarize and constrict intact cerebral arteries (13, 28, 38). In light of these observations, the present study examined whether mechanical stimuli could inhibit K_{IR} and whether this resulting suppression arose from PKC activation. To mechanically stimulate cerebral arterial smooth muscle, isolated cells were exposed to a hypotonic challenge. We and others have previously employed this swelling stimulus to activate mechanically sensitive inward currents and initiate myogenic-like responses in intact cerebral arteries (8, 11, 38, 43, 44). Hypotonic challenge induced a rapid inhibition of K_{IR} that was sustained over time (Fig. 7). This suppression displayed a degree of specificity, since whole cell K_{DR} activity was unaffected by the swelling stimulus (Fig. 8). Like past vascular studies that have employed hypotonicity to modulate mechanically sensitive inward currents (38, 45), PKC mobilization appears essential to initiating K_{IR} suppression (Fig. 9). This was principally revealed through the ability of 1) PMA to modulate K_{IR} , and 2) calphostin C (a PKC inhibitor) to prevent hypotonic bath solutions from inhibiting this K^+ conductance. At present, the PKC isoform responsible for inhibiting smooth muscle K_{IR} channels remains unclear. However, given that free pipette Ca^{2+} concentration was ~ 20 nM in the present study, a role for a Ca^{2+} -independent isoform of PKC is likely. This view is consistent with past vascular work noting that the activation of swelling-activated Cl^- currents is dependent on PKC ϵ (45). While this study is the first in smooth muscle to indicate that K_{IR} channels are mechanically sensitive, these observations are not without broader precedent. Indeed, both stretch and shear stress have been reported previously to alter current flow through K_{IR} channels in ventricular myocytes and endothelial cells, respectively (4, 16).

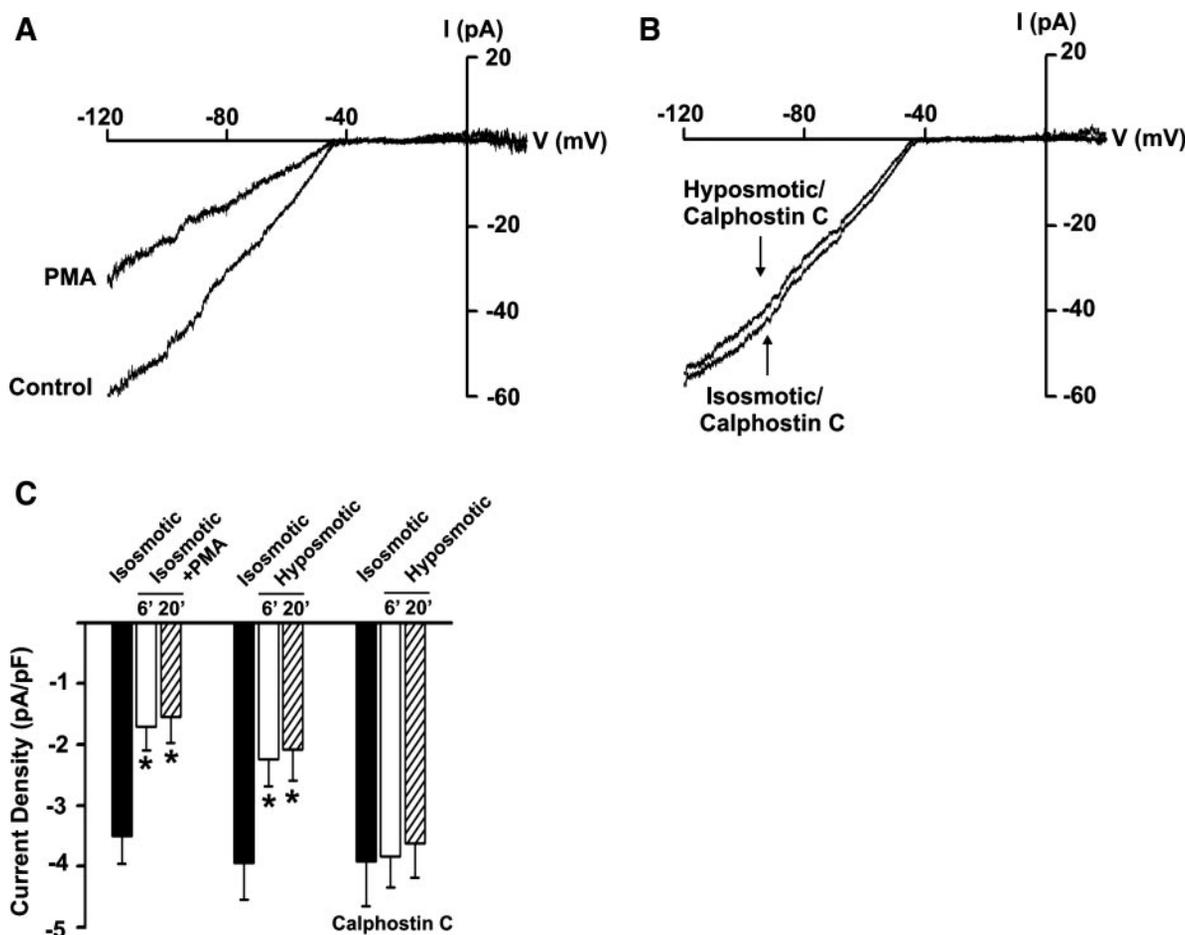


Fig. 9. Hyposmotic challenge inhibits the K_{IR} current through a transduction process involving PKC. *A*: representative traces of the Ba^{2+} -sensitive K_{IR} current before and after a 6-min exposure to phorbol-12-myristate-13-acetate (PMA; 50 nM). *B*: representative traces of the Ba^{2+} -sensitive K_{IR} current before and after 6 min of hyposmotic challenge + calphostin C (300 μ M). *C*: summary data illustrating the effects of PMA ($n = 6$), hyposmolarity ($n = 6$), and hyposmolarity \pm calphostin C ($n = 6$) on the Ba^{2+} -sensitive K_{IR} current at -120 mV. Data are means \pm SE. *Significant difference from isosmotic conditions.

Functional implications. A century ago, Bayliss (1) described the ability of resistance arteries to constrict to elevated intravascular pressure. In the cerebral circulation, pressure-induced vasoconstriction is an essential regulatory response that fundamentally depends on smooth muscle cell depolarization and influx of Ca^{2+} through voltage-operated Ca^{2+} channels (15, 22, 43). Traditionally, studies probing the basis of pressure-induced depolarization have concentrated on inward conductances. This includes a swelling-activated Cl^{-} current (11, 44) as well as nonselective cation currents comprised of various members of the transient receptor potential channel family (9, 26, 42). Such studies have, however, generally overlooked the potential contribution of K^{+} conductances except for those functioning in a negative-feedback mode (6, 18, 21). By illustrating the mechanosensitivity of the K_{IR} current, this study suggests for the first time that these channels could play a role in initiating pressure-induced depolarization. Designing a functional experiment to definitively link K_{IR} modulation with myogenic tone development is difficult and limits the present investigation. One must contend with the fact that K_{IR} inhibition substantively changes V_M and diameter, which makes group comparisons problematic. Likewise, agents that prevent hyposmolarity from inhibiting K_{IR} channels (i.e.,

PKC inhibitors) also impair the activation of mechanosensitive inward currents (8, 38, 45).

In summary, this study explored whether K_{IR} channels in cerebral arterial smooth muscle cells were suppressed by vasoconstrictor stimuli. Vasoconstrictor agonists previously shown to depolarize and constrict cerebral arteries did not inhibit K_{IR} channels likely composed of $K_{IR2.1}$ and $K_{IR2.2}$. In contrast, K_{IR} activity was suppressed by a hyposmotic challenge, a stimulus that activates mechanically sensitive currents in vascular smooth muscle cells. Subsequent experiments confirmed the dependency of current suppression on PKC activation. Given the apparent mechanosensitivity of K_{IR} , we propose a role for K_{IR} in the development of myogenic depolarization and constriction.

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