Kv2 channels oppose myogenic constriction of rat cerebral arteries

Gregory C. Amberg and Luis F. Santana

Department of Physiology and Biophysics, University of Washington, Seattle, Washington

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Amberg, Gregory C., and Luis F. Santana. Kv2 channels oppose myogenic constriction of rat cerebral arteries. Am J Physiol Cell Physiol 291: C348–C356, 2006.—By hyperpolarizing arterial smooth muscle, voltage-gated, Ca2+-independent K+ (Kv) channels decrease calcium influx and thus oppose constriction. However, the molecular nature of the Kv channels function in arterial smooth muscle remains controversial. Recent investigations have emphasized a predominant role of Kv1 channels in regulating arterial tone. In this study, we tested the hypothesis Kv2 channels may also significantly regulate tone of rat cerebral arteries. We found that Kv2.1 transcript and protein are present in cerebral arterial smooth muscle. In addition, our analysis indicates that a substantial component (~50%) of the voltage dependencies and kinetics of Kv current in voltage-clamped cerebral arterial myocytes is consistent with Kv2 channels. Accordingly, we found that stromatoxin, a specific inhibitor of Kv2 channels, significantly decreased Kv currents in these cells. Furthermore, stromatoxin enhanced myogenic constriction of pressurized arterial segments. We also found that during angiotensin II-induced hypertension, Kv2 channel function was reduced in isolated myocytes and in intact arteries. This suggests that impaired Kv2 channel activity may contribute to arterial dysfunction during hypertension. On the basis of these novel observations, we propose a new model of Kv channel function in arterial smooth muscle in which Kv2 channels (in combination with Kv1 channels) contribute to membrane hyperpolarization and thus oppose constriction.

RESISTANCE ARTERIES respond to increased intraluminal pressure by contracting (6). This intrinsic feature of small arteries, termed myogenic tone, regulates blood flow and influences blood pressure. The degree of myogenic tone depends on the level of intracellular Ca2+ ([Ca2+]i) of the smooth muscle cells lining the walls of these arteries (19). [Ca2+]i in arterial myocytes is largely determined by their membrane potential. Depolarization increases the open probability (P_o) of dihydropyridine-sensitive, voltage-gated L-type Ca2+ channels, which increases Ca2+ influx, hence increasing [Ca2+]i, and causing constriction (15, 19, 29). Accordingly, hyperpolarization relaxes arterial smooth muscle by decreasing L-type Ca2+ channel activity, which decreases [Ca2+]i, thus inducing relaxation.

Outward K+ currents hyperpolarize physiological membranes. Voltage-gated, Ca2+-independent K+ (Kv) channels, which open upon membrane depolarization, are important regulators of membrane potential of arterial smooth muscle cells (20, 26, 28). Kv currents are formed by a family of ion channels that give rise to an array of functionally distinct voltage-dependent currents. Much attention has recently been paid to the relationship between Kv1 (Shaker) channel function and myogenic tone in systemic resistance arteries (1, 11, 27). A general implication of these efforts is that Kv1 channels produce the physiologically relevant Kv current, which regulates arterial myocyte membrane potential and tone of these arteries.

In contrast, non-Kv1 voltage-dependent channels have also been identified in arterial smooth muscle. We (4) and others (16, 24, 33) have demonstrated Kv2 (Shab) channel expression in arterial smooth muscle, and there is evidence of Kv2 channel function in aortic (34), mesenteric (24), and pulmonary arterial (33) smooth muscle. These observations imply that Kv2 channels may be physiologically important, which may seem at odds with the above-mentioned view that Kv1 channels predominate in the smooth muscle cells of resistance arteries. Indeed, to the best of our knowledge, the functionality of Kv2 channels in the regulation of myogenic tone has not been examined.

In this study, we resolved this issue by performing the first systematic examination of Kv2 channel function in cerebral arteries and directly tested the hypothesis that, by hyperpolarizing arterial smooth muscle, Kv2 channels oppose myogenic constriction. We found that a substantial portion (~50%) of the Kv current in cerebral arterial myocytes is produced by Kv2 channels. We also elaborated on our previous findings regarding Kv2.1 channel expression (4) by showing decreased Kv2 channel function during angiotensin II-dependent hypertension. Finally, we found that inhibition of Kv2 channels constricted pressurized intact cerebral arteries. From these observations we conclude that Kv2 channels, together with Kv1 channels, regulate arterial tone by opposing myogenic constriction.

MATERIALS AND METHODS

Angiotensin II infusion and smooth muscle cell isolation. Male Sprague-Dawley rats (~250 g) were implanted with subcutaneous osmotic minipumps (Alza) that delivered either angiotensin II (300 ng·kg-1·min-1) or Ringer solution. After 7 days, the systolic blood pressure of sham rats was 131 ± 5.8 mmHg (n = 5), whereas the systolic blood pressure of rats receiving angiotensin II was 197 ± 8.3 mmHg (n = 5), as determined by tail-cuff plethysmography (5). The rats were then euthanized with pentobarbital sodium (250 mg/kg ip), and smooth muscle cells were isolated from basilar and cerebral arteries as previously described (5). The animals were handled in strict accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee.

Intact artery measurements. Midcerebral arteries were cannulated and mounted in a close-working-distance arteriograph. The endothelium was disrupted when air bubbles were passed through the artery. The arteriograph was placed on the stage of an inverted microscope and extraluminally (not intraluminally) perfused (3–6 ml/min) at 37°C with a bicarbonate-based physiological saline solution (PSS) composed of (in mM) 119 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 1.6 CaCl2, 1.2 MgSO4, and 11 glucose, with the pH set to 7.4 by}

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bubbling with a gas mixture of 95% O₂-5% CO₂. After equilibration (~20 min), intravascular pressure was increased to 80 mmHg. Endothelial damage was confirmed by the absence of a dilator response to acetylcholine (5 μM) after the development of tone (data not shown). Internal arterial diameters were measured from live video images with the length-calibrated edge-detection function of IonOptix imaging software at a sampling rate of 2 Hz. Experiments began after a stable level of myogenic tone was obtained and were terminated by the addition of 30 μM diltiazem in nominally Ca²⁺-free bicarbonate-based PSS to obtain the passive diameter of the artery. Induced constriction was defined as the percent decrease in internal diameter after the application of contractile stimuli from that observed after the development of tone under control conditions.

Single myocyte RT-PCR and Western blot analysis. A single isolated arterial smooth muscle cell free from visible debris was visualized on an inverted microscope and carefully aspirated into large-bore glass pipettes, as described previously (12) with ~10 μl of PSS, transferred into sterile 0.5-ml tubes containing 10 μl RT reaction mixture (Invitrogen) with random primers, including RNase OUT (20 μg/ml), and additional RNase OUT (20 μg/ml), reverse transcriptase was carried out by incubating at 25°C for 10 min, followed by 50°C for 120 min. The RT reaction was terminated by incubating at 85°C for 5 min, followed by application of RNase H (2 units) at 37°C for 20 min. RT products were stored at −70°C until used. Two negative controls were performed in parallel: 1) isolated myocytes were subjected to the RT protocol in the absence of Superscript III RT (RT− control) and 2) aspirated PSS (without an accompanying myocyte) was subjected to the full RT protocol (non-template control). PCR amplification was never observed with these negative control samples.

We purified cDNA using a protocol modified from the one described by Liss (23): GlycoBlue (1.5 μg; Ambion), poly C RNA and poly dC DNA (250 ng each; Midland), and a 1/10 volume of 3 M sodium acetate (Ambion) were added to the RT products; cDNA was precipitated overnight with 3.5 volumes 100% ethanol at −20°C. Samples were then centrifuged for 60 min at 4°C (14,000 g), and the supernatant were discarded. One hundred microliters of 75% ethanol were added to the RT products; cDNA was precipitated with 30 μl of diethyl pyrocarbonate water and stored at −70°C. Samples were incubated at 65°C for 6 min and placed on ice for 1 min. After the addition of 30 μl of dNTPs (Invitrogen) with random primers, including RNase OUT (20 μg/ml), and additional RNase OUT (20 μg/ml), reverse transcriptase was carried out by incubating at 25°C for 10 min, followed by 50°C for 120 min. The RT reaction was terminated by incubating at 85°C for 5 min, followed by application of RNase H (2 units) at 37°C for 20 min. RT products were stored at −70°C until used. Two negative controls were performed in parallel: 1) isolated myocytes were subjected to the RT protocol in the absence of Superscript III RT (RT− control) and 2) aspirated PSS (without an accompanying myocyte) was subjected to the full RT protocol (non-template control). PCR amplification was never observed with these negative control samples.

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Real-time PCR was performed on purified cDNA samples with TaqMan reagents on an ABI Prism 7700 sequence detection system (Applied Biosystems) following the manufacturer’s instructions. The rat Kv2.1-specific (GenBank accession no. NM_013186) primers and probe used were the following: forward, NT 2292–2312; probe, NT 2318–2343; and reverse, NT 2381–2358.

Western blot analysis was performed on whole cerebral arteries as described in a previous study (4). Briefly, cerebral arteries were homogenized in phosphatase-buffered saline with 5 mM EDTA, 1% Triton X-100, and a mammalian protease inhibitor mixture (Sigma). The homogenate was centrifuged at 10,000 g at 4°C for 10 min, and the protein concentration of the supernatant was determined using the bicinchoninic acid method. Ten micrograms of total protein were loaded on a 4–15% Tris-HCl polyacrylamide gel and run with an appropriate molecular weight standard at 100 mV for 1 h. Fractionated protein was then transferred to a polyvinylidene difluoride membrane using a Mini-trans Blot Cell (Bio-Rad) at 100 mV for 1 h at 4°C. The blots were blocked in TBS/Tween solution composed of 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20, with 5% nonfat milk for 1 h at 25°C. Blots were then incubated with a primary antibody specific to Kv2.1 (Sigma) and TBS/Tween with 1% nonfat milk at 4°C overnight. After being washed three times with TBS/Tween for 10 min, blots were incubated with a horseradish peroxidase-conjugated secondary antibody diluted in TBS/Tween for 2 h. This was followed by incubation with SuperSignal West Femto Chemiluminescent Substrate (Pierce). Under our experimental conditions, immunoblots showed a single band at the expected molecular mass of native Kv2.1 proteins (~130 kDa), indicating that cross-reactivity of this antibody with other proteins was negligible. Note that antibodies raised against this epitope have been characterized in detail previously (36).

Channel expression in human embryonic kidney-293 cells. Human embryonic kidney-293 cells were transfected with Kv1.2 and Kv1.5 (kindly provided by Dr. William C. Cole), Kv1.5, Kv1.2 (kindly provided by Dr. Steven L. Archer), and heterologously expressed L-type Ca²⁺ channels (Cav1x1.2, Cav2x1.1, and Cav3x3a) (kindly provided by Dr. Diane Lipscombe) constructs using Lipofectamine 2000, as described by the manufacturer.

Electrophysiology. Kv currents were measured using the conventional whole cell patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments). Only healthy, relaxed, spindle-shaped smooth muscle cells (see Ref. 27) with a mean capacitance of 19.6 ± 0.57 pF were used. The cells were continuously superfused at room temperature (22–25°C) with a nominally Ca²⁺-free Tyrode solution containing (in mM) 130 NaCl, 5 KCl, 3 MgCl₂, 10 glucose, 10 HEPES, adjusted to pH 7.4 with NaOH. The patch pipettes were filled with a solution composed of (in mM) 87 K+ aspartate, 20 KCl, 1 CaCl₂, 1 MgCl₂, 5 MgATP, 10 EGTA, and 10 HEPES, adjusted to pH 7.2 with KOH. For all whole cell experiments, the time between sweeps was 15 s, and 100 nM ibotenate (IbTx) was included in the external solution. Multiple recordings were obtained for each cell and averaged before analysis. Excised (inside-out) patch, single-channel recordings were performed as previously described (2).

Chemicals and statistics. All chemicals were from Sigma, unless stated otherwise. Data are presented as means ± SE. Statistical significance was evaluated by Student’s t-test; P values <0.05 were considered significant.

RESULTS

Inhibition of Kv2 channels constricts cerebral arteries. To examine Kv2 channel function in intact vessels, we pressurized freshly isolated midcerebral arterial segments to 80 mmHg. After myogenic tone was developed, the inner diameter of the pressurized artery presented in Fig. 1A was 82 μm (passive diameter = 109 μm; 24.7% constriction). We then applied 10 nM of the Kv2 channel inhibitor stromatoxin (Fig. 1, A and B). This concentration is close to the reported IC₅₀ (~12 nM) for inhibition of Kv2 channels by native stromatoxin (13). As shown in Fig. 1A, even at this relatively low concentration, stromatoxin induced robust constriction of cerebral arteries. Indeed, on average, 10 nM stromatoxin induced a 12.6 ± 0.8% constriction (n = 5). The effect of stromatoxin on arterial diameter was partially reversible, an observation that is consistent with the slowly reversible nature of stromatoxin inhibition of Kv2 channels (13). These data suggest that Kv2 channels oppose myogenic constriction in cerebral arteries.

To place the Kv2-mediated vasodilation into physiological perspective, we compared the effects of stromatoxin on myogenic tone to those of the large-conductance Ca²⁺-activated K⁺ (BK) channel inhibition with IbTx (100 nM). Note that 100 nM IbTx completely eliminates BK currents in cerebral arterial myocytes and robustly constricts cerebral arteries (8, 25). At this concentration, IbTx constricted (16.7 ± 2.2%; n = 5, data not shown) cerebral arteries to the same extent as 10 nM stromatoxin (12.6 ± 0.8%, n = 5, P = 0.13; Fig. 1B). These data suggest that Kv2 channels play a significant role in
opposing vasoconstriction and that the contribution of these channels to relaxation is, at a minimum, comparable to that of BK channels in cerebral arteries.

To substantiate this conclusion, we performed control experiments examining the specificity of stromatoxin. Although native stromatoxin has been shown to be without effect on Kv1 channels (Kv1.1-Kv1.6), including Kv1.2/1.5 heteromultimers (13), we tested the effect of the commercially available recombinant form of the toxin used in this study on heterologously expressed Kv2.1 and Kv1.2/1.5 channels (Fig. 1C). As expected, stromatoxin (100 nM) inhibited expressed Kv2.1 currents (by 75 ± 2.3%, n = 5) but had no effect on Kv1.2/1.5 currents, which were inhibited by 1 mM 4-aminopyridine (n = 5). Monomeric Kv1.5 currents were also insensitive to 100 nM stromatoxin (n = 5, data not shown).

We also tested the effect of stromatoxin on cerebral arterial myocyte BK channels in excised inside-out patches (Fig. 1D). BK channels produce easily identifiable currents of ~10 pA at +40 mV with symmetrical 140 mM K+ (2) with a steady-state (t > 5 min) P0 of 0.329 ± 0.033 (n = 10) at 1 μM Ca2+ at the cytosolic side of the membrane (32). Similar to structurally related hanatoxin (22), 100 nM stromatoxin did not modify L-type Ca2+ channel activity, which was sensitive to nifedipine (1 μM; n = 5). We conclude that 100 nM stromatoxin has no effect on Kv1.2/1.5, Kv1.5, BK, or L-type Ca2+ channels and thus is a specific blocker of Kv2.1 channels in arterial smooth muscle. Together, our data
strongly support the hypothesis that stromatoxin-sensitive Kv2.1 channels oppose constriction of cerebral arteries.

To further support our conclusion, we examined Kv2.1 channel expression in cerebral arteries. We observed robust Kv2.1 channel protein expression in cerebral arteries (Fig. 1F, top). Although arterial smooth muscle cells are the most abundant cell type in resistance arteries, Kv2.1 channels may also be expressed in non-smooth muscle cells (e.g., endothelial cells or neurons) present in the tissue. Thus we probed for smooth muscle cell-specific Kv2.1 expression by performing real-time RT-PCR on single isolated arterial myocytes. Clear amplification of Kv2.1 transcript was observed in 5 of 7 independent experiments using single smooth muscle cells subjected to RT-PCR (Fig. 1F, bottom). To control spurious amplification (i.e., non-smooth muscle cell debris and genomic and/or reagent contamination), we performed two parallel negative controls where either the reverse transcriptase was omitted or the desired template (smooth muscle cells) was not included (non-template control). Physiological saline aspirated from an area adjacent to dispersed smooth muscle cells served as our non-template control. Amplification was never observed in these negative controls (n = 7 each). From these data we conclude that Kv2.1 channels are expressed and functional in cerebral arterial smooth muscle.

Kv2 channel function is decreased in angiotensin II-dependent hypertension. After establishing that Kv2 channels are expressed and functional in cerebral arteries, we examined the role of these channels in the development arterial dysfunction during angiotensin II-dependent hypertension. We tested the effect of stromatoxin on pressurized midcerebral arterial segments from angiotensin II-infused rats. After myogenic tone was developed, the inner diameter of the pressurized artery (see Fig. 2A) was 76 μm (passive diameter = 107 μm; 28.9% constriction). Similar to control (sham) arteries, stromatoxin (10 nM) induced constriction in pressurized (80 mmHg) arteries from angiotensin II-infused rats (Fig. 2A). However, the effect of stromatoxin was reduced by ~50% in arteries from angiotensin II-infused rats (Fig. 2, A and B; 5.7 ± 1.1%; n = 5) compared with control arteries (12.6 ± 0.8%; P < 0.05, n = 5; data from Fig. 1). Indeed, inhibition of BK channels with IbTx (100 nM) in the continued presence of stromatoxin was required to constrict hypertensive artery segments to the same extent as stromatoxin alone constricted control arteries (dotted line in Fig. 2A). The smaller effect of stromatoxin on arteries from angiotensin II-infused rats was not due a diminished ability of these arteries to respond to contractile stimuli because depolarization with 60 mM KCl constricted sham (55.6 ± 1.6; n = 5) and hypertensive arteries (53.0 ± 2.3%; n = 5) to the same extent (P > 0.05; data not shown). From these data, we conclude that the reduced effect of stromatoxin on cerebral arterial smooth muscle during angiotensin II-dependent hypertension resulted from decreased Kv2 channel function.

If the decreased effect of stromatoxin on pressurized arteries from angiotensin II-infused rats results from decreased Kv2 channel function, then it is reasonable to predict that the stromatoxin-sensitive (Kv2) current should be reduced in arterial myocytes from these hypertensive animals. Thus we examined the stromatoxin sensitivity of Kv currents in cerebral arterial myocytes from control (sham) and angiotensin II-infused rats. We used a relatively high concentration of stromatoxin (100 nM) to maximally inhibit Kv2 currents without sacrificing specificity (13). Figure 2C shows the effect of stromatoxin (100 nM) on cerebral arterial myocyte Kv currents from control saline-infused (sham) and angiotensin II-infused rats. In sham myocytes at step depolarizations to +40 mV, approximately one-half of the Kv current (8.7 ± 1.8 pA/pF; n = 8) was sensitive to inhibition by stromatoxin. Consistent with our observations in intact arteries above and our previous findings on Kv2.1 expression (4), following angiotensin II infusion, Kv2 currents (i.e., stromatoxin-sensitive currents) were reduced by ~50% (3.5 ± 0.9 pA/pF; n = 8) compared with control (P < 0.05). The stromatoxin-insensitive component of the Kv current (i.e., Kv1) was not different between control myocytes and those from angiotensin II-infused animals (P > 0.05). These data are consistent with the view that...
Kv2, but not Kv1, channel function is impaired during angiotensin II hypertension.

Note that for sham myocytes (see Fig. 2C, left), the time course of inactivation over 500 ms was well described by two exponential terms \((\tau_1 = 89.4 \pm 5.3\% and \tau_2 = 339.8 \pm 29.6\%\, ms; n = 8)\). However, following strong inhibition of Kv2 channels with 100 nM stromatoxin (13), the time course of inactivation over 500 ms was well fit with a single exponential of 368.4 ± 54.2 ms for sham myocytes \((n = 8)\), suggesting that the more rapidly inactivating component is produced by Kv2 channels. Similarly, the rate of activation of sham currents was best fit with a biexponential function (see below); however, a single exponential \((5.4 \pm 1.2\, ms)\) was sufficient in the presence of stromatoxin, thus indicating that the slowly activating component is formed by Kv2 channels. These observations suggest that two populations of Kv channels, one of which is formed by stromatoxin-sensitive Kv2-containing channels, are functional in cerebral arterial myocytes.

The biophysical profile of cerebral arterial smooth muscle Kv currents is consistent with currents produced by Kv1 and Kv2 channels. If constriction of cerebral arteries and the decrease in whole cell Kv current by stromatoxin result from inhibition of Kv2 channels in arterial smooth muscle cells, then a substantial portion of the Kv currents of these myocytes should have properties that are consistent with Kv2 currents. Thus we used the conventional whole cell patch-clamp technique to characterize the Kv currents of cerebral arterial myocytes. IbTx (100 nM) was included in the nominally Ca2+-free external solution for all experiments to minimize contamination from Ca2+- and BK currents, the other major voltage-dependent K+ current in these cells (28, 37).

Note that 100 nM stromatoxin, which strongly inhibits Kv2 currents (13), reduced the total Kv current by ~50% (Fig. 2C, left). This suggests that approximately one-half of the composite current is mediated by Kv2-containing channels. With this in mind, we tested the hypothesis that the Kv current in cerebral arterial myocytes is produced by two populations of channels (i.e., Kv1 and Kv2) contributing equally \((i.e., \approx 50\%\) to the total Kv current. If the Kv current does indeed have distinct Kv1 and Kv2 components, then each parameter analyzed (kinetics and voltage dependencies) should reflect these two components. That is, the data should be well described by two components of about equal amplitude; one presumably corresponding to the Kv1 component (1) and the other to the Kv2 component.

We first analyzed the rate of decay of composite Kv currents in voltage-clamped cerebral arterial myocytes. As shown in Fig. 3A, 15-s step depolarizations to potentials between ~60 and +40 mV from a holding potential of ~70 mV evoked slowly inactivating outward Kv currents \((n = 16)\). The extent of inactivation, given as a percentage, during 15-s step depolarizations to +40 mV was 67.4 ± 1.6% \((n = 16)\). The time course of inactivation at +40 mV was well fit with an equally weighted biexponential function (see Fig. 3A, C and D) with \(\tau_1 = 1.05 \pm 0.08\) s and \(\tau_2 = 5.79 \pm 0.28\) s \((n = 16)\). A shorter voltage protocol was used to measure the kinetics of activation and deactivation (Fig. 3D). The kinetics of activation were faster at more depolarized test potentials (see Fig. 3D). At +40 mV; activation was well fit with an equally weighted biexponential function with \(\tau_1 = 5.71 \pm 0.44\) ms and \(\tau_2 = 19.74 \pm 1.38\) ms \((n = 19)\). As evident in tail currents recorded at ~30 mV (see Fig. 3, D and F), these Kv currents deactivated relatively slowly. The time constants of the deactivation at ~30 mV after step depolarizations to +40 mV currents were \(\tau_1 = 55.91 \pm 4.17\) ms and \(\tau_2 = 134.20 \pm 5.04\) ms \((n = 19)\).

These analyses illustrate that the kinetic profile of Kv currents in cerebral arterial smooth muscle cells resembles that of currents formed by Kv1 and Kv2 channels. For example, the fast components of activation and deactivation \((\tau_1\) in each case) resemble those of Kv1.2/Kv1.5 heteromultimers (18, 30), whereas the slow components \((\tau_2)\) are similar to values reported for Kv2.1/Kv5.1 heteromultimers (21). Although the rates of inactivation were clearly best fit with a double-exponential function, the results were less informative with respect to Kv1 and Kv2 values reported in the literature than those of activation and deactivation. This may result from variance in the apparent rate of inactivation due to differences in test pulse length.

To assess the voltage dependency of activation, peak Kv currents were transformed into conductances, normalized, and plotted as a function of voltage (Fig. 3H). These data were fit with a two-component Boltzmann equation, in which the voltage at 50% current \((V_{1/2})\) and slope factor \((k)\) values were fixed using values corresponding to Kv1.2/1.5 and Kv2.1/5.1 heteromultimers reported in the literature (see Fig. 3 for details). This analysis revealed that the conductance-voltage relationship of Kv currents in cerebral arteries was well fit \((r^2 = 0.98, n = 35)\) with \(V_{1/2}\) and \(k\) values for Kv1.2/1.5 and Kv2.1/5.1 channels. The voltage dependency of steady-state inactivation was determined using 15-s conditioning potentials between ~70 and +40 mV, followed by a test potential of +40 mV (Fig. 3G). As with conductance, the voltage dependency of steady-state inactivation was well fit \((r^2 = 0.99, n = 14)\) with \(V_{1/2}\) and \(k\) values corresponding to Kv1.2/1.5 and Kv2.1/5.1 heteromultimers.

To further support our hypothesis that cerebral arterial myocyte Kv currents are produced by two populations of channels, we repeated the biophysical analysis presented above on Kv currents from myocytes isolated from rats infused with angiotensin II (data not shown). Currents were obtained using the same voltage protocols as for the sham myocytes in Fig. 3. For these analyses, the amplitude of the components putatively corresponding to Kv2 channels was reduced in half to reflect the ~50% reduction of Kv2 current, as determined by stromatoxin sensitivity above. Thus the amplitude of \(\tau_2\) for activation and deactivation and the amplitude of \(\tau_1\) for inactivation \((i.e., the Kv2 component)\) were reduced to 0.33 and the amplitudes of \(\tau_1\) for activation and deactivation and the amplitude of \(\tau_2\) for inactivation \((Kv1 component)\) were increased to 0.66 to account for the increased proportional contribution of Kv1 current. The results from these analyses are included in Table 1. In all cases, myocytes from angiotensin II-infused rats had Kv currents that were well fit with bi-exponential functions containing a reduced Kv2 channel component and yielded \(\tau\) values that were not different from those obtained from sham myocytes fitted with two equally weighted exponentials \((P > 0.05\) for all parameters).

**DISCUSSION**

In this study, we investigated the functionality of Kv2 channels in cerebral arterial smooth muscle. Three important
conclusions may be drawn from our observations. First, Kv2 channels are expressed and functional in cerebral arterial smooth muscle. Second, Kv2 channels oppose myogenic constriction of cerebral arteries. Third, Kv2 channel function is impaired in cerebral arteries during angiotensin II-dependent hypertension. Taken together, our findings provide compelling evidence supporting the conclusion that Kv2 channels are physiologically relevant regulators of cerebral arterial smooth muscle function.

The evidence suggesting Kv2 channel function in cerebral arterial smooth muscle is manifold. First, we observed Kv2.1 transcript and protein expression in arterial smooth muscle. Second, stromatoxin, an specific inhibitor of Kv2 channels (see below), decreased the amplitude of Kv currents in single arterial myocytes and constricted pressurized intact arterial segments following the development of myogenic tone. Third, the biophysical profile of cerebral arterial Kv currents contains a component that is consistent with that of Kv2 channels. Taken together, these data provide strong support to the view that cerebral arterial smooth muscle cells express functional Kv2 channels, which play a significant role in the regulation of membrane potential of these cells.

Many of the conclusions reached in this study depend on the Kv2 channel specificity of stromatoxin. Stromatoxin is a member of a well-characterized family of spider toxins composed of structurally conserved short peptide sequences possessing multiple disulfide bridges; other members include the hanatoxins, heteropodotoxins, and phrixotoxins (10). This family of toxins inhibits ion channels not by pore occlusion but rather by specific modification of gating kinetics (14). At 100 nM, stromatoxin does not inhibit Kv1 channels (Kv1.1–1.6), including Kv1.2/1.5 heteromultimers (13). This final point is
properties of the native toxin. We also found that stromatoxin used in this study retains the pharmacological channels suggest that the commercially available recombinant channels (e.g., Kv2.1/Kv9.3) (13). Although stromatoxin has no inactivating "A-type" currents produced by Kv4 channels are many smooth muscles (3); however, the rapidly activating and inactivation were increased to 0.66 to reflect the increased proportional of myocytes examined. Kv, voltage-gated Ca²⁺-independent K⁺ channel; τ₁, fast activation/deactivation component; τ₂, slow activation/deactivation component. For sham Kv currents, kinetic parameters were modeled with two components of equal amplitude (amplitudes of τ₁ and τ₂ = 0.5). For Kv currents from angiotensin II-treated rats, the amplitude of τ₂ for activation and deactivation and the amplitude of τ₁ for inactivation were reduced to 0.33 to reflect the reduction in Kv2 current during hypertension; accordingly, the amplitudes of τ₁ for activation and deactivation and the amplitude of τ₂ for inactivation were increased to 0.66 to reflect the increased proportional contribution of Kv1 current. *P < 0.05, no parameter was significantly different between sham and angiotensin II Kv currents.

Table 1. Comparison of Kv current kinetics in myocytes isolated from sham and angiotensin II-infused rats

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<th>Activation, ms</th>
<th>Deactivation, ms</th>
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<td>Sham*</td>
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<td>τ₁</td>
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<td>τ₂</td>
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<td>τ₁</td>
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<td>0.98 ± 0.11 (7)</td>
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<td>τ₂</td>
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Values are means ± SE. Numbers given in parentheses indicate the number of myocytes examined. Kv, voltage-gated Ca²⁺-independent K⁺ channel; τ₁, fast activation/deactivation component; τ₂, slow activation/deactivation component. For sham Kv currents, kinetic parameters were modeled with two components of equal amplitude (amplitudes of τ₁ and τ₂ = 0.5). For Kv currents from angiotensin II-treated rats, the amplitude of τ₂ for activation and deactivation and the amplitude of τ₁ for inactivation were reduced to 0.33 to reflect the reduction in Kv2 current during hypertension; accordingly, the amplitudes of τ₁ for activation and deactivation and the amplitude of τ₂ for inactivation were increased to 0.66 to reflect the increased proportional contribution of Kv1 current. *P < 0.05, no parameter was significantly different between sham and angiotensin II Kv currents.

particularly relevant because Kv1.2/1.5-containing heteromultimers are thought to underlie the Kv1 current in cerebral arterial smooth muscle (1). In contrast, stromatoxin potently inhibits Kv2.1-, Kv2.2-, and Kv2-containing heteromeric channels (e.g., Kv2.1/Kv9.3) (13). Although stromatoxin has no effect on Kv3 channels, it is a potent inhibitor of Kv4.2 channels (IC₅₀ ≈ 1 nM) (13). Kv4 channels are present in many smooth muscles (3); however, the rapidly activating and inactivating "A-type" currents produced by Kv4 channels are not a component of the Kv current of cerebral arterial myocytes (see Fig. 2D, and Refs. 1, 4, 28). Our data showing inhibition of heterologously expressed Kv2.1 but not Kv1.5 or Kv1.2/1.5 channels suggest that the commercially available recombinant stromatoxin used in this study retains the pharmacological properties of the native toxin. We also found that stromatoxin had no effect on BK channels or L-type Ca²⁺ currents. Thus, with respect to cerebral arterial smooth muscle cell Kv currents, stromatoxin appears to be a selective inhibitor of Kv2 channels.

Two recent studies (1, 27) studies have suggested that Kv1 channels underlie the Kv1 current regulating membrane potential and myogenic tone in systemic resistance arteries. The biophysical properties of cerebral arterial smooth muscle Kv currents are consistent with and do not exclude a significant contribution from Kv1 channels to the Kv current ensemble. However, the same can also be said for Kv2 channels because of overlapping patterns of Kv channel properties. Consistent with this, strong inhibition of Kv1 channels with 1 μM crotoxolide (1) and Kv2 channels with 100 nM stromatoxin (this study) resulted in substantial but less than complete inhibition of cerebral arterial smooth muscle Kv currents in each case. Thus, we propose that both Kv1 and Kv2 channels contribute to membrane hyperpolarization and thus relaxation of cerebral arterial smooth muscle. Furthermore, we were able to model the kinetic and voltage-dependent properties of the composite Kv current with two distinct equally weighted components that correspond to Kv1 and Kv2 channels.

As our analyses indicate, for the Kv current kinetics (activation, inactivation, and deactivation) and voltage dependencies (activation and inactivation) we always observed two numerically distinct components that varied little between individual cells with an r² value > 0.95. These data suggest that the biophysical profile of Kv currents in cerebral arterial smooth muscle cells is consistent with a model, in which composite currents in cerebral arterial myocytes are formed by roughly equal contributions of Kv1 and Kv2 channels. Note, however, that there is a great deal of overlap between Kv1 and Kv2 current features in the literature. Indeed, a Kv1 current with properties that resemble a Kv2 current in many respects has been described previously (27). Furthermore, the kinetics of a homogenous population of channels are often described by multieponential functions. This highlights the inherent problems associated with assigning molecular correlates to endogenous currents on the basis of biophysical properties alone. From our data, however, we can conclude that the biophysical properties of Kv currents in cerebral arteries are consistent with currents formed by a roughly equal contribution of Kv1 and Kv2 channels.

Because, in addition to Kv2.1, stromatoxin also inhibits Kv2.2 and Kv2-containing channels with modulatory subunits (13), we cannot conclude that the stromatoxin-sensitive current observed is due exclusively to inhibition of Kv2.1 channels. Indeed, the rates of activation and deactivation are very similar to that of Kv2.1 channels co-expressed with "silent" nonconducting Kv5.1 subunits; Kv5.1 does not markedly change the rate of inactivation of Kv2.2 currents (21, 31). However, our previous finding of a 50% decrease in Kv2.1 protein during angiotensin II-dependent hypertension (4) coupled with the 50% decrease in the stromatoxin-sensitive current here indicates that the majority of the channels sensitive to stromatoxin contain Kv2.1 subunits. Although Kv5.1 transcripts have been detected in smooth muscle (35) and we have detected Kv5.1 transcripts in cerebral arterial tissue (G. C. Amberg and L. F. Santana, unpublished observation), we cannot conclude that Kv5.1 channels interact with Kv2.1 channels in cerebral arterial smooth muscle at this time. To investigate this intriguing issue is beyond the scope of this study and acceptance or rejection of this hypothesis would not change our conclusion that Kv2 channels are important regulators of excitability in cerebral arterial smooth muscle.

In contrast to the stromatoxin-sensitive (Kv2) current, the amplitudes of the stromatoxin-insensitive component (i.e., Kv1 component) of the composite Kv currents was similar in control and angiotensin II myocytes. These data indicate that Kv2, but not Kv1, channel function is decreased during the development of angiotensin II-dependent hypertension. These findings are consistent with our recent study (4), demonstrating "≈50% reduction of Kv2.1 protein after angiotensin II infusion; we did not detect a change in Kv1.2 or Kv1.5 channel expression. Note that Kv1.2 and Kv1.5 are thought to underlie the Kv1 current in cerebral arterial myocytes (1). It is important to mention that downregulation of Kv1.5 channels in mesenteric arterial smooth muscle has been reported in a different model of acquired hypertension (7); unfortunately, Kv2.1 expression was not examined. Future studies should examine changes in Kv2 and Kv1 channel function in other models of hypertension was well as different arterial beds.

The effect of stromatoxin on the arterial diameter of pressurized arteries was striking. At a concentration only approaching the IC₅₀ (≈12 nM) for Kv2.1 channels (10 nM), stromatoxin induced arterial constriction to a similar extent as near-
complete inhibition of BK channels (8, 25) with IbTx. Although quantitative comparisons between Kv2 and BK channel function based on pharmacological inhibition would be speculative at best, from our data, we can conclude qualitatively that Kv2 channels oppose myogenic constriction at least to a similar extent as BK channels under physiological conditions. These findings are particularly interesting because impaired BK channel function due to loss of the accessory β1 subunit is associated with enhanced vasoconstriction and hypertension (2, 9). In this context, it is intriguing to speculate that, as with BK channels, downregulation (~50%) of Kv2.1 function could, in principle, contribute to arterial dysfunction during hypertension. Future studies should address this important issue.

It is possible that the effects of stromatoxin on pressurized arteries resulted from inhibition of neuronal (we removed the endothelium) Kv2 or Kv4.2 channels. Such inhibition could potentially cause neuronal depolarization and release of vasoactive factors (e.g., norepinephrine). However, Knot and Nelson (20) concluded that vasoconstriction induced by inhibition of cerebral arterial Kv currents with the nonspecific K⁺ channel blocker 4-aminopyridine was myogenic in origin because it was unaffected by pharmacological blockade of muscarinic, adrenergic, serotonergic, and histaminergic receptors. Furthermore, our dual observation of a 50% decrease in smooth muscle cell stromatoxin-sensitive (i.e., Kv2) current and a 50% decrease in stromatoxin-mediated constriction in smooth muscle from angiotensin II-infused rats is consistent with stromatoxin acting directly on the Kv2 channels located on the arterial smooth muscle cells.

Our data showing a 50% decrease in stromatoxin-mediated constriction in arteries from angiotensin II-infused rats is consistent with a decrease in Kv2 channel function. However, an important consideration must be addressed before reaching such a conclusion. It has been suggested that during hypertension cerebral arteries are depolarized from approximately −50 to −35 mV (37). This could confound our observation of decreased stromatoxin effect if the number of available Kv2 channels is reduced as a result of greater steady-state inactivation at the more depolarized potential. However, examination of the conductance and steady-state inactivation curves presented in Fig. 3 reveals that this is unlikely. Depolarization from −50 to −35 mV would result in only a 33% (~0.75% to 0.50%) decrease in Kv channels available because of greater steady-state inactivation. Furthermore, the same 15-mV depolarization would also increase the proportion of channels activated because this voltage range is at the shoulder of the K⁺ conductance curve. Thus the 33% decrease in the number of channels available would be partially offset by an increase in the proportion of channels activated. Therefore, decreased Kv2 channel function as a result of increased steady-state inactivation cannot by itself account for the 50% reduction in the stromatoxin response in arteries from angiotensin II-infused rats. However, our electrophysiological data showing a decrease of Kv2 current amplitude by 50% in cerebral arterial myocytes from angiotensin II-infused rats could account for the decreased effect of stromatoxin in these arteries. Note that nonspecific depolarization of normo- and hypertensive arteries resulted in the same degree of constriction (~50% for each). This indicates that arteries from angiotensin II-infused animals are able to respond to contractile stimuli to the same extent as arteries from normotensive animals.

In conclusion, we have provided multiple, independent lines of evidence supporting the conclusion that Kv2 channels are physiologically opponents of myogenic constriction in cerebral arterial smooth muscle. Furthermore, we have shown that during angiotensin II-dependent hypertension, Kv2 channel function is disrupted. This observation demonstrates that the level of Kv2 channel function is dynamic and suggests that Kv2 channels may contribute to the electrical plasticity of cerebral arterial smooth muscle in health and disease.

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