Voltage-gated K⁺ (Kv) channels control the excitability of arterial smooth muscle. However, the molecular mechanisms regulating Kv channel function in smooth muscle remain unclear. We examined the hypothesis that the vasoactive peptide angiotensin II (Ang II) regulates arterial smooth muscle Kv channel function via calcineurin-dependent activation of the transcription factor NFAT. We found that sustained administration of Ang II decreased Kv currents (I_Kv) by reducing the expression of Kv2.1 K⁺ channel subunits. This effect of Ang II was independent of pressure but required Ca²⁺ influx through L-type Ca²⁺ channels. Consistent with our hypothesis, we found that calcineurin and NFAT3 are obligatory components of the signaling cascade mediating reduced I_Kv by Ang II. We conclude that sustained Ang II exposure increases smooth muscle Ca²⁺, which leads to activation of calcineurin and NFAT3, culminating in decreased Kv2.1 expression and reduced I_Kv function. These results support the novel concept that NFAT3 controls the excitability of arterial smooth muscle by regulating Kv2.1 expression.

Angiotensin II is a potent vasoconstrictor. In addition to increasing contractility (1), the effects of Ang II on arterial smooth muscle include stimulation of myocyte proliferation (2) and regulation of gene expression (3). Ang II activates arterial smooth muscle by increasing intracellular Ca²⁺ ([Ca²⁺]i). Two mechanisms may contribute to increased [Ca²⁺]i during Ang II stimulation. First, Ang II activates Ca²⁺ release from intracellular stores (4–6). Second, Ang II decreases the activity of large conductance, Ca²⁺-activated K⁺ (BK) (7) and Kv channels (8) through a mechanism that involves the activation of protein kinase C (PKC). Decreases in outward BK and Kv currents depolarize arterial smooth muscle. Depolarization increases Ca²⁺ influx through voltage-gated Ca²⁺ channels, which increases [Ca²⁺]i, thereby causing contraction. Accordingly, sustained in vivo administration of Ang II leads to hypertension (9).

A recent study (3) suggested that Ang II may regulate gene expression in arterial smooth muscle by activating the Ca²⁺/calmodulin-dependent phosphatase calcineurin (CaN). Although the precise mechanisms by which Ca²⁺ controls CaN activation are unclear, the prevailing view holds that sustained increases in global Ca²⁺ are necessary (10). Upon activation, CaN dephosphorylates the ubiquitous transcription factor NFAT, which translocates to the nucleus and interacts with another transcription factor, GATA6 (11–13). Formation of this heterodimer results in modification of gene expression. Four (NFATc1–c4) of the five known NFAT isoforms are regulated by CaN (12). Expression profiling indicates that NFAT3 predominates in cerebral vascular smooth muscle (14, 15). NFAT3 may function as an activator (16) or as a repressor of gene expression (17). However, the functional effects of CaN/NFAT signaling and the role it plays in changing arterial function during Ang II signaling are unclear.

The aim of this study was to examine the effects of Ang II and CaN/NFAT signaling on the function of K⁺ channels in cerebral arterial smooth muscle. We found that in vivo administration of Ang II reduced Kv channel function in cerebral arteries by reducing the expression of Kv2.1 K⁺ channel subunits. The effects of Ang II on Kv currents were independent of blood pressure. Most importantly, we observed that CaN/NFAT3 activity is necessary and sufficient for Ang II-dependent regulation of Kv currents in arterial smooth muscle. We conclude that activation of NFAT3 by prolonged Ang II signaling decreases Kv channel function by decreasing Kv2.1 K⁺ channel subunit expression.

**Experimental Procedures**

**In Vivo Angiotensin II Administration and Blood Pressure Measurements**—We implanted osmotic minipumps (Alzet) delivering Ang II (250 ng/kg/day) subcutaneously into male Sprague-Dawley rats. Blood pressure measurements were taken using a tail-cuff plethysmograph from Narco Biosystems as described previously (9). Animals were handled in strict accordance to the guidelines of the University of Washington Institutional Animal Care and Use Committee.

**Organ Culture and Isolation of Arterial Myocytes**—Rats and mice (male wild type BALB/c and NFAT3 knock-out, ~25 g) were euthanized with a lethal dose of sodium pentobarbital (100 mg/kg; intraperitoneally). Cerebral artery segments were cultured as described before (18). Briefly, basilar, middle, and posterior cerebral arteries were dissected and put into sterile, ice-cold buffer composed of the following (in mM): 137 NaCl, 5.6 KCl, 2.6 CaCl₂, 1 MgCl₂, 4.17 NaHCO₃, 0.42 NaH₂PO₄, 0.44 NaH₂PO₄, 5 glucose, 10 HEPES, supplemented with penicillin/streptomycin (1% v/v), Eagle’s minimum essential medium amino acid solution (2% v/v), and MEM vitamin solution (pH 7.4). Arteries were cleaned of connective tissue, cut into pieces (~2 mm in length), and placed in sterile 35-mm Petri dishes containing 2 ml of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 0.5% albumin (v/v) and 1% penicillin/streptomycin (v/v). The arteries were stored for 48 h at 37 °C in a humidified incubator fed with a gas mixture of 95% O₂ and 5% CO₂. Smooth muscle cells were prepared from freshly dissected and cultured cerebral arteries as described previously (19).

**Reversible Permeabilization**—Cerebral arteries were reversibly permeabilized using a derivative of a protocol described previously (20).
Briefly, arteries were incubated for 20 min at 4 °C in the following solution (in mM): 120 KCl, 2 MgCl₂, 10 EGTA, 5 Na₃ATP, 20 TES (pH 6.8). Samples were then placed in this solution supplemented with the calcineurin auto-inhibitory peptide (Cip, 100 μM) and M, 3000 Calcium Green³⁻/dextran conjugate (100 μM; Molecular Probes) or Calcium Green³⁻ alone (200 μM) for 60 min at 4 °C followed by a similar CIP/Calcium Green³⁻-containing solution with elevated MgCl₂ (10 mM) for 30 min at 4 °C. Permeabilization was reversed by placing the arteries for 30 min at 22 °C in a MOPS-buffered physiological solution containing the following (in mM): 140 NaCl, 5 KCl, 10 MgCl₂, 5 glucose, 2 MOPS, pH 7.1. Ca²⁺ was then gradually increased in this solution from nominally Ca²⁺-free to 0.01, 0.1, and 1.8 mM over 45 min. Arteries were then cultured as described above for 48 h.

Adenoviral Infection—The adenoviral construct encoding a constitutively active form of NFAT with an N-terminal EGFP tag (NFAT/EGFP) (21) was kindly provided by Dr. Martin F. Schneider. Briefly, the adenoviral construct encodes a constitutively active form of NFAT with an N-terminal EGFP tag (NFAT/EGFP) (21) before (left) and after (center) application of Ibtx (100 nM). Western blots of Kv1.2, -1.5, and -2.1 channel proteins in sham and Ang II cerebral arteries. The bar plot shows the relative amount (compared with sham) of Kv channel protein after Ang II infusion. *p < 0.05 versus sham. C, Western blots of Kv1.2, -1.5, and -2.1 channel proteins in sham and Ang II cerebral arteries. The bar plot shows the relative amount (compared with sham) of Kv channel protein after Ang II infusion. *p < 0.05 versus sham.

Western Blots—Cerebral arteries were homogenized with a Dounce homogenizer for 30–60 s in phosphate-buffered saline with 5 mM EDTA, 1% Triton X-100, and a mammalian protease inhibitor mixture (Sigma). The homogenate was cleared of cellular debris by centrifugation at 10,000 × g at 4 °C for 10 min. Protein concentration of the supernatant was measured using the bicinchoninic acid method (22) with bovine serum albumin as a standard. 10 μg of total protein was loaded on a 4–15% Tris-HCl polyacrylamide gel and run with an appropriate molecular weight standard at 100 mM for 1 h. Fractionated protein was transferred to a polyvinylidene difluoride membrane using a Mini-trans Blot Cell (Bio-Rad) at 100 V for 60 min at 4 °C. The blots were blocked in TBS/Tween (20 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat milk for 1 h at 25 °C. After blocking, blots were incubated with primary antibodies specific to Kv1.2 (Sigma), Kv1.5 (Sigma), and Kv2.1 (Sigma) and TBS/Tween with 1% nonfat milk at 4 °C overnight. Following incubation with the primary antibody, blots were washed three times with TBS/Tween for 10 min and incubated with a horseradish peroxidase-conjugated secondary antibody diluted in TBS/Tween for 2 h. This was followed by incubation with SuperSignal West Fermo Chemiluminescent Substrate (Pierce). Kv channel protein was quantified by densitometry using an Alphaimager 2200 analysis system and software (Alpha Innotech Corp).

Confocal Microscopy and [Ca²⁺]ᵢ Imaging—Confocal images were acquired using Bio-Rad Radiance 2100 confocal system coupled to an inverted Nikon TE-300 microscope equipped with a Nikon Plan Apo (60×, NA = 1.4) oil-immersion lens. Images were acquired and analyzed with Lasersharp 4.0 (Bio-Rad) software. For [Ca²⁺]ᵢ imaging experiments, cells were loaded with the acetoxymethyl form of the fluorescent Ca²⁺ indicator fluo-4 (5 μM) as described previously (19). In these experiments, background-subtracted fluorescence signals were normalized by dividing the fluorescence (F) intensity at each time point by the resting fluorescence (Fᵢ). Images were pseudo-colored (when appropriate) using Corel Photopaint 11. All imaging experiments were performed at room temperature (22–25 °C).

Chemicals and Statistics—Unless otherwise stated, all chemicals were from Sigma. Data are presented as mean ± S.E. Two sample comparisons were made by using Student’s t test. Multigroup comparisons were made by one-way analysis of variance followed by Tukey’s multiple comparison when appropriate. A p value less than 0.05 was considered significant. The asterisk is used in the figures to illustrate a significant difference between groups. n values represent number of cells (electrophysiology) or animals (Western analysis) studied.

RESULTS

In Vivo Administration of Angiotensin II Decreases Kv Current in Arterial Smooth Muscle—To examine the effects of systemic Ang II on K⁺ channel function, we implanted subcutaneous osmotic minipumps delivering Ang II (250 ng/kg/day) to male Sprague-Dawley rats. We used systolic blood pressure...
NFATc3 Controls Smooth Muscle Excitability

**Fig. 2. Ang II-mediated reduction of I_{Kv} is independent of arterial pressure.** A, representative K^+ currents from control (medium only, top row) and Ang II-treated cells (bottom row) recorded before (left) and after z (center) the application of Ibtx (100 nM). The traces to the right represent the Ibtx-sensitive (I_{Btx}) component of these currents. B, current-voltage relationships of I_{Kv}, I_{Kv1.2}, and I_{Btx} in control (medium only) and Ang II (top row) cells. *p < 0.05 versus medium. Western blots of Kv1.2, Kv1.5, and Kv2.1 channel proteins in control and Ang II arteries. The bar plot shows the relative amount (compared with medium) of Kv channel protein in cerebral arteries cultured in the presence of Ang II. *p < 0.05 versus medium.

| Kv protein       | Control | Ang II |
|------------------|---------|--------|
| Kv1.2            | 100 kDa | 60 kDa |
| Kv1.5            | 60 kDa  | 120 kDa|
| Kv2.1            | 120 kDa | 60 kDa |

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**Kv Current Is Independent of Pressure**—We investigated the signaling mechanism(s) by which increased systemic Ang II reduced I_{Kv} in arterial smooth muscle. First, we tested the hypothesis that the effect of Ang II on arterial smooth muscle I_{Kv} was independent of arterial pressure. We assayed the effects of sustained Ang II exposure on I_{Kv} by culturing cerebral arteries from normotensive rats for 48 h in the presence or absence of Ang II (100 nM). An important advantage of this approach is that, unlike cultured dissociated cells, myocytes isolated from intact arteries do not undergo significant phenotypic drift during short term (48 h) culture (18).

To establish success of the infusion. Similar to others (9), 7 days after pump implantation, Ang II-infused animals were hypertensive (systolic pressure = 210 ± 10 mm Hg, n = 6) whereas control animals infused with saline (sham) remained normotensive (systolic pressure = 120 ± 10 mm Hg, n = 6). After confirmation of Ang II delivery, we examined K^+ currents in cerebral arterial smooth muscle cells from control and Ang II-infused rats (Fig. 1). K^+ currents (I_K) were evoked in sham and Ang II myocytes by applying 1-s depolarizing pulses from a holding potential of −70 mV to voltages ranging from −80 to +70 mV. In cerebral arterial smooth muscle cells, I_K values are primarily composed of voltage-activated (I_Kv) and large conductance, BK (I_{BK}) currents (23). To separate I_K from I_{BK}, we recorded K^+ currents before and after application of iboterin (Ibotx; 100 nM), a specific blocker of BK channels.

**Fig. 1A** shows two sets of representative I_K traces recorded from myocytes isolated from sham-treated and Ang II-infused animals. We found that I_K was smaller in Ang II cells than in sham cells from sham arteries (n = 10, p < 0.05). Application of Ibotx (100 nM) reduced the amplitude of K^+ currents in sham and Ang II cells. However, the amplitude of the Ibotx-insensitive current, which we designated as the voltage-dependent K^+ current (I_{Kv}), was smaller in Ang II cells than in sham cells (p < 0.05). In contrast, the Ibotx-sensitive K^+ currents (i.e. I_{Btx}) were similar in Ang II and sham cells (p > 0.05). Fig. 1B shows the current-voltage relationships of I_{Kv}, I_{Kv1.2}, and I_{Btx} in these cells. Note that I_{Kv} and I_{Kv1.2} values were smaller in Ang II cells than in sham cells at most voltages examined. At +70 mV, the amplitude of I_{Kv} was 35.2 ± 5.1 pA/pF (n = 10) and 15.2 ± 5.1 pA/pF (n = 10, p < 0.05) in sham and Ang II cells, respectively. At +70 mV, I_{Kv1.2} was ~60% smaller in Ang II (10.2 ± 5.1 pA/pF, n = 10) than in sham (25.2 ± 5.1 pA/pF, n = 10, p < 0.05) cells. Conversely, I_{BK} was similar in Ang II and sham cells at all voltages (p > 0.05). These data show that in vivo administration of Ang II decreases I_{Kv} because of a reduction in the amplitude of I_{Kv1.2} but not I_{BK}.

In vascular smooth muscle, I_{Kv} is thought to be produced by Kv1.2, Kv1.5 (24, 25), and Kv2.1 (25) K^+ channel subunits. We used Western blot analysis to examine Kv1.2, Kv1.5, and Kv2.1 protein expression in sham-treated and Ang II arteries (Fig. 1C). In accordance with the ~60% reduction of I_{Kv} observed with our electrophysiological measurements (e.g. Fig. 1A and B), Western blot analysis revealed a 50 ± 10% reduction in Kv2.1 protein expression (Fig. 1B; n = 5, p < 0.05). Most interestingly, Ang II infusion did not change the amount of Kv1.2 and Kv1.5 protein (p > 0.05). These data suggest that the reduction of I_{Kv} observed following Ang II infusion resulted from decreased expression of Kv2.1 channel subunits.

**Angiotensin II-mediated Decrease of Arterial Smooth Muscle Kv Current Is Independent of Pressure**—We investigated the signaling mechanism(s) by which increased systemic Ang II reduced I_{Kv} in arterial smooth muscle. First, we tested the hypothesis that the effect of Ang II on arterial smooth muscle I_{Kv} was independent of arterial pressure. We assayed the effects of sustained Ang II exposure on I_{Kv} by culturing cerebral arteries from normotensive rats for 48 h in the presence or absence of Ang II (100 nM). An important advantage of this approach is that, unlike cultured dissociated cells, myocytes isolated from intact arteries do not undergo significant phenotypic drift during short term (48 h) culture (18).

Following 48 h of organ culture, we dissociated myocytes from these arteries and recorded K^+ currents as described above. Similar to cells from Ang II-infused rats, I_{Kv} was smaller in myocytes from arteries cultured with Ang II than in myocytes from control (culture medium only) arteries (Fig. 2, A and B). At +70 mV the amplitude of I_{Kv} was 37.7 ± 3.8 pA/pF (n = 6) and 19.1 ± 3.6 (n = 7, p < 0.05) in control and Ang II cells, respectively. Consistent with this, at this potential I_{Kv} was ~60% smaller in cultured Ang II (10.8 ± 1.6 pA/pF) than in control cells (28.4 ± 2.1 pA/pF; p < 0.05). As with Ang II infusion, culturing cerebral arteries in the presence of Ang II did not alter I_{BK} (p < 0.05).

We used Western blot analysis once again to examine whether a decrease in Kv1.2, Kv1.5, or Kv2.1 protein accompanied the observed reduction of I_{Kv} in vitro. Similar to arteries from Ang II-infused rats, we found that Kv2.1 protein levels
Ang II and Ang II/H11010/H11011 calcineurin plays a critical role in the control of $I_{Kv}$. A, representative $K^+$ currents (at +70 mV) from cells isolated from arteries cultured in the presence of medium alone, with CsA (1 μM), or with CsA plus Ang II (100 nM). $K^+$ currents were recorded in these cells before and after the application of Ibx (100 nM). The traces to the right represent the Ibx-sensitive ($I_{Kv,i}$) component of these currents. B, representative transmitted and fluorescent images of a nonloaded control (saline; left) myocyte and a Ca$^{2+}$-Green-loaded myocyte (right). C, representative $K^+$ currents (at +70 mV) from cells isolated from arteries cultured with medium alone or in the presence of Ang II (100 nM) plus Ca$^{2+}$-Green dextran (Ang II + dextran; 200 μM) and Ang II plus CiP (100 μM) and Ca$^{2+}$-Green dextran (100 μM; Ang II + CiP). $K^+$ currents were recorded in these cells before and after the application of Ibx (100 nM). The traces to the right represent the Ibx-sensitive ($I_{Kv,i}$) component of these currents. D, current-voltage relationships of $I_{Kv}$ in medium, CsA, Ang II + CsA, Ang II + Ca$^{2+}$-Green dextran, and Ang II + CiP cells. *, $p < 0.05$ versus medium, CsA, Ang II + CsA, and Ang II + CiP. E, Western blots of $K_v$1.2, -1.5, and -2.1 proteins in medium (control), Ang II, and Ang II + CsA arteries. The bar plot shows the relative amount (compared with medium) of $K_v$ protein in arteries cultured in the presence of Ang II and Ang II + CsA. *, $p < 0.05$ versus medium.

were about 50% lower in arteries cultured with Ang II than in control (Fig. 2B; n = 10, $p < 0.05$). $I_{Kv,1.2}$ and $I_{Kv,1.5}$ protein levels were unchanged in Ang II-incubated arteries ($p > 0.05$). These data suggest that sustained Ang II signaling is sufficient to decrease $I_{Kv}$ by a reduction in $K_v$2.1 and $I_{Kv}$ are independent of changes in arterial pressure.

Activation of calcineurin is necessary for Angiotensin II-induced Reduction of $K_v$ Current in Arterial Smooth Muscle—Ang II has been shown to stimulate the activity of the Ca$^{2+}$-dependent phosphatase CaN in vascular smooth muscle cells (26). We tested the hypothesis that activation of CaN underlies the actions of Ang II on $I_{Kv}$ in arterial smooth muscle. $K^+$ currents were measured from cells isolated from cerebral arteries cultured with the CaN inhibitor cyclosporin A (CsA; 1 μM) in the presence or absence of Ang II (100 nM; see Fig. 3, A and D). CsA alone had no effect on $I_{Kv}$, $I_{Kv,K} + I_{Kv,B}$ (n = 5, $p > 0.05$). However, we found that with CsA in the culture medium, Ang II no longer reduced $I_{Kv}$ (n = 5, $p > 0.05$), suggesting that calcineurin plays a critical role in the control of $I_{Kv}$ by Ang II. To confirm that the effect of CsA resulted from CaN inhibition, as opposed to pharmacological nonspecificity, we performed similar experiments, but with the highly specific CaN auto-inhibitory peptide (CiP; 100 μM). CiP was introduced into arterial smooth muscle cells prior to culturing with Ang II (100 nM) using a reversible permeabilization technique. For identification of successful permeabilization, cells were loaded with an $M_r$ 30000 Calcium Green(TM)-dextran conjugate (200 μM for control) or co-loaded with Ca$^{2+}$-Green-dextran and CiP (100 μM, each). Permeabilized cells were identified by their Ca$^{2+}$-Green fluorescence (see Fig. 3B), and only positive cells were used for electrophysiological experiments. As with CsA, inhibition of CaN with CiP prevented the reduction of $I_{Kv}$ following sustained Ang II exposure (Fig. 3, C and D, n = 6, $p > 0.05$). These results indicate that activation of a CaN-dependent pathway is necessary for Ang II-mediated reduction of $I_{Kv}$. Consistent with this conclusion, CsA prevented the reduction in $K_v$2.1 (or $K_v$1.2 and $K_v$1.5) protein produced by Ang II (Fig. 3E; n = 5, $p > 0.05$). In addition, we found no difference between the levels of $K_v$2.1 protein expression arteries incubated in media alone or with CsA ($p > 0.05$; data not shown).

Angiotensin II-mediated Reduction of $K_v$ Current Requires Ca$^{2+}$ Influx through L-type Ca$^{2+}$ Channels—An increase in [Ca$^{2+}$], is a necessary step for Ang II-dependent activation of...
Indeed, Ang II increases $[\text{Ca}^{2+}]_i$ in portal vein smooth muscle by enhancing $\text{Ca}^{2+}$ influx through L-type $\text{Ca}^{2+}$ channels (27). Thus, we investigated if the actions of Ang II on $I_{KV}$ described above require $\text{Ca}^{2+}$ influx through L-type $\text{Ca}^{2+}$ channels. We first examined the effects of Ang II on $[\text{Ca}^{2+}]_i$ in freshly dissociated cerebral arterial myocytes. Fig. 4A shows that Ang II (100 nM) evoked a small but sustained ($n = 5, p < 0.05$) increase in global $[\text{Ca}^{2+}]_i$ as determined by confocal microscopy. However, in the presence of the L-type $\text{Ca}^{2+}$ channel blocker diltiazem (10 $\mu$M), application of Ang II had no effect on $[\text{Ca}^{2+}]_i$ ($n = 5, p < 0.05$). This suggests that Ang II increases $[\text{Ca}^{2+}]_i$ in cerebral arterial smooth muscle in an L-type $\text{Ca}^{2+}$ channel-dependent manner.

Accordingly, we tested the hypothesis that $\text{Ca}^{2+}$ influx through L-type channels is necessary for Ang II-dependent reduction of $I_{KV}$ (Fig. 4B). In these experiments, we measured $I_{Kv}$ and $I_{BK}$ amplitudes in myocytes from arteries cultured for 48 h with Ang II (100 nM) in the presence or absence of diltiazem (10 $\mu$M). Fig. 4B, shows that diltiazem abolished the decrease in $I_{KV}$ produced by 48 h of exposure to Ang II at all voltages examined ($n = 5, p < 0.05$). This indicates that $\text{Ca}^{2+}$ influx via L-type $\text{Ca}^{2+}$ channels is required, presumably in order to activate CaN, for Ang II down-regulation of $I_{KV}$ in arterial smooth muscle.

Expression of Activated NFATc3 Is Sufficient in Reducing Kv Current in Arterial Smooth Muscle—Members of the NFAT family of transcription factors are among the best characterized substrates for CaN (11–13). Furthermore, Ang II has been shown to activate NFATc3 in vascular smooth muscle (3, 14). We investigated whether NFATc3, the primary isoform expressed in cerebral arteries (14), underlies the actions of Ang II on $I_{KV}$ in arterial smooth muscle. We infected intact cerebral arteries with an adenovirus construct expressing a constitutively active, EGFP-tagged, NFATc3 mutant (ΔNFAT). Arteries exposed to an adenovirus expressing only the EGFP tag served as control. Infected myocytes were identified from noninfected myocytes by
their EGFP fluorescence (see Fig. 5A). Consistent with a biologically active form of NFATc3, cells expressing the ΔNFAT mutant showed high levels of nuclear fluorescence (Fig. 5A). In contrast, myocytes expressing EGFP alone showed a diffuse, largely cytoplasmic, pattern of fluorescence.

Expression of ΔNFAT reduced the amplitude of $I_K$ (Fig. 5, B and C; $n = 5$, $p < 0.05$) to the same extent as Ang II ($p > 0.05$). Furthermore, like Ang II, $I_{Kv}$ ($p < 0.05$) but not $I_{Ko}$ ($p < 0.05$) was smaller in ΔNFAT-expressing cells than in EGFP-positive control cells. These findings suggest that Ang II-mediated activation of NFATc3, we cultured cerebral arteries from transgenic mice lacking functional NFATc3 expression (c3-KO) (28) to activate NFATc3, we cultured cerebral arteries from transgenic mouse V12⁄2 (mouse V12⁄2). In addition, the voltage at which $I_K$ reached 50% ($V_{1/2}$) was smaller in the presence of Ang II than with culture medium alone. These data support the novel conclusion that CaN/NFATc3 is necessary for Ang II-mediated reduction of $I_{Ko}$ via activation of NFATc3.

NFATc3 Is Necessary for Angiotensin II-mediated Reductions of $K_v$ Current in Arterial Smooth Muscle—To provide direct support for our hypothesis that Ang II reduces $I_{Ko}$, through activation of NFATc3, we cultured cerebral arteries from transgenic mice lacking functional NFATc3 expression (c3-KO) (28) in the presence or absence of Ang II (100 nM). Fig. 6A shows that control wild type and c3-KO myocytes produced robust $I_{Ko}$, $I_{Kv}$, and $I_{BK}$. Note that the density of these currents in wild type mice was similar to that observed in rat myocytes ($p < 0.05$). Furthermore, kinetic analysis showed that the rate of activation (at $+40$ mV, mouse $\tau_{max} = 20.0 \pm 1.6$ ms, $n = 9$ versus rat $\tau_{max} = 17.5 \pm 2.3$ ms, $n = 16$) and the rate of inactivation (during a 15-s pulse to $+40$ mV, mouse $\tau_{inact} = 2.9 \pm 0.3$ s, $n = 5$ versus rat $\tau_{inact} = 3.1 \pm 0.7$ s, $n = 6$) of $I_{Kv}$ in wild type mouse cells were similar to those in rat cells ($p < 0.05$; data not shown). In addition, the voltage at which $I_{Kv}$ reached 50% ($V_{1/2}$) of its maximal conductance (mouse $V_{1/2} = +17.9 \pm 0.3$ mV, $n = 5$, versus rat $V_{1/2} = +15.5 \pm 1.2$ mV, $n = 25$) and steady-state inactivation ($V_{1/2} = -35.0 \pm 1.1$ mV, $n = 5$, versus rat $V_{1/2} = -39.6 \pm 3.2$ mV, $n = 16$) were not different between wild type mouse and rat myocytes ($p < 0.05$; data not shown). Thus, $I_{Kv}$ values in rat and mouse cerebral arterial myocytes are likely produced by similar molecular entities and permit comparison between the two species.

We examined the effects of sustained in vitro Ang II exposure on $I_{Kw}$ in wild type and c3-KO cerebral arterial smooth muscle cells. Similar to rat cerebral arteries, incubation of wild type mouse cerebral arteries in the presence of Ang II (100 nM) decreased the amplitude of $I_{Ko}$ and $I_{Kv}$ (Fig. 6, A and B; $n = 7$, $p < 0.05$). At $+70$ mV, the amplitude of $I_{Ko}$ and $I_{Ko}$ was ~45% smaller in the presence of Ang II than with culture medium alone. Similar to rat, Ang II did not change $I_{Ko}$ in myocytes from wild type mice ($p > 0.05$). These data show that, like rat cerebral arteries, sustained exposure to Ang II reduced $I_{Ko}$ in mouse cerebral smooth muscle by decreasing $I_{Kv}$. In striking contrast, incubation of c3-KO arteries for 48 h in the presence of Ang II (100 nM) did not reduce the amplitude of $I_{Ko}$, $I_{Kv}$, or $I_{BK}$ (Fig. 6, A and B). These results demonstrate that activation of NFATc3 is necessary for Ang II-mediated reduction of $I_{Ko}$ in cerebral arterial smooth muscle.

**DISCUSSION**

In this study, we examined the molecular mechanisms by which Ang II regulates $K^+$ channel function in cerebral arterial smooth muscle. We report three major findings. First, sustained Ang II exposure decreased $I_{Ko}$ by reducing Kv2.1 expression. Second, down-regulation of $I_{Ko}$ by Ang II was dependent on Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels. Third, CaN and NFATc3 are obligatory components in the signaling pathway that leads to decreased $I_{Ko}$ during sustained Ang II exposure. These data support the novel conclusion that CaN/NFATc3 tunes the excitability of arterial smooth muscle by regulating expression of Kv2.1 channels.

**Physiological Consequences of Angiotensin II-induced Reduction of Smooth Muscle $K_v$ Current—**Classical biophysics predicts that, in the absence of other changes, decreasing an out-
ward K⁺ conductance results in membrane depolarization (29). Consistent with this, Kv channel blockers (e.g. 4-aminopyridine) have been shown to depolarize arterial smooth muscle and cause constriction of pressurized cerebral arteries (30). Membrane depolarization results in contraction of arterial smooth muscle by increasing the open probability of voltage-gated L-type Ca²⁺ channels, which increases Ca²⁺ influx and raises global [Ca²⁺]ᵢ (31). In addition to decreasing Kv channel expression, Ang II may reduce Iᵥ via activation of PKC (8). Thus, Ang II-mediated reduction of Iᵥ may occur on acute (i.e. PKC) and delayed (i.e. expression; see below) time scales. Synergism between these mechanisms may greatly reduce Iᵥ during chronic Ang II exposure resulting in depolarization and arterial constriction. Enhanced constriction, in turn, may contribute to vascular dysfunction during hypertension.

Ang II may induce membrane depolarization by other mechanisms. For example, activation of PKC by Ang II may inhibit Ca²⁺ sparks thereby decreasing BK channel activity (7). Furthermore, we recently showed that in vivo Ang II administration reduced expression of the BK channel β1 subunit but not the pore-forming α subunit (32). The functional consequence of this change in molecular composition of BK channels is an uncoupling of these channels from Ca²⁺ sparks, their physiological activators, thus decreasing their hyperpolarizing influence on membrane potential. In summary, increased Ang II activity may depolarize and thereby constrict arterial smooth muscle by reducing Iᵥ, inhibiting Ca²⁺ sparks, and decreasing sensitivity of BK channels to activation by Ca²⁺.

We found that Iᵥ was similar in Ang II and control myocytes. However, as mentioned above, Ang II infusion reduces the sensitivity of BK channels to physiological changes in [Ca²⁺], by reducing β1 subunit expression (32). Although these data may appear to be at odds, note that this is precisely what one would predict to occur under our experimental conditions. In the present series of experiments, we measured Iᵥ, while [Ca²⁺] was buffered to less than 100 nM. At this Ca²⁺ concentration there is little functional interaction between BK channel α and β1 subunits (33-35). Thus, our data showing similar Iᵥ in Ang II and control cells indicates that these cells have a similar number of functional α subunits with comparable intrinsic voltage and Ca²⁺ sensitivities. This is consistent with our observation that the amount of α transcript as well as the number of functional BK channels in Ang II and control cells was the same (32). Future experiments will have to examine the mechanisms by which Ang II regulates expression of the BK channel β1 subunit in arterial smooth muscle.

**Mechanism of Smooth Muscle Kᵥ Current Reduction by Angiotensin II**—We observed that Ang II-induced reduction of Iᵥ...
NFATc3 Controls Smooth Muscle Excitability

requires Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels and activation of CaN and NFATc3. We propose a positive feedback model that integrates these data with findings by others to explain how Ang II and CaN/NFATc3 signaling control \( \text{I}_{\text{Kv}} \) in vascular smooth muscle (Fig. 7). In this model, Ang II binding to type 1 receptors (AT1) increases Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels resulting in a small but sustained increase in global [Ca\textsuperscript{2+}]). This increase in global [Ca\textsuperscript{2+}], activates CaN, which dephosphorylates NFATc3, resulting in nuclear translocation where it reduces Kv2.1 gene expression. Down-regulation of Kv2.1 decreases \( \text{I}_{\text{Kv}} \), thereby depolarizing the arterial smooth muscle. Membrane depolarization causes further Ca\textsuperscript{2+} influx into these cells and thereby enhances contraction. This self-perpetuating loop may be interrupted pharmacologically, as in this study, by blockade of L-type Ca\textsuperscript{2+} channels or inhibition of CaN.

Previous reports (10) lend credence to this model. First, CaN/NFAT signaling is activated by sustained elevations in global [Ca\textsuperscript{2+}], similar to the one reported here. Second, Ang II has been shown to activate and induce nuclear translocation of CaN (26) and NFATc3 in vascular smooth muscle (3, 14). Third, in ventricular myocytes, CaN/NFATc3 activation decreased expression of Kv channel subunits, including Kv2.1 (36). This later finding, in association with the present study, suggests the provocative hypothesis that activation of CaN/NFATc3 may be a general mechanistic point of convergence among stimuli that regulate expression of Kv channels in the cardiovascular system.

NFAT activation is generally associated with an increase in gene expression (5, 16). However, previous reports (17) have shown that NFAT may also function as a repressor of gene expression. Analysis of the sequence 5’ to the Kv2.1 gene (GenBank\textsuperscript{TM} accession number NM_008417; –1040 to –1202) and Kv1.5 (GenBank\textsuperscript{TM} accession number NM_145983; –77 to –1240) genes, yet Ang II did not decrease the expression of these proteins. There are at minimum three scenarios that may explain this observation. 1) Unlike ventricular myocytes (36), activation of NFAT is not sufficient to decrease expression of Kv1.2 and Kv1.5 in cerebral arterial smooth muscle. 2) A post-translational mechanism compensates for NFAT-induced decrease in Kv1.2 and Kv1.5 expression. 3) Relative to Kv2.1, protein levels of Kv1.2 and Kv1.5 may be low, thus limiting our ability to detect a significant decrease in expression. In support of the latter, the kinetics and voltage dependences of activation and steady-state inactivation of \( \text{I}_{\text{Kv}} \) reported here resemble those of heterologously expressed Kv2.1 channels (37) or native currents attributed to Kv2.1 channels (25). Furthermore, the reduction of \( \text{I}_{\text{Kv}} \) was proportionally similar to the reduction of Kv2.1 protein (~50% for each). These observations suggest that Kv2.1 is a major contributor to \( \text{I}_{\text{Kv}} \) in cerebral arterial smooth muscle. Note, however, that a recent study concluded that Kv1.2 and Kv1.5 make a substantial contribution to \( \text{I}_{\text{Kv}} \) in middle cerebral arteries (24). Regardless, Kv2.1 has been shown to be expressed and hyperpolarize vascular smooth muscle from mesenteric (38), tail (25), aortic (39), and pulmonary (40) arteries. Thus, activation of CaN and NFAT may modulate the excitability of numerous arterial beds by regulating Kv2.1 expression.

It is intriguing to speculate that by down-regulating Kv2.1, CaN/NFATc3 activation may contribute to the development of hypertension. Indeed, decreased \( \text{I}_{\text{Kv}} \) has been reported in arterial myocytes from other models of hypertension, including spontaneously hypertensive (41, 42) and Dahl salt-sensitive rats (43). Future experiments should examine whether activation of CaN/NFATc3 is responsible for the reduction of \( \text{I}_{\text{Kv}} \) in these models.

Conclusions—The results of this study suggest a signaling pathway by which Ang II regulates \( \text{I}_{\text{Kv}} \) in arterial smooth muscle. Our data indicate that sustained activation of Ang II receptors increases intracellular Ca\textsuperscript{2+} thus stimulating CaN/NFATc3. Activation of NFATc3 decreases \( \text{I}_{\text{Kv}} \) in arterial

Fig. 7. Model for NFATc3-mediated reduction of Kv2.1 during Ang II signaling. Binding of Ang II to AT1 receptors increases Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels thereby increasing global [Ca\textsuperscript{2+}]. This activates calcineurin, which dephosphorylates NFATc3, thus permitting nuclear translocation where NFATc3 reduces Kv2.1 expression. Down-regulation of Kv2.1 decreases \( \text{I}_{\text{Kv}} \) and hence causes depolarization and induces further Ca\textsuperscript{2+} influx. This positive feedback loop may be interrupted blocked by pharmacological blockade of L-type Ca\textsuperscript{2+} channels or inhibition of calcineurin.

Putative NFAT-binding elements are also found 5’ to the Kv1.2 (GenBank\textsuperscript{TM} accession number NM_008417; –84 to –1202) and Kv1.5 (GenBank\textsuperscript{TM} accession number NM_145983; –77 to –1240) genes, yet Ang II did not decrease the expression of these proteins. There are at minimum three scenarios that may explain this observation. 1) Unlike ventricular myocytes (36), activation of NFAT is not sufficient to decrease expression of Kv1.2 and Kv1.5 in cerebral arterial smooth muscle. 2) A post-translational mechanism compensates for NFAT-induced decrease in Kv1.2 and Kv1.5 expression. 3) Relative to Kv2.1, protein levels of Kv1.2 and Kv1.5 may be low, thus limiting our ability to detect a significant decrease in expression. In support of the latter, the kinetics and voltage dependences of activation and steady-state inactivation of \( \text{I}_{\text{Kv}} \) reported here resemble those of heterologously expressed Kv2.1 channels (37) or native currents attributed to Kv2.1 channels (25). Furthermore, the reduction of \( \text{I}_{\text{Kv}} \) was proportionally similar to the reduction of Kv2.1 protein (~50% for each). These observations suggest that Kv2.1 is a major contributor to \( \text{I}_{\text{Kv}} \) in cerebral arterial smooth muscle. Note, however, that a recent study concluded that Kv1.2 and Kv1.5 make a substantial contribution to \( \text{I}_{\text{Kv}} \) in middle cerebral arteries (24). Regardless, Kv2.1 has been shown to be expressed and hyperpolarize vascular smooth muscle from mesenteric (38), tail (25), aortic (39), and pulmonary (40) arteries. Thus, activation of CaN and NFAT may modulate the excitability of numerous arterial beds by regulating Kv2.1 expression.

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smooth muscle by down-regulating Kv2.1 expression. Finally, our findings support the view that CaN and NFATc3 signaling may be a general mechanism for the control of Kv channel expression in excitable cells.

Acknowledgments—We thank Drs. Laurie Glimcher and Martin F. Schneider for providing the NFATc3 knock-out mice and ΔNFAT adenovirus, respectively. We also thank Dr. Stephen M. Schwartz for assistance with pump implantation and blood pressure measurements.

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