Angiotensin II Inhibits bTREK-1 K⁺ Channels in Adrenocortical Cells by Separate Ca²⁺- and ATP Hydrolysis-dependent Mechanisms*

Bovine adrenocortical cells express bTREK-1 K⁺ channels that set the resting membrane potential (V_m) and couple angiotensin II (AngII) and adrenocorticotropin hormone (ACTH) receptors to membrane depolarization and corticosteroid secretion. In this study, it was discovered that AngII inhibits bTREK-1 by separate Ca²⁺- and ATP hydrolysis-dependent signaling pathways. When whole cell patch clamp recordings were made with pipette solutions that support activation of both Ca²⁺- and ATP-dependent pathways, AngII was significantly more potent and effective at inhibiting bTREK-1 and depolarizing adrenal zona fasciculata cells, than when either pathway is activated separately. External ATP also inhibited bTREK-1 through these two pathways, but ACTH displayed no Ca²⁺-dependent inhibition. AngII-mediated inhibition of bTREK-1 through the novel Ca²⁺-dependent pathway was blocked by the AT₁ receptor antagonist losartan, or by including guanosine-5’-O-(2-thiodiphosphate) in the pipette solution. The Ca²⁺-dependent inhibition of bTREK-1 by AngII was blunted in the absence of external Ca²⁺ or by including the phospholipase C antagonist U73122, the inositol 1,4,5-trisphosphate receptor antagonist 2-amino-ethoxycyclic olate borate, or a calmodulin inhibitory peptide in the pipette solution. The activity of unitary bTREK-1 channels in inside-out patches from adrenal zona fasciculata cells was inhibited by application of Ca²⁺ (5 or 10 μM) to the cytoplasmic membrane surface. The Ca²⁺-ionophore ionomycin also inhibited bTREK-1 currents through channels expressed in CHO-K1 cells. These results demonstrate that AngII and selected paracrine factors that act through phospholipase C inhibit bTREK-1 in adrenocortical cells through simultaneous activation of separate Ca²⁺- and ATP hydrolysis-dependent signaling pathways, providing for efficient membrane depolarization. The novel Ca²⁺-dependent pathway is distinctive in its lack of ATP dependence, and is clearly different from the calmodulin kinase-dependent mechanism by which AngII modulates T-type Ca²⁺ channels in these cells.

Bovine adrenocortical cells, including cortisol-secreting AZF¹ cells and aldosterone-secreting AZG cells express bTREK-1 leak-type K⁺ channels that function pivotally in the physiology of corticosteroid secretion (1, 2). bTREK-1 belongs to the mechanogated, thermo- and fatty acid-sensitive subgroup of two-pore/four-transmembrane fragment family of K⁺ channels (3–9). In the adrenal cortex, bTREK-1 channels couple hormonal signals originating at the cell membrane to depolarization-dependent Ca²⁺ entry (1, 2, 10, 11). bTREK-1 channels are inhibited by AngII and ACTH at concentrations identical to those that trigger membrane depolarization and corticosteroid secretion (1, 12). Other paracrine factors, including ATP, which stimulates cortisol secretion through a G protein-coupled P2Y₃ receptor, also inhibit bTREK-1 and depolarize AZF cells with similar potency (13, 14).

The signaling pathways that link the peptide hormones and paracrine factors to bTREK-1 inhibition are only partially understood. In particular, adrenocortical cells express two pharmacologically distinct types of AngII receptors (15–17). Losartan-sensitive AT₁ receptors are coupled to multiple signaling pathways (17–19). Most physiological responses, including AngII-stimulated corticosteroid secretion, are mediated through AT₁ receptor-dependent activation of PLC, which catalyzes the synthesis of inositol trisphosphates (IP₃) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (17, 20). Adrenocortical cells also express losartan-insensitive AT₂ receptors, which comprise about 20% of the AngII receptors in these cells (15, 17). The signaling pathways and function of these receptors in the physiology of corticosteroid-secreting cells is unknown.

In whole cell patch clamp recordings from bovine AZF and AZG cells, AngII maximally inhibited bTREK-1 by 72–77% with an IC₅₀ of ~145 pm, provided that ATP was present in the recording pipette at millimolar concentrations (1, 11, 12). AngII-mediated inhibition of bTREK-1 was eliminated when ATP in the pipette solution was replaced by the non-hydrolyzable ATP analog AMP-PNP, or UTP (12, 21). The kinase or ATPase that mediates AngII inhibition through this ATP hydrolysis-dependent pathway has not been identified.

In these whole cell recording studies, [Ca²⁺²], was buffered to 22 nm with 11 mM BAPTA. In a separate study, we found that

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¹ The abbreviations used are: AZF, bovine adrenal fasciculata; AZG, bovine adrenal glomerulosa; V_m, resting membrane potential; AngII, angiotensin II; ACTH, adrenocorticotropin hormone; PLC, phospholipase C; 2-APB, 2-aminoethoxydiphenyl borate; CIP, calmodulin inhibitory peptide; CHO, Chinese hamster ovary; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; IP₃, inositol 1,4,5-trisphosphate; AMP-PNP, 5’-adenyl-β,γ-imidodiphosphate; GDPβS, guanosine-5’-O-(2-thiodiphosphate); 8-PEt-cAMP, 8-(4-chlorophenylthio) adenosine 3’,5’-cyclic monophosphosphate.

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bTREK-1 in AZF cells was inhibited by raising the [Ca²⁺]-, in the pipette solution from 22 mM to 2 µM (22). bTREK-1 was also inhibited by superfusing cells with the Ca²⁺ ionophore ionomycin, provided that [Ca²⁺]-, was buffered by 2 mM, rather than 11 mM BAPTA. At the single channel level, in excised inside-out patch recordings, the application of saline containing 35 µM Ca²⁺ to the cytoplasmic face of the membrane markedly inhibited bTREK-1 channel activity. In these experiments, 5 mM ATP was present at the cytoplasmic face of the membrane.

Taken together, the results of these studies could suggest that AngII inhibits bTREK-1 through a single Ca²⁺- and ATP hydrolysis-dependent signaling pathway that requires the activation of a Ca²⁺-dependent kinase. In bovine AZG cells, AngII enhances the activity of T-type Ca²⁺ channels through the activation of Ca²⁺/calmodulin-dependent kinase II (23–25). Alternatively, the effective inhibition of bTREK-1 by AngII in whole cell recordings with [Ca²⁺]-, strongly buffered to 22 mM using 11 mM BAPTA suggests that AngII may inhibit bTREK-1 by multiple signaling pathways, only one of which is Ca²⁺-dependent.

In whole cell and single channel patch clamp recordings from bovine adrenocortical cells, we discovered that AngII does inhibit bTREK-1 through a separate Ca²⁺- and ATP hydrolysis-dependent signaling pathway. Simultaneous activation of both pathways by AngII leads to near complete inhibition of bTREK-1 current and pronounced depolarization of bovine adrenocortical cells. Molecular features of the new Ca²⁺-dependent mechanism were identified.

### MATERIALS AND METHODS

Tissue culture media, antibiotics, fibronectin, and fetal bovine sera were obtained from Invitrogen. Coverslips were from Belco (Vineland, NJ). Enzymes, BAPTA, MgATP, Na₂ATP, GDPβS, ACTH (1–24), AngII, 8-PCPT-cAMP, ionomycin, and EGTA were obtained from Sigma. U73122, U73343, and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Biomol (Plymouth Meeting, PA). The calmodulin inhibitory peptide (CIP) was obtained from Calbiochem (La Jolla, CA). AngII receptor antagonists, losartan and PD123319, were kindly provided by Dr. Ronald Smith (Merck Pharmaceutical Co.) and Dr. Joan Keiser (Parke-Davis), respectively.

### Isolation and Culture of AZF Cells

Bovine adrenal glands were obtained from steers (age 2–3 years) at a local slaughterhouse. Isolated AZF and AZG cells were obtained and prepared as previously described (26). After isolation, cells were either resuspended in Dulbecco’s modified Eagle’s medium/F-12 (1:1) with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and the antioxidants 1 mM BAPTA, 20 mM sodium azide, and 0.1 mM 1,2-EDTA or 100 µM ascorbic acid (Dubelco’s modified Eagle’s medium/F-12 + ) and plated for immediate use, or resuspended in fetal bovine serum, 5% MeSO, divided into 1-ml aliquots, and stored in liquid nitrogen for future use. For patch clamp experiments, cells were plated in Dulbecco’s modified Eagle’s medium/F-12+ in 35-mm dishes containing 9-mm² glass coverslips. Coverslips were treated with fibronectin (10 µg/ml) at 37°C for 30 min then rinsed with warm, sterile phosphate-buffered saline immediately before adding cells. Cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO₂.

### Transient Transfection and Visual Identification of CHO-K1 or COS-7 Cells Expressing bTREK-1

For whole cell patch clamp recordings of bTREK-1 in CHO-K1 or COS-7 cells, bTREK-1 current amplitudes were measured to monitor the activity of bTREK-1 in transfected cells. Transfected cells were identified based on decoration with the beads. Whole cell and single channel bTREK-1 currents were recorded from transfected cells as described below for AZF cells.

### Patch Clamp Experiments

Patch clamp recordings of K⁺ channel currents were made in the whole cell and inside-out patch configuration from bovine AZF cells. Although the results reported in this study were obtained by recording currents from AZF cells, preliminary recordings from AZG cells showed no difference in bTREK-1 currents with respect to modulation by AngII. For whole cell recordings, the standard external solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 5 mM glucose, with pH adjusted to 7.3 using NaOH. The standard pipette solution consisted of (in mM): 120 KCl, 2 MgCl₂, 10 HEPES, and 0.2 Tris, with pH titrated to 6.8 using KOH. The buffering capacity of pipette solutions was varied by adding combinations of Ca²⁺ and BAPTA or EGTA using the Bound and Determined software program (28). Low and high capacity Ca²⁺ buffering solutions contained 0.5 mM EGTA and 11 mM BAPTA, respectively. The low capacity Ca²⁺ buffering solution was normally Ca²⁺-free. [Ca²⁺]-, was buffered to 22 mM in the high capacity buffering solution. Nucleotides, including MgATP, NaUTP, and AMP-PNP were added to pipette or bath solutions as noted in the text. For inside-out patch recordings, the standard external and pipette solutions used for whole cell recordings were switched.

Patch clamp recordings of IₚCa were made in the whole cell configuration. The standard pipette solution was in mM: 120 CsCl, 2 MgCl₂, 2 NaUTP, 0.5 EGTA, 0.2 GTP, 10 HEPES with pH titrated to 7.2 using CsOH. The external solution contained (in mM): 117 tetraethylammonium (NE), 5 CsCl, 20 CaCl₂, 2 MgCl₂, 5 HEPES, with pH adjusted to 7.3 using tetraethylammonium-OH. All solutions were filtered through 0.22-µm cellulose acetate filters.

### Recording Conditions and Electronics

AZF cells were used for patch clamp experiments 2–12 h after plating. Typically, cells with diameters <15 µm and capacitances of 10–15 pF were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume: 1.5 ml) that was continuously perfused by gravity at a rate of 3–5 ml/min. For whole cell recordings, patch electrodes with resistances of 1.0–2.0 MΩ were fabricated from Cornin 0010 glass (World Precision Instruments, Sarasota, FL). These electrodes routinely yielded access resistances of 1.5–4.0 MΩ and an average capacitance of 200 pF. For single channel recordings, patch electrodes with higher resistances (3–5 MΩ) were used. Current records were rejected if cell amplitudes were recorded at room temperature (22–25°C) according to the procedure of Hamill et al. (29) using a List EPC-7 patch clamp amplifier.

Pulse generation and data acquisition were done using a personal computer and PCLAMP software with TL-1 interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 2–10 KHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using summed scaled hyperpolarizing steps of 1/2 to 1/4 pulse amplitude. Data were analyzed using PCLAMP (CLAMPFIT 9.2, FETCHAN 6.04, and PSAT 6.04) and SigmaPlot (version 8.0) software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve. p values were calculated using Student’s t test.

### RESULTS

AngII Inhibits bTREK-1 by Separate Ca²⁺ and ATP-dependent Mechanisms

Bovine AZF cells express two types of K⁺ channels, voltage gated, rapidly inactivating Kv1.4 channels, and leak-type bTREK-1 channels (2, 30, 31). In whole cell recordings, bTREK-1 amplitude often increases spontaneously over a period of minutes, provided that the recording pipette contains ATP or other nucleotide triphosphates at millimolar concentrations (1, 26).

The absence of time- and voltage-dependent bTREK-1 inactivation allows the corresponding membrane current to easily isolated in whole cell recordings, using either of two voltage clamp protocols. When voltage steps of 300 ms duration are applied from a holding potential of −80 mV, bTREK-1 can be measured near the end of a voltage step when the transient Kv1.4 current has inactivated (Fig. 1, A–C, left traces). Alternatively, bTREK-1 can be selectively activated by an identical voltage step, after a 10-s prepulse to −20 mV has fully inactivated Kv1.4 channels (Fig. 1, A–C, right traces). Measurement of bTREK-1 by either method yielded nearly identical results.

In previous whole cell recording studies on AZF cells using patch electrodes containing 2–5 mM MgATP and [Ca²⁺]-, strongly buffered to 22 mM with 11 mM BAPTA, AngII inhibited...
bTREK-1 by a maximum of 77–82% with an IC$_{50}$ of 145 mM. However, when MgATP in the pipette was replaced with UTP, which is not a substrate for kinases or ATPases, AngII was ineffective (1, 12, 21).

In the present study, AngII at a maximally effective concentration (10 nM), inhibited bTREK-1 by only 12.0 ± 4.5% ($n = 11$) when pipette solutions contained 2 mM UTP and 11 mM BAPTA (Fig. 1, A and D). However, when [Ca$^{2+}$]$_i$ was weakly buffered by substituting 0.5 mM EGTA for 11 mM BAPTA, inhibition of bTREK-1 by AngII was restored, even in the

![Diagram A](image1.png)

**FIG. 1.** AngII inhibits bTREK-1 through separate Ca$^{2+}$- and ATP-dependent pathways. The inhibition of bTREK-1 in bovine AZF cells by AngII was measured in whole cell patch clamp recordings using pipette solutions that permitted or blocked activation of Ca$^{2+}$- and ATP-dependent signaling. K$^+$ currents were recorded from bovine AZF cells at 30-s intervals in response to voltage steps to +20 mV, applied from a holding potential of −80 mV with or without 10-s prepulses to −20 mV. After bTREK-1 reached a stable maximum amplitude, cells were superfused with saline containing AngII (2 or 10 nM). A–C, K$^+$ currents were recorded with (right traces) or without (left traces) depolarizing prepulses. bTREK-1 amplitudes recorded with (open circles) or without (closed circles) prepulses are plotted against time. Numbers on traces correspond to those on the plot. Pipette solution containing: A, 2 mM UTP, 11 mM BAPTA; B, 2 mM UTP, 0.5 mM EGTA; or C, 5 mM MgATP, 0.5 mM EGTA. D, summary of experiments as in A–C. Bars indicate percent of bTREK-1 remaining after steady state block by AngII (2 or 10 nM). Values are mean ± S.E. for the indicated number of determinations.
presence of 2 mM UTP. With \([Ca^{2+}]\), weakly buffered by 0.5 mM EGTA, AngII (2 or 10 nM) inhibited bTREK-1 by 67.4 ± 2.5% \((n = 33)\) (Fig. 1, B and D). With weak \([Ca^{2+}]\), buffering, AngII also inhibited bTREK-1 by 58.0 ± 7.5% \((n = 8)\) when the pipette contained the non-hydrolyzable ATP analog AMP-PNP (2 mM) (Fig. 1D).

These results suggest that AngII-mediated inhibition of bTREK-1 may occur through separate \(Ca^{2+}\)- and ATP hydrolysis-dependent signaling pathways. If AngII modulates bTREK-1 by parallel mechanisms, each of which produces only partial inhibition, it is likely that simultaneous activation of both pathways would more effectively suppress this current. Accordingly, when bTREK-1 currents were recorded with a pipette solution whose composition supported activation of both ATP- and \(Ca^{2+}\)-dependent pathways \((0.5 \text{ mM EGTA, } 5 \text{ mM MgATP})\), AngII (2 mM) was significantly more effective, inhibiting bTREK-1 by 92.9 ± 0.7% \((n = 8)\) (Fig. 1, C and D).

Not only was AngII more effective at inhibiting bTREK-1 at both the \(Ca^{2+}\)- and ATP pathways when available, it was also more potent. To compare the potency of AngII as an inhibitor of bTREK-1 through \(Ca^{2+}\)- and combined pathways, AZF cells were superfused with AngII at various concentrations and bTREK-1 currents were recorded with pipette solutions containing 0.5 mM EGTA and either 2 mM NaUTP or 5 mM MgATP. Inhibition curves constructed from data obtained in these experiments clearly showed that AngII was more potent, as well as more effective, at inhibiting bTREK-1 when \(Ca^{2+}\)- and ATP-dependent pathways were available (Fig. 2A, left).

With both pathways available for activation, AngII inhibited bTREK-1 almost completely, with an IC\(_{50}\) of 63 pM. By comparison, with only the \(Ca^{2+}\) pathway available for activation, AngII inhibited bTREK-1 by a maximum of ~70%, with an IC\(_{50}\) of 149 pM (Fig. 2A). The marked enhancement of bTREK-1 inhibition by AngII when both \(Ca^{2+}\)- and ATP-dependent pathways were activated was most pronounced at lower AngII concentrations. At low AngII concentrations, bTREK-1 inhibition was partially reversible with washing (Fig. 2A).

Activation of \(Ca^{2+}\)- and ATP-dependent Pathways Correlate with Membrane Depolarization—bTREK-1 channels are largely responsible for setting the resting potential of bovine adrenocortical cells (1, 12). In the absence of a significant inward current, the Goldman-Hodgkin-Katz voltage equation predicts that a large fraction of the bTREK-1 channels would have to be inhibited to effectively depolarize these cells. The simultaneous activation of both the \(Ca^{2+}\)- and ATP-dependent pathways should therefore provide a more efficient means of depolarizing adrenocortical cells. Accordingly, in combined current and voltage clamp experiments, AngII inhibited bTREK-1 and strongly depolarized AZF cells only when both the \(Ca^{2+}\)- and ATP-dependent pathways were available for activation.

When recordings were made with pipette solutions that sustained only activation of the \(Ca^{2+}\)-dependent pathway, AngII (2 nM) inhibited bTREK-1 current by 62.2 ± 5.7%, whereas these cells were depolarized by an average of 11.9 ± 2.4 mV \((n = 5)\) from their resting potential of −68.8 ± 4.4 mV (Fig. 2B). By comparison, when recordings were made with pipette solutions that allowed activation of both \(Ca^{2+}\)- and ATP-dependent pathways, AngII (2 nM) inhibited bTREK-1 almost completely by 96.8 ± 1.0%, and depolarized AZF cells by an average of 44.3 ± 4.3 mV \((n = 4)\) from their resting potential of −63.3 ± 3.2 mV (Fig. 2C).

ATP, but Not ACTH or cAMP, Inhibits bTREK-1 through Both \(Ca^{2+}\)- and ATP-dependent Pathways—Previously, we demonstrated that externally applied ATP and UTP inhibited bTREK-1 channels through activation of a G protein-coupled purinergic receptor with a P2Y\(_{3}\) agonist profile (32). In that study where \([Ca^{2+}]\), was strongly buffered with 11 mM BAPTA, ATP (100 \mu M) inhibited bTREK-1 by a maximum of 71.3 ± 3.2% and this inhibition was eliminated by substituting AMP-PNP for MgATP in the pipette solution. Thus, similar to AngII, externally applied ATP inhibits bTREK-1 through a ATP hydrolysis-dependent mechanism.

P2Y\(_{3}\) receptors are coupled to PLC activation and the release of \([Ca^{2+}]\), (33, 34). To determine whether externally applied ATP also inhibited bTREK-1 through a \(Ca^{2+}\)-dependent mechanism, inhibition was studied with a pipette solution designed to permit activation of the \(Ca^{2+}\), but not the ATP-dependent pathway. With \([Ca^{2+}]\), weakly buffered by 0.5 mM EGTA, ATP at 10 and 100 \mu M inhibited bTREK-1 by 26.2 ± 9.3 \((n = 7)\) and 42.8 ± 4.4% \((n = 6)\), respectively (Fig. 3, A and D).

When considered in conjunction with the previously mentioned study (32), these results indicate that similar to AngII, external ATP inhibits bTREK-1 through separate \(Ca^{2+}\)- and ATP hydrolysis-dependent mechanisms. Accordingly, when AZF cells were superfused with ATP under conditions where \(Ca^{2+}\)- and ATP-dependent pathways could be activated, bTREK-1 was inhibited by 85.6 ± 5.8% \((n = 4)\) (Fig. 3D).

ACTH also potently inhibits bTREK-1 in bovine AZF cells with an IC\(_{50}\) of ~5 pM (1). Although the signaling pathways are only partially understood, inhibition likely involves both protein kinase A-dependent and -independent actions of cAMP (5, 35). Inhibition by ACTH or cAMP is eliminated when AMP-PNP or UTP is substituted for ATP in the pipette solution with \([Ca^{2+}]\), strongly buffered by 11 mM BAPTA (26, 35). Experiments were done to determine whether, in addition to the ATP-dependent mechanism, ACTH or cAMP could also inhibit bTREK-1 by a \(Ca^{2+}\)-dependent process.

In contrast to AngII, ACTH failed to inhibit bTREK-1 in whole cell recordings made with pipette solutions containing 2 mM UTP and 0.5 mM EGTA. In the experiment illustrated in Fig. 3B, bTREK-1 grew to a stable maximum amplitude before the cell was superfused with 200 pM ACTH. ACTH produced no measurable inhibition of bTREK-1. When this same cell was superfused with 2 mM AngII, bTREK-1 was inhibited by 72.7% within 3 min. Overall, in similar experiments, ACTH inhibited bTREK-1 by 9.5 ± 5.3% \((n = 9)\) (Fig. 3D). Subsequent superfusion of AngII (2 nM) in 7 of these cells inhibited bTREK-1 by 72.1 ± 7.6%.

Because cAMP is the principle intracellular messenger for ACTH, the failure of ACTH to inhibit bTREK-1 in the above experiments could be because of a requirement for cAMP synthesis, previous to inhibition through a \(Ca^{2+}\)-dependent pathway. However, when AZF cells were superfused with the membrane-permeable cAMP analog 8-pct-cAMP (250 \mu M) while recording bTREK-1 with pipettes containing 0.5 mM EGTA and 2 mM UTP, no significant inhibition of bTREK-1 was observed (Fig. 3, C and D). Pre-exposure to 8-pct-cAMP also did not affect inhibition of bTREK-1 by AngII (Fig. 3D).

Properties of the \(Ca^{2+}\)-dependent Inhibitory Pathway—Results to this point have identified a new \(Ca^{2+}\)-dependent pathway by which hormones and paracrine factors inhibit a specific background K\(^+\) current and depolarize bovine adrenocortical cells. Because AngII physiologically regulates the secretion of aldosterone and cortisol, it is important to identify the molecular components of the signaling pathway that links AngII receptors to TREK-1 channels and membrane depolarization.

The \(Ca^{2+}\)-dependent Inhibition of bTREK-1 Is Voltage-independent and -specific—Although bTREK-1 K\(^+\) channels are active at negative membrane potentials, they display weak voltage dependence (35). The \(Ca^{2+}\)-dependent inhibition of bTREK-1 by AngII was independent of voltage over a wide range of test potentials. The experiment illustrated in Fig. 4 shows current-
voltage relationships recorded before and after superfusing the cell with AngII (2 nM) under conditions where only the Ca²⁺/ATP-dependent pathway could be activated. In this experiment, bTREK-1 was inhibited by greater than 90% over the range of test potentials where it could be accurately measured (−20 to +40 mV). Inhibition through the Ca²⁺-dependent pathway was entirely selective for bTREK-1. The voltage-gated Kv1.4 current was not altered in this and three similar experiments.

Ca²⁺-dependent Inhibition of bTREK-1 Is Mediated through an AT₁ Receptor and Is G Protein-dependent—Bovine adrenocortical cells express both AT₁ and AT₂ AngII receptors (15, 17). In a previous study, we demonstrated that AngII-mediated inhibition of bTREK-1 through the ATP-dependent pathway occurred through activation of an AT₁ receptor (12). In the present study, we found that AngII-dependent inhibition of bTREK-1 through the Ca²⁺ pathway is also mediated through

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**FIG. 2.** Properties of AngII-mediated inhibition of bTREK-1 and membrane depolarization by Ca²⁺- and combined Ca²⁺/ATP-dependent pathways. A, potency and effectiveness. The inhibition of bTREK-1 by AngII at various concentrations was determined with pipette solutions designed to allow activation of only the Ca²⁺-dependent pathway (2 mM NaUTP, 0.5 mM EGTA) or both Ca²⁺ and ATP-dependent pathways (5 mM MgATP, 0.5 mM EGTA). Left, time-dependent inhibition of bTREK-1 by 200 pM AngII through combined pathways. Right, inhibition curves. Unblocked bTREK-1, expressed as percent of control, is plotted against the AngII concentration under conditions where only the Ca²⁺-dependent or both the Ca²⁺- and ATP-dependent pathways were available for activation as indicated. Data were fit with an equation of the form: \( I/I_{\text{max}} = 1/(1 + (B/K_D)^X) \), where \( B \) is the AngII concentration, \( K_D \) is the equilibrium dissociation constant, and \( X \) is the Hill coefficient. Values are mean ± S.E. of from 4 to 23 determinations. B and C, AngII-mediated bTREK-1 inhibition and membrane depolarization. K⁺ currents were recorded from AZF cells using the voltage protocols described in the legend of Fig. 1 with pipette solutions designed to allow activation of the Ca²⁺ (B), or the combined Ca²⁺/ATP-dependent pathways (C). When bTREK-1 reached a stable maximum value, membrane potential was then recorded after switching to current clamp, and AngII (2 nM) was superfused as indicated. When membrane potential reached a stable value, K⁺ currents were recorded to determine the extent of bTREK-1 inhibition. Membrane potential was plotted against time at the right. Numbers on traces correspond to currents recorded before (1) and after (2) superfusing AngII.
a losartan-sensitive AT$_1$ receptor and is G protein-dependent. When whole cell recordings were made with pipette solution that permitted selective activation of the Ca$_{2+}$/H$_{11001}$-dependent pathway, the AT$_1$ receptor antagonist losartan suppressed inhibition of bTREK-1 by AngII (Fig. 5, A and D). Cells treated with ACTH or 8-pcpt-cAMP were then superfused with AngII (2 nM). A–C, K$^+$ currents recorded with (right traces) or without (left traces) depolarizing prepulses and associated plots of current amplitudes for cells treated with NaATP (A), ACTH followed by AngII (B), or 8-pcpt-cAMP followed by AngII (C). Numbers on the traces correspond to those on plots at right. D, summary of experiments as in A–C. Bars indicate fraction of bTREK-1 remaining after the steady-state block was achieved. White bar shows block by external ATP (100 μM*) through combined Ca$_{2+}$- and ATP hydrolysis-dependent pathways. Values are mean ± S.E. for the indicated number of determinations.

**FIG. 3.** ATP but not ACTH or 8-pcpt-cAMP displays Ca$_{2+}$-dependent inhibition of bTREK-1. Whole cell K$^+$ currents were recorded from AZF cells at 30-s intervals in response to voltage steps to +20 mV applied from a holding potential of −80 mV with or without a 10-s prepulse to −20 mV. Standard pipette solution was supplemented with 0.5 mM EGTA, 2 mM UTP, or 5 mM MgATP, and 200 μM GTP. After bTREK-1 reached a stable maximum amplitude, cells were superfused with saline containing NaATP (10 or 100 μM), ACTH (200 pM), or 8-pcpt-cAMP (250 μM). Numbers on the traces correspond to those on plots at right. D, summary of experiments as in A–C. Bars indicate fraction of bTREK-1 remaining after the steady-state block was achieved. White bar shows block by external ATP (100 μM*) through combined Ca$_{2+}$- and ATP hydrolysis-dependent pathways. Values are mean ± S.E. for the indicated number of determinations.

Overall, in a total of nine experiments, losartan (500 nM) alone inhibited bTREK-1 by 12.2 ± 4.9%. In the presence of 500 nM losartan, 2 nM AngII inhibited bTREK-1 by only 13.8 ± 5.3%. In five of these experiments, subsequent superfusion of AngII alone inhibited bTREK-1 by 77.1 ± 5.9% (Fig. 5, A and D).

The specific AT$_2$ antagonist PD123319 failed to blunt AngII-mediated inhibition of bTREK-1 (Fig. 5, B and D). In the presence of 500 nM PD123319, AngII (2 nM) inhibited bTREK-1

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bTREK-1 through this Ca\(^{2+}\) inhibition of bTREK-1 by strongly buffering [Ca\(^{2+}\)]\(_{i}\) rather than by a direct effect of Ca\(^{2+}\) on the channel. However, when whole cell recordings were made with [Ca\(^{2+}\)]\(_{i}\), buffered to a resting physiological concentration of 100 nM with 11 mM BAPTA, AngII (2 nM) remained ineffective, inhibiting bTREK-1 by 10.6 ± 5.9% (n = 4). Thus, at physiological [Ca\(^{2+}\)]\(_{i}\), which supports the activation of PLC, AngII does not inhibit bTREK-1 in the presence of strong Ca\(^{2+}\) buffering.

In addition, it was observed that raising [Ca\(^{2+}\)]\(_{i}\) in the pipette to 100 nM did not alter the expression of bTREK-1. At [Ca\(^{2+}\)]\(_{i}\) of 22 and 100 nM, maximum bTREK-1 current densities were 50.8 ± 7.9 and 57.1 ± 8.9 pA/pF (n = 5), respectively. Apparently, Ca\(^{2+}\) does not affect bTREK-1 activity at concentrations up to 100 nM.

To further determine whether the Ca\(^{2+}\)-dependent inhibition of bTREK-1 by AngII is mediated through PLC, whole cell recordings were made with pipette solutions containing the PLC antagonist U73122 (42). U73122 (3 μM) suppressed inhibition of bTREK-1 by AngII (2 nM) from the control value of 66.0 ± 2.9% (n = 22) to 48.2 ± 7.3% (n = 16) (Fig. 6, A and C). In contrast, the inactive analog of U73122, U73343, failed to blunt inhibition by AngII. With U73343 (3 μM) in the patch pipette, AngII inhibited bTREK-1 by 73.2 ± 2.6% (n = 6) (Fig. 6C). At concentrations of 5 μM and higher, U73122 was found to markedly inhibit the expression of the bTREK-1 current in whole cell recordings.

To determine whether AngII-mediated inhibition of bTREK-1 through the Ca\(^{2+}\)\(_{i}\)-dependent inhibition of bTREK-1 was blunted, but not eliminated (Fig. 6, B and C).

In the experiment illustrated in Fig. 6B, currents were initially recorded in control saline, after which the cell was sequentially superfused with saline without added Ca\(^{2+}\), followed by this “zero Ca\(^{2+}\)” solution containing 2 nM AngII, and finally by one containing AngII as well as 2 mM Ca\(^{2+}\). The amplitude of the bTREK-1 current rapidly increased in the absence of external Ca\(^{2+}\), and then decreased by 30% upon superfusion of AngII. Superfusion of saline containing 2 mM AngII and 2 mM Ca\(^{2+}\) further reduced bTREK-1 current amplitude to 29% of its maximum value within 10 min.

Overall, in 6 cells, AngII (2 nM) inhibited bTREK-1 by an average of 45.1 ± 8.7% (n = 6) in the absence of added external Ca\(^{2+}\), compared with 66.0 ± 2.9% (n = 22) in control saline (Fig. 6C). In three cells where inhibition of bTREK-1 by AngII was first measured in the absence, and then in the presence of external Ca\(^{2+}\), inhibition increased from 28.4 to 66.2%.

The Ca\(^{2+}\)-dependent inhibition of bTREK-1 by AngII may be mediated in part by the IP\(_3\)-stimulated release of Ca\(^{2+}\) by intracellular stores. 2-APB inhibits IP\(_3\)-stimulated release of Ca\(^{2+}\) from intracellular stores with an IC\(_{50}\) of ~40 μM (43). Including 2-APB (80 μM) in the recording pipette significantly reduced the inhibition of bTREK-1 by AngII (2 nM) to 32.9 ± 11.3% (n = 7) (Fig. 6C).

Mechanism of Ca\(^{2+}\)-dependent Inhibition of bTREK-1—The results presented thus far indicate that Ca\(^{2+}\) entering through the plasma membrane and released from intracellular stores may both contribute to AngII-mediated inhibition of bTREK-1. The molecular mechanism by which Ca\(^{2+}\) inhibits the activity of bTREK-1 channels is not known. Because the Ca\(^{2+}\) pathway functions in the absence of hydrolyzable ATP, it is likely that Ca\(^{2+}\) interacts directly with the bTREK-1 channel or an associated subunit or protein. If Ca\(^{2+}\) interacts directly with the bTREK-1 channel protein, then agents that increase intracellular Ca\(^{2+}\) should effectively inhibit the activity of cloned bTREK-1 channels expressed in a cell line. The Ca\(^{2+}\) ionophore ionomycin (1 and 5 μM) inhibited the activity of bTREK-1 channels expressed in CHO-K1 cells by 54.5 ± 18.6 and 75.6 ± 5.6%, respectively (Fig. 7, A and C).
The inhibition of bTREK-1 channels expressed in CHO-K1 cells by ionomycin suggests a direct interaction with the channel or a Ca\(^{2+}\)-dependent protein expressed in a wide range of cells. The Ca\(^{2+}\)-dependent modulation of a number of ion channels requires a calmodulin intermediate. In some instances Ca\(^{2+}\)-free apocalmodulin is constitutively bound to the channel, whereas in others, only the Ca\(^{2+}\)-calmodulin complex binds to the channel with high affinity (44, 45). To determine whether the Ca\(^{2+}\)-dependent inhibition of bTREK-1 is calmodulin dependent, the inhibition of bTREK-1 by AngII was studied in whole cell recordings with pipettes containing a CIP. This 17-residue peptide mimics the calmodulin-binding domain of myosin light chain kinase and binds to the calmodulin with a \(K_d\) of 6 pM (46).

In the presence of 0.5 \(\mu\)M CIP, AngII (2 nM) inhibited bTREK-1 by 45.7 ± 7.4% \((n = 7)\), compared with the control value of 66.0 ± 2.8% \((n = 22)\) (Fig. 7, B and C). Thus, the presence of CIP at concentrations nearly 100,000 times its \(K_d\) for calmodulin blunted, but did not eliminate, AngII-mediated inhibition of bTREK-1.

**Effect of Ca\(^{2+}\) on Unitary bTREK-1 Currents**—Whole cell patch clamp experiments indicated that Ca\(^{2+}\)-mediated inhibi-
tion of bTREK-1 might occur through a direct interaction of Ca\(^{2+}\) with the channel or a channel-associated protein. Single channel recording experiments in which Ca\(^{2+}\) was applied directly to the cytoplasmic surface of excised inside-out patches in the absence of ATP were consistent with this model for inhibition. In these experiments, AZF cell membrane patches were excised into an internal solution where [Ca\(^{2+}\)] was buffered to 22 nM with 11 mM EGTA at a test potential of \(+40\) mV (inside positive). At this potential, all Kv1.4 K\(^{+}\) channels are inactivated, leaving only bTREK-1 channels active in the membrane patch. With 2 mM UTP applied to the cytoplasmic face of the patch, bTREK-1 activity typically increased spontaneously and continuously during prolonged recordings.

In the experiments illustrated in Fig. 8A, histogram analysis of unitary current amplitudes showed a major peak with a mean of \(-3.8 \pm 0.6\) pA, and two additional peaks with means of approximately twice \((-7.3 \pm 2.8\) pA) and three times \((-12.3 \pm 1.7\) pA) the unitary amplitude. Superfusion of the
cytoplasmic membrane surface with saline containing 5 μM Ca²⁺ inhibited channel activity by 53.3%. Inhibition was reversible upon switching back to low Ca²⁺ saline. In this patch, bTREK-1 activity was restored to 161% of control. Similar results were obtained in each of four experiments with 5 μM Ca²⁺.

The inhibition of bTREK-1 by Ca²⁺ was concentration dependent. Superfusion of membrane patches with saline containing 10 μM Ca²⁺ produced near complete inhibition of channel activity. In the experiment illustrated in Fig. 8B, 10 μM Ca²⁺ inhibited unitary bTREK-1 activity by 94.5%. Similar results were obtained in four separate cells. Histogram analysis showed that neither 5 nor 10 μM Ca²⁺ altered unitary

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**Fig. 7. Effect of ionomycin and CIP on cloned and native bTREK-1 channels.**

**A**, inhibition of cloned bTREK-1 channels by ionomycin. K⁺ currents were recorded from bTREK-1-transfected CHO-K1 cells in response to voltage steps to +20 mV applied at 30-s intervals from a holding potential of −80 mV. After recording currents in control saline, cell was superfused with ionomycin (5 μM). Numbers on current traces correspond to those on the plot of bTREK-1 amplitudes at the right. **B**, effect of CIP on bTREK-1 inhibition by AngII. Whole cell K⁺ currents were recorded from an AZF cell at 30-s intervals in response to voltage steps to +20 mV with or without depolarizing prepulses using a pipette solution that contained 500 nM CIP. After bTREK-1 reached a stable maximum, cell was superfused with 2 nM AngII. Numbers on current traces correspond to those on the plot of bTREK-1 amplitudes at the right. **C**, summary of experiments as in A and B. Bars indicate fraction of bTREK-1 remaining after steady state block by ionomycin (1 or 5 μM) or AngII (2 nM). Values are mean ± S.E. for the indicated number of separate determinations. *, p value for AngII + CIP = 0.0046.
bTREK-1 amplitudes, excluding the possibility that inhibition occurs through a pore-blocking mechanism. Ca\(^{2+}\) (10 μM) also inhibited the activity of unitary bTREK-1 channels expressed in COS-7 cells, although clear, reversible inhibition was observed in only 2 of 4 cells (Fig. 8C).

**AngII Does Not Modulate the T-type Ca\(^{2+}\) Current through the Ca\(^{2+}\)-dependent Pathway—**AngII has been reported to enhance the T-type Ca\(^{2+}\) current of bovine AZG cells by a Ca\(^{2+}\)-dependent mechanism that includes a leftward shift in the voltage dependence of channel activation, and requires the activation of a Ca\(^{2+}\)/calmodulin-dependent protein kinase (23, 25). Experiments were done to determine whether AngII could modulate T-type Ca\(^{2+}\) currents in bovine adrenal cortical cells by the Ca\(^{2+}\)-dependent, but kinase-independent pathway.

Nearly all freshly plated bovine AZF cells express only low voltage-activated, slowly deactivating T-type Ca\(^{2+}\) currents (47). In the experiment illustrated in Fig. 9A, T-type deactivating “tail” currents were recorded with pipette solutions containing 2 mM NaUTP in place of MgATP, with Ca\(^{2+}\) weakly buffered by 0.5 mM EGTA. Currents were recorded at −80 mV after

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**Fig. 8. Inhibition of unitary bTREK-1 currents by Ca\(^{2+}\).** Unitary bTREK-1 currents were recorded from inside-out AZF cell membrane patches excised from AZF cells (A and B) or COS-7 cells transfected with bTREK-1 (C) using pipette and bath solutions described under “Materials and Methods.” Patches were excised into solution containing 22 mM Ca\(^{2+}\). Unitary bTREK-1 currents were continuously recorded at +30 mV in control solution and after switching to saline containing 5 (A) or 10 μM (B) free Ca\(^{2+}\). Amplitude histograms were constructed from unitary currents recorded at +30 mV over a 50-s period. Current amplitudes were distributed into bins 0.2 pA in width. Currents were sampled at 5 KHz and filtered at a cutoff frequency of 2 KHz. A, unitary currents (top) and corresponding amplitude histograms (bottom) from recordings in control solution (22 mM Ca\(^{2+}\)) and after superfusion of 10 μM Ca\(^{2+}\), as indicated. B, unitary currents and amplitude histograms in control solution (22 mM Ca\(^{2+}\)) and after superfusion of 10 μM Ca\(^{2+}\), as indicated. C, unitary currents and amplitude histograms from COS-7 patch recordings in control saline (22 mM Ca\(^{2+}\)), 10 μM Ca\(^{2+}\), and again in control saline (wash).
activation by 10-ms depolarizing pulses to 0 mV. AngII (2 nM) failed to increase the amplitude of the T-type Ca\(^{2+}\) current in this or any of six similar experiments. AngII also failed to significantly shift the voltage dependence of T channel activation under similar conditions (Fig. 9B).

**DISCUSSION**

In this study, we identified a new Ca\(^{2+}\)-dependent pathway by which AngII inhibits bTREK-1 K\(^{+}\) channels and depolarizes bovine adrenocortical cells. The simultaneous activation of the novel Ca\(^{2+}\)-dependent pathway and a previously described ATP hydrolysis-dependent pathway by AngII provides a fail-safe mechanism for potent and effective bTREK-1 inhibition and depolarization of AZF cells (Fig. 10). Other paracrine factors, including ATP, which activate PLC-coupled receptors and stimulate corticosteroid secretion from bovine adrenocortical cells also inhibit bTREK-1 K\(^{+}\) channels through these parallel pathways. In contrast, ACTH which functions through G\(_{s}\) and cAMP inhibits bTREK-1 through ATP hydrolysis, but not through Ca\(^{2+}\)-dependent mechanisms.

In addition to AngII, ATP also inhibited bTREK-1 through the Ca\(^{2+}\)-dependent pathway at concentrations that stimulate large increases in cortisol production from bovine AZF cells (13). In a previous study, we showed that ATP also inhibited bTREK-1 by an ATP hydrolysis-dependent mechanism through a nucleotide receptor with a P2Y\(_{3}\) agonist profile (14). These two results indicate that activation of the PLC-coupled P2Y\(_{3}\) receptor in AZF cells inhibits bTREK-1 by the same two pathways utilized by AngII. Accordingly, externally applied ATP was significantly more effective at inhibiting bTREK-1 when recordings were made under conditions that supported activation of both the Ca\(^{2+}\)- and ATP-dependent paths.

In contrast to AngII and external ATP, ACTH and cAMP failed to inhibit bTREK-1 when only the Ca\(^{2+}\)-dependent mechanism was available for activation. Thus, although ACTH and cAMP both inhibit bTREK-1 by protein kinase A-dependent and-independent mechanisms, neither of these is mediated by Ca\(^{2+}\) (35).

**The Ca\(^{2+}\)-dependent Signaling Pathway**—The effective inhibition of bTREK-1 by AngII in whole cell recordings with pipettes containing UTP or AMP-PNP in place of MgATP precluded the involvement of a kinase or ATPase in the Ca\(^{2+}\)-dependent response, because UTP and AMP-PNP are not

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**Fig. 9.** AngII does not modulate T-type Ca\(^{2+}\) current through the Ca\(^{2+}\)-dependent pathway. The effect of AngII on T-type Ca\(^{2+}\) current amplitude and voltage-dependent activation were measured in AZF cells. A, Ca\(^{2+}\) currents were activated by 10-ms voltage steps to −5 mV applied at 30-s intervals from a holding potential of −80 mV. After recording Ca\(^{2+}\) currents in standard saline, cell was superfused with saline containing 2 nM AngII. "Tail current" amplitudes recorded at −80 mV are plotted at the right. B, activation curves. The effect of AngII on the voltage dependence of T-type Ca\(^{2+}\) channel activation was measured by recording tail currents at a constant potential (−80 mV) after applying activating voltage steps of 10-ms duration in 10-mV increments to potentials between −60 and +30 mV before (●) and after (○) superfusing cells with 2 nM AngII. Tail current amplitudes were normalized to the maximum value and plotted as the fraction of open channels against test potential. Data points were fit with a Boltzmann function of the form: Fraction open = 1/[1 + \(\exp((v_{1/2} - \nu)/k))\], where \(v_{1/2}\) is the voltage at which one-half of the channels are in the open conformation and \(k\) is the slope factor.
Ang II inhibits bTREK-1 through Dual Pathways

substrates for these enzymes. However, the molecular components of the Ca\(^{2+}\)-dependent signaling pathway remained to be identified.

Experiments in this study in combination with those from a previous report (12) demonstrate that inhibition of bTREK-1 by AngII through both the Ca\(^{2+}\)- and ATP hydrolysis-dependent pathways are mediated by a losartan-sensitive AT\(_1\) receptor through a G-protein-dependent process. Although most physiological responses mediated through AT\(_1\) receptors require a G-protein intermediate, several studies have shown that activated AT\(_1\) receptors interact directly with different effector proteins (17, 36, 48).

AT\(_1\) receptors in many cells, including adrenocortical cells, are coupled to PLC through the GTP-binding protein G\(_o\) (17). U73122 (3 \(\mu M\)) significantly reduced, but did not eliminate, AngII-mediated inhibition of bTREK-1. This result is consistent with a model where the Ca\(^{2+}\)-dependent inhibition of bTREK-1 depends on PLC activation. U73122 inhibits agonist-induced PLC\(\beta\) activation in intact cells with IC\(_{50}\) values in the micromolar range (42). At concentrations above 3 \(\mu M\), U73122 significantly inhibited the expression of bTREK-1 in whole cell recordings as has been previously reported for other K\(^+\) channels, precluding the use of higher concentrations in our experiments (49). In this regard, it is significant that the inactive U73122 analog U73343 (3 \(\mu M\)) had no effect on AngII-mediated inhibition of bTREK-1.

PLC activation in adrenocortical cells leads to IP\(_3\)-induced release of intracellular Ca\(^{2+}\), as well as Ca\(^{2+}\) influx through plasma membrane channels (20, 37, 39). Superfusing AZF cells with Ca\(^{2+}\)-free saline initially increased bTREK-1 current and significantly reduced the inhibition of this current by AngII, indicating that Ca\(^{2+}\) influx through plasma membrane channels contributes to the regulation of channel activity. Bovine adrenocortical cells express TRPC4 non-selective cation channels that may be activated by AngII (37, 38). Ca\(^{2+}\) entering through these TRPC4 channels could be linked to the inhibition of nearby bTREK-1 channels.

The rapid increase in bTREK-1 current observed upon superfusion of Ca\(^{2+}\)-free saline could result from a reduction in the basal Ca\(^{2+}\) influx through the plasma membrane or by a change in the effective transmembrane voltage. Removal of extracellular Ca\(^{2+}\) shifts the voltage dependence of channel activation in the hyperpolarizing direction by effectively reducing the electric field strength across the membrane (50). Consequently, because bTREK-1 channels display weak voltage dependence, the absence of [Ca\(^{2+}\)]\(_i\) could enhance current by increasing channel open probability (26).

Bovine adrenocortical cells also express T-type voltage-gated Ca\(^{2+}\) channels that may represent the other major pathway for Ca\(^{2+}\) entry in these cells (10, 47, 51). However, because AngII failed to increase Ca\(^{2+}\) current through T-type Ca\(^{2+}\) channels under the conditions of our experiment, it is unlikely that Ca\(^{2+}\) entering through these channels contributed to inhibition of bTREK-1.

The reduction in AngII-stimulated inhibition of bTREK-1 current produced by 2-APB (80 \(\mu M\)) is consistent with a model wherein inhibition is mediated in part by IP\(_3\)-stimulated release of Ca\(^{2+}\) from intracellular stores. In this regard, 2-APB inhibits IP\(_3\)-stimulated Ca\(^{2+}\) release with an IC\(_{50}\) of 42 \(\mu M\), but increases Ca\(^{2+}\) release from microsomes at concentrations above 90 \(\mu M\) (43). Therefore, 2-APB was not used at concentrations above 80 \(\mu M\) in our studies. Overall, the results indicate that the Ca\(^{2+}\)-dependent inhibition of bTREK-1 by AngII involves both PLC-dependent Ca\(^{2+}\) influx and release from intracellular stores.

Molecular Mechanism for Ca\(^{2+}\)-Inhibition of bTREK-1—The results of experiments using ionomycin, CIP, and Ca\(^{2+}\) applied directly to excised membrane patches provide insight into the molecular mechanism by which Ca\(^{2+}\) inhibits bTREK-1. The absence of MgATP in all of these experiments ruled out the participation of protein kinases, simplifying the problem. However, Ca\(^{2+}\) modulates the gating of a number of K\(^+\) channels by multiple kinase-independent mechanisms. Several Ca\(^{2+}\)-activated K\(^+\) channels are gated by Ca\(^{2+}\) binding directly to the channel (52, 53). For other K\(^+\) channels, modulation by Ca\(^{2+}\) proceeds through a calmodulin intermediate. Calmodulin can be constitutively associated with the channel, or it may bind to the channel only after first binding Ca\(^{2+}\) (44, 45, 54, 55).

Ionomycin inhibited bTREK-1 K\(^+\) current in transfected cells by a maximum of 75.6 \(\pm\) 5.6%, indicating that as many as three-fourths of these channels retain their Ca\(^{2+}\) sensitivity even when expressed in a foreign cell type. Similarly, Ca\(^{2+}\) (10 \(\mu M\)) inhibited the activity of bTREK-1 channels expressed in COS-7 cells in half of the patches tested. If Ca\(^{2+}\)-mediated inhibition of bTREK-1 required an AZF cell-specific subunit or other protein not expressed in other eukaryotic cells, ionomycin and Ca\(^{2+}\) would have been ineffective as inhibitors of the transfected channels. However, if the Ca\(^{2+}\)-dependent inhibition of bTREK-1 were mediated through a ubiquitously expressed protein such as calmodulin, ionomycin and Ca\(^{2+}\) would likely retain their effectiveness in transfected cells. The complete inhibition of transfected bTREK-1 channels by ionomycin and Ca\(^{2+}\) in whole cell and single channel recordings may suggest that not all bTREK-1 channels become associated with the Ca\(^{2+}\)-dependent protein in CHO-K1 or COS-7 cells.

The reduced inhibition of bTREK-1 observed when the patch pipette contained CIP supports a role for calmodulin in the inhibition of this channel by AngII. However, because this peptide binds to calmodulin with extremely high affinity (\(K_d = 6\) pM), it is perhaps surprising that CIP did not more effectively suppress the AngII response. This may suggest that apocalmodulin is constitutively and tightly bound to the native bTREK-1 channel in a Ca\(^{2+}\)-independent fashion. Near its
carboxyl-terminal end (amino acids 340–354), bTREK-1 protein contains a sequence that conforms partially to the consensus "IQ" motif calmodulin-binding site found in several other K⁺ channels where calmodulin is constitutively associated (54, 56). The inhibition of bTREK-1 by Ca²⁺ in excised inside-out AZF cell patches indicates that if Ca²⁺-dependent inhibition of bTREK-1 is mediated through calmodulin, then calmodulin must be constitutively associated with the bTREK-1 channel.

Comparison of Signaling Pathways for AngII Inhibition of bTREK-1 and Activation of T-type Ca²⁺ Channels—In bovine AZG cells, AngII enhances current through Ca₃.₂ T-type Ca²⁺ channels by activation of Ca²⁺/calmodulin-dependent kinase II, which phosphorylates these channels at a specific site (25, 57). Interestingly, we have shown that AngII inhibits bTREK-1 channels in bovine adrenocortical cells by two entirely different pathways. Furthermore, AngII does not modulate Ca₃.₂ T-type Ca²⁺ channels in bovine adrenocortical cells by either of the two mechanisms that inhibit bTREK-1 channels. In a previous study, we showed that AngII has no effect on the T-type Ca²⁺ current in bovine AZG cells through the ATP-dependent pathway (11). Finally, the results of the current study do not rule out the possibility that AngII may also inhibit bTREK-1 by a third pathway involving a Ca²⁺-dependent kinase such as Ca²⁺/calmodulin kinase or kinase C.

Modulation of Cloned Neuronal TREK-1 Channels—The inhibition of rat brain TREK-1 channels by Gi-coupled metabotropic glutamate receptors has also been reported, and the signaling pathways mediating rat brain TREK-1 regulation have been explored in transfected cell lines (58). Glutamate-mediated inhibition of TREK-1 was blocked by U73122, indicating the involvement of PLC. However, in contrast with our studies, these whole cell experiments were done with [Ca²⁺]₀ buffered by 5 mM EGTA and without ATP in the pipette solution. In this system, evidence is presented indicating that inhibition occurred through a direct effect of the PLC-generated second messenger diacylglycerol, rather than through Ca²⁺- or ATP-dependent pathways. It appears that TREK-1 K⁺ channels in various cell types are modulated by a range of signaling pathways, involving kinases, diacylglycerol, cAMP, and Ca²⁺.

The differences that we have observed in the signaling pathways that modulate native bTREK-1 channels compared with cloned TREK-1 channels from other species could arise through differences in channel-associated proteins, or signaling pathways present in the native or host cells. For example, the availability of calmodulin for interaction with TREK-1 may be quite different in a transfected cell, when compared with a native cell.

Physiological Significance—bTREK-1 channels function as a central control point in bovine adrenocortical cells where hormonal and paracrine signals originating at the cell membrane are coupled to depolarization-dependent Ca²⁺ entry. We have shown that bovine AZF cells have developed a fail-safe mechanism for potent and effective inhibition of bTREK-1 current and membrane depolarization by AngII through the activation of parallel Ca²⁺- and ATP hydrolysis-dependent signaling pathways (Fig. 10).

In neurons and other excitable cells, bTREK-1 and other four transmembrane/two-pore K⁺ channels act as a brake on electrical activity (5, 59). It remains to be seen whether, under physiological conditions, bTREK-1 inhibition by AngII triggers Ca²⁺-dependent action potentials in bovine adrenocortical cells. Action potentials have been recorded in adrenocortical cells from several species (60–62). In the absence of bTREK-1, opposing T-type Ca²⁺ currents and A-type K⁺ currents could give rise to action potentials.

In addition to AngII, external ATP and perhaps other paracrine factors inhibit bTREK-1 through these same two pathways. Inhibition of specific ion channels by simultaneous activation of multiple signaling pathways may be a common theme when near total inactivation is required. Accordingly, ACTH- and cAMP-mediated inhibition of bTREK-1 by protein kinase A-dependent and -independent mechanisms may be yet another example (5, 35). Overall, ACTH and AngII inhibit bTREK-1 through at least four different signaling pathways.

Although it is clear that AngII acts through an AT₁ receptor by a G protein-dependent mechanism to inhibit bTREK-1 via separate Ca²⁺- and ATP-dependent pathways, the underlying molecular events are incompletely understood. Specifically, our results do not demonstrate that the inhibition of bTREK-1 by Ca²⁺ is entirely dependent on PLC. Furthermore, although Ca²⁺ entry through the plasma membrane contributes to bTREK-1 inhibition, the molecular identity of the plasma membrane channel has not been determined. Finally, the specific role of calmodulin in TREK-1 inhibition by Ca²⁺ remains to be defined.

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