TREK-1 K⁺ Channels Couple Angiotensin II Receptors to Membrane Depolarization and Aldosterone Secretion in Bovine Adrenal Glomerulosa Cells

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ABSTRACT

Bovine adrenal glomerulosa (AZG) cells were shown to express bTREK-1 background K\(^+\) channels that set the resting membrane potential and couple Angiotensin II (AngII) receptor activation to membrane depolarization and aldosterone secretion. Northern blot and in situ hybridization studies demonstrated that bTREK-1 mRNA is uniformly distributed in the bovine adrenal cortex, including zona fasciculata and zona glomerulosa, but is absent from the medulla. TASK-3 mRNA which codes for the predominant background K\(^+\) channel in rat AZG cells is undetectable in the bovine adrenal cortex.

In whole cell voltage clamp recordings, bovine AZG cells express a rapidly inactivating voltage-gated K\(^+\) current and a non-inactivating background K\(^+\) current with properties that collectively identify it as bTREK-1. The outwardly rectifying K\(^+\) current was activated by intracellular acidification, ATP, and by superfusion of bTREK-1 openers including arachidonic acid (AA) and cinnamyl 1-3,4-dihydroxy-\(\alpha\)-cyanocinnamate (CDC). Bovine chromaffin cells did not express this current.

In voltage and current clamp recordings, AngII (10 nM) selectively inhibited the noninactivating K\(^+\) current by 82.1 ± 6.1% and depolarized AZG cells by 31.6 ± 2.3 mV. CDC and AA overwhelmed AngII-mediated inhibition of bTREK-1 and restored the resting membrane potential to its control value even in the continued presence of AngII. Vasopressin (50 nM) which also physiologically stimulates aldosterone secretion inhibited the background K\(^+\) current by 73.8 ± 9.4 %.

In contrast to its potent inhibition of bTREK-1, AngII failed to alter the T-type Ca\(^{2+}\) current (I\(_{\text{T-Ca}}\)) measured over a wide range of test potentials using pipette solutions of identical nucleotide and Ca\(^{2+}\)-buffering compositions. AngII also failed to alter the voltage-dependence of
T channel activation under these same conditions. Overall, these results identify bTREK-1 K\(^+\) channels as a pivotal control point where AngII receptor activation is transduced to depolarization-dependent Ca\(^{2+}\) entry and aldosterone secretion.

**Key Words:** adrenal glomerulosa, TREK-1 K\(^+\) channels, patch clamp, Angiotensin II, aldosterone
List of Non-standard Abbreviations:

Angiotensin II - AngII

Arachidonic acid - AA

AZG - bovine adrenal zona fasciculata

BAPTA - 1,2-bis-(2-aminophenoxy)ethane-N,N',N,N"-tetraacetic acid

BSA – bovine serum albumin

cinnamyl 1-3,4-dihydroxy-α-cyanocinnamate - CDC

DMEM/F12+ - DMEM/F12 (1:1) with 10 % FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and the antioxidants 1 μM tocopherol, 20 nM selenite and 100 μM ascorbic acid.

FBS - fetal bovine sera

2P/4TMS - two pore/four transmembrane segment

PBS – phosphate buffered saline

T-type Ca$^{2+}$ current - $I_{T-Ca}$
INTRODUCTION

Angiotensin II (AngII) is a principal physiological stimulus for aldosterone secretion by bovine AZG cells (3,41). Although AngII-stimulated aldosterone secretion is mediated through the activation of a losartan-sensitive AT₁ receptor, the specific signaling pathways involved are only partially understood. In particular, the roles of specific ion channels and depolarization-dependent Ca²⁺ entry in the process have not been clarified. In this regard, both bovine and rat AZG cells maintain negative resting potentials and express both voltage-gated T- and L-type Ca²⁺ channels, as well as voltage-gated and background K⁺ channels (26,41,46) (4,11,25,29,31,43).

AngII-stimulated aldosterone secretion depends, at least in part, on Ca²⁺-entry through voltage-gated T- and L-type Ca²⁺ channels (6,24,25,46). Several studies indicate that AngII enhances the activity of low voltage-activated T-type Ca²⁺ channels in AZG cells (6,9,26,31). The enhanced activity of T-type Ca²⁺ channels was associated with an approximate 10 mV negative shift in the voltage dependence of T channel activation (6,31). These actions of AngII may occur through activation of an AT₁ receptor through a mechanism that involves calmodulin-dependent protein kinase II (CaMKII) (1,27).

In contrast to the above findings, other patch clamp studies on rat and bovine AZG cells reported that AngII either has no effect, or actually inhibits T-type Ca²⁺ channels in these cells (25,45). Further, AngII does not directly activate high voltage-activated L-type Ca²⁺ channels in AZG cells (24,25,30). Overall, it is unlikely that AngII enhances Ca²⁺ entry in AZG cells solely through a direct action on voltage-gated Ca²⁺ channels.

Other studies reported that AngII depolarizes murine, feline, bovine, and human AZG cells by inhibiting unidentified background K⁺ channels, thereby suggesting a specific mechanism for the indirect activation of voltage-gated Ca²⁺ channels (4,25,41,43). However, until recently, K⁺
channels that could set the resting potential of AZG cells and whose inhibition by AngII would be coupled to depolarization-dependent Ca\(^{2+}\) entry have not been identified. In recent years, more than one dozen two pore/four transmembrane (2P/4TMS) background K\(^+\) channels have been identified. These background K\(^+\) channels exhibit little voltage dependence, remain open at negative membrane potentials, and set the resting potential of a wide range of cells (19,39). Recently, rat AZG cells were shown to express the 2P/4TMS K\(^+\) channels TASK-1 and TASK-3 (10,11). TASK-3 was reported to be the dominant background K\(^+\) channel in these cells (10). However, neither TASK-1 nor TASK-3 were shown to set the resting potential of AZG cells, nor was inhibition of either channel by AngII shown to mediate membrane depolarization.

Cortisol-secreting bovine adrenal zona fasciculata (AZF) cells express bTREK-1 background channels that are inhibited through activation of multiple native G-protein coupled receptors. These include receptors for the peptides ACTH and AngII, as well as P2Y nucleotide and multiple P1 adenosine receptors (17,32,33,51,52). Inhibition of bTREK-1 through all of these receptors is coupled to AZF cell depolarization.

We now report that bovine AZG cells also robustly express bTREK-1 K\(^+\) channels and that, in these cells, they set the resting membrane potential. Inhibition of these channels by AngII is tightly coupled to membrane depolarization. Specific activators of bTREK-1 channels reverse AngII-mediated depolarization and suppress aldosterone secretion. Under conditions where AngII produced near complete inhibition of bTREK-1, this peptide had no effect on T-type Ca\(^{2+}\) current.
MATERIALS AND METHODS

Materials

Tissue culture media, antibiotics, fibronectin, and fetal bovine sera (FBS) were obtained from Invitrogen (Carlsbad, CA). Coverslips were from Bellco (Vineland, NJ). Phosphate-buffered saline (PBS), enzymes, 1,2, bis-(2-aminophenoxy) ethane-N,N,N,N’’-tetraactic acid (BAPTA), ATP, arachidonic acid (AA), AMP-PNP, and AngII were from Sigma (St. Louis, MO). Baicalein and cinnamyl 1-3,4-dihydroxy-α-cyanocinnamate (CDC) were obtained from Biomol (Plymouth Meeting, PA). rTASK-3 cDNA was the kind gift from both Dr. D. Kim (Department of Physiology and Biophysics, Finch University of Health Sciences/The Chicago Medical School) and Dr. R. Preisig-Muller (Institut fur Normale und Pathologische Physiologie, Marburg University). Marathon-ready cDNA from normal human adrenals was obtained from Clontech (Palo Alto, CA).

Methods

Isolation and Culture of AZG cells

Bovine adrenal glands were obtained from steers (age range 2 to 3 years) within 1 hr of slaughter at a local slaughterhouse. Fatty tissue was removed immediately and the glands were transported to the laboratory in ice-cold PBS containing 0.2% dextrose. Isolated AZG cells were obtained and prepared as previously described (40) with some modifications. Briefly, glomerulosa cells were isolated from adrenal capsular tissue and cells adherent to the capsule. Capsular tissue was cut into small (0.2-0.5 cm³) pieces. Tissue was digested for 1 h at 37°C in DMEM/F12 (1:1) with dispase (10 mg/ml), BSA (1%, wt/vol) and 50 μg/ml DNase. After digestion, the tissue suspension was strained through 2 layers of cheesecloth, and cells were either resuspended in DMEM/F12 (1:1) with 10 % FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and the
antioxidants 1 μM tocopherol, 20 nM selenite and 100 μM ascorbic acid (DMEM/F12+) and plated for immediate use, or resuspended in FBS/5% DMSO, divided into aliquots, and stored in liquid nitrogen for future use. Cells were plated in 35 mm dishes for secretion experiments, or 35 mm dishes containing 9 mm² glass coverslips for electrophysiology experiments. Dishes or coverslips were treated with fibronectin (10 μg/ml) at 37°C for 30 minutes, and then rinsed with warm, sterile PBS immediately before adding cells. Cells were plated in DMEM/F12+ and were maintained at 37°C in a humidified atmosphere of 95% air-5% CO₂.

**Patch Clamp Experiments**

Patch clamp recordings of K⁺ channel currents were made in the whole cell configuration. The standard pipette solution consisted of 120 mM KCl, 1 mM CaCl₂, 2mM MgCl₂, 11 mM BAPTA, 10 mM HEPES, 1 mM ATP, and 200 μM GTP, with pH titrated to 7.1 using KOH. Pipette solution of this composition yielded a free Ca²⁺ concentration of 2.2 X 10⁻⁸ M, as determined by the Bound and Determined software program (5). In some experiments, MgATP in the pipette solution was raised to 5 mM and pH was lowered to 6.4, as noted in the text. The 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 7.4 using NaOH. All solutions were filtered through 0.22 μm cellulose acetate filters.

**Recording Conditions and Electronics**

AZG cells were used for patch clamp experiments 2-12 h after plating. Since AZG cells are significantly smaller than AZF cells, cells with capacitances of 7-12 pF were selected for recording. Coverslips were transferred from 35 mm culture dishes to the recording chamber (volume: 1.5 ml)
that was continuously perfused by gravity as a rate of 3-5 ml/min. Patch electrodes with resistances of 2-3 MΩ were fabricated from Corning 0010 glass (World Precision Instruments, Sarasota, FL). These electrodes routinely yielded access resistances of 1.5 -5.0 MΩ and voltage-clamp time constants of <100 µs. K+ currents were recorded at room temperature (22-25 °C) according to the procedure of Hamill et al (22) 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, USA). Linear leak and capacity currents were subtracted from current records using summed scaled hyperpolarizing steps of 1/3 to 1/4 pulse amplitude. Data were analyzed using PCLAMP (CLAMPFIT 6.04) and SigmaPlot (ver 8.0) software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

**Measurement of bTREK-1 K+ Currents**

The absence of time and voltage-dependent inactivation of the bTREK-1 K+ current allowed it to be easily isolated for measurement in whole cell recordings from AZG cells, using either of two voltage clamp protocols. When voltage steps of 300 ms duration were applied from a holding potential of -80 mV to a test potential of +20 mV, bTREK-1 could be selectively measured near the end of the voltage step, where the rapidly inactivating bKv1.4 K+ current had completely inactivated. Alternatively, bTREK-1 was selectively activated with an identical voltage step, after a 10 s prepulse to -20 mV had fully inactivated bKv1.4 K+ current (see Figure 2A).
Aldosterone Secretion Experiments

AZG cells were cultured on fibronectin-coated 35 mm dishes at a density of 1.5 X 10^6 cells/dish in defined media (DMEM/F12 (1:1), 50 µg/ml BSA, 100 µM ascorbic acid, 1 µM tocopherol, 10 nM insulin, and 10 µg/ml transferrin). After 1 h, the media was aspirated and changed to defined media (DMEM/F12 with 50 µg/ml BSA, 100 µM ascorbic acid, 1 µM tocopherol, 0.15 µg/ml insulin, and 10 µg/ml transferrin) either without (control), or with CDC, or AngII, or these two in combination. Drugs were added directly to media in dishes from concentrated stock. 200 µl samples of media were collected at selected times and frozen at -20 ºC for later assay. Aldosterone concentration was determined using a solid phase radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). Experiments were performed in triplicate, and assayed for aldosterone in duplicate.

Northern Blot Analysis

RNeasy columns (Qiagen, Valencia, CA) that had been treated with RNase-free DNase (Qiagen, Valencia, CA) to remove genomic contamination were used to extract total RNA from AZG and AZF cells that had been cultured in DMEM/F12+ for 8 hours.

Total RNA was separated on a denaturing 8% formaldehyde, 1.0% agarose gel, and transferred to a nylon membrane (Gene Screen Plus, NEN). RNA was fixed to the membrane by UV-crosslinking using a Stratalinker (Stratagene, La Jolla, CA). Northern blot was prehybridized in heat-sealable plastic bag for 2 hrs at 42ºC in ULTRAhyb (Ambion, Austin, TX), then hybridized with a random-primed 32P-alpha-dCTP radiolabeled 379 bp EcoRI fragment of bTREK-1 or a full-length TASK-3 cDNA (Prime-it-II, Stratagene, La Jolla, CA), overnight in minimal volume of hybridization solution at 42ºC. After 18-24 hrs, blots were washed twice at room temperature in 2X
SSPE for 15 min, twice at 40°C in 1X SSPE, 1% SDS for 30 min. For TREK-1 hybridization, a more-stringent wash, twice at 65°C with 0.1 X SSPE, 1% SDS for 15 minutes was necessary. Autoradiograms were obtained by exposing blots to Kodak X-O-Mat AR film at -70 °C for 1 h (bTREK-1) or 24 h (TASK-3).

In Situ Hybridization

Tissue Preparation

Bovine tissue, obtained as described above, was immersed in 4% paraformaldehyde/0.1M sodium phosphate buffer pH 7.4 at 4°C for 1-3 h to preserve morphology, then embedded in OCT embedding matrix for frozen sectioning in embedding molds. Frozen blocks were allowed to equilibrate with cryostat chamber at -7°C. Tissue was cut into 10 µm sections, then thaw-mounted onto charged slides (SuperFrost/Plus). Slides were stored at -80°C. Before hybridization, slides were allowed to equilibrate to room temperature, and then fixed in a 4% paraformaldehyde solution. Slides were then subjected to a series of washes in 0.1 M PBS (pH 7.4), acetic anhydride (0.25% I 0.1 M triethanolamine, 0.9% NaCl, pH 8.0), and subsequently dehydrated and delipidated in a series of ethanol washes. The hybridization reaction was carried out overnight at 37°C in the presence of 60 µl of hybridization buffer (Amresco) with 3’terminal-35S-labeled oligo, 100 mM DTT, and 250 µg/ml yeast t-RNA. Slides were washed in SSC of increasing stringency, dehydrated by a series of ethanol washes, then exposed to Biomax Film (Kodak) for 3 days to evaluate signal.

Oligonucleotide Probes

A bovine TREK-1 probe was designed using template region bp 1167-1203 where there is much less sequence similarity to other 2 pore/4 transmembrane-spanning (2P/4TMS) channels such
as TREK-2, TASK, or TRAAK. A sense probe was used to assay non-specific binding. A bovine CYPIIB 32 nt oligo (5’ GTC CAG CTG GGA TGT GGT AGT TCT GCA GCA CC 3’) was used as a positive control to provide morphological identification of adrenal tissue regions. Specific bTREK-1 probe sequences were as follows: antisense: 5’ CTT GTC ATA AAT CTC CAC GCT CAG CCG CCT CCT GGT T 3’ (37 nt), and sense control: 5’ AAC CAG GAG GCG GCT GAG CGT GGA GAT TTA TGA CCA G 3’(37 nt). Oligos were synthesized and PAGE purified by IDT (Coralville, IA). Probe sequences were checked against sequences in GenBank to ensure no cross-reactivity with other two-pore K⁺ channel gene products or sequences in the database.
RESULTS

bTREK-1 mRNA Expression in Bovine Adrenal

Northern blot analysis was used to characterize the relative expression of mRNA coding for TREK-1 and TASK-3 in bovine AZF and AZG cells. Figure 1A shows that AZF and AZG cells both express bTREK-1 mRNA which is present in 3 transcripts of 4.9, 3.6, and 2.8 Kb. Further, as previously shown in AZF cells, bTREK-1 mRNA is also markedly induced in AZG cells by a 20 h treatment with forskolin (5 µM) (14).

In contrast to TREK-1, TASK-3 mRNA was poorly expressed in these same bovine AZF and AZG cells. TASK-3 was undetectable in the RNA from both control and forskolin-treated cells after exposing the film for 24 h, while bTREK-1 mRNA was easily detected with a 1 h exposure.

In situ hybridization experiments confirmed the finding that bTREK-1 mRNA is robustly expressed throughout the bovine adrenal cortex, including the glomerulosa and fasciculata. These experiments also indicated that bTREK-1 is undetectable in the adrenal medulla. The experiment illustrated in Figure 1B shows that mRNAs for bTREK-1 and CYP11B, a steroid hydroxylase in the pathways that convert cholesterol to cortisol and aldosterone (35), are both strongly expressed in bovine AZF and AZG tissue, but not in the adrenal medulla. In this regard, no distinction could be made between the level of bTREK-1 expression in the subcapsular glomerulosa and the adjacent fasciculata. The presence of bTREK-1 mRNA in AZF as well as AZG cells suggests that the corresponding TREK-1 K⁺ channels are expressed in glomerulosa cells.

bTREK-1 K⁺ Channels are Expressed in Bovine AZG Cells

AZG cells were isolated for patch clamp recording, and K⁺ currents from these cells were
activated by two different voltage clamp protocols as described in the Methods. Whole cell patch clamp recordings showed that bovine AZG cells uniformly expressed two types of K+ currents that were similar to those previously described in bovine AZF cells (15,17,34). These included a rapidly inactivating A-type K+ current, and a non-inactivating K+ current with a large instantaneous and a smaller time-dependent component.

bTREK-1 is distinctive among 2P/4TMS channels in its activation by ATP and intracellular acidification (16,17,53). The non-inactivating K+ current in AZG cells was activated by both ATP and low pH. In the experiment illustrated in Figure 2, whole cell K+ currents were recorded with pipette solutions containing 5 mM MgATP at pH 6.4 or 1 mM MgATP at pH 7.1. When recordings were made with 5 mM MgATP at pH 6.4, the non-inactivating K+ current spontaneously increased in amplitude for a period of minutes before it reached a stable maximum value (Figure 2). In contrast, when the K+ current was recorded at pH 7.1 with 1 mM MgATP in the pipette, the noninactivating current was less prominent and failed to grow significantly beyond its initial amplitude. Overall, with acidified pipette solution containing 5 mM MgATP, the putative bTREK-1 reached a maximum current density of 27.9 ± 4.8 pA/pF (n=24). By comparison with standard pipette solution, this current reached a maximum current density of only 11.1 ± 2.2 pA/pF (n=12).

In situ hybridization experiments showed that bovine adrenal chromaffin cells express little or no bTREK-1 mRNA. Accordingly, bTREK-1 current was undetectable in whole cell patch clamp recordings from enzymatically-dissociated chromaffin cells using acidified pipette solution containing 5 mM ATP. In recordings from 8 cells, only voltage-gated K+ currents similar to those previously described were observed (28) (data not shown).
Arachidonic Acid Activates the Background K⁺ Current in AZG Cells

Although the non-inactivating current enhanced by acidified pipette solution containing 5 mM MgATP resembled bTREK-1, additional evidence was needed to establish its identity. Of the more than one dozen background K⁺ channels characterized thus far, only the mechanogated subgroup including TREK-1, TREK-2, and TRAAK are activated by arachidonic acid (AA) and other polyunsaturated fatty acids (13,18,19,39). In AZG cells, AA triggered a pronounced increase in the amplitude of a non-inactivating current with voltage-dependent rectification indistinguishable from bTREK-1 (17).

In the experiment illustrated in Figure 3A, whole cell K⁺ currents were activated with and without depolarizing prepulses, using pipettes containing standard internal solution (pH 7.1, 1 mM MgATP). After the non-inactivating current reached a stable amplitude, the cell was superfused with 10 µM AA which increased this K⁺ current more than 17 fold within 8 min, while the rapidly inactivating A-type current was completely inhibited.

The increase in amplitude of the non-inactivating K⁺ current was rapidly reversed while inhibition of the A-type K⁺ current was poorly reversible upon superfusion of control saline (Figure 3, A and B). Overall, 10 µM AA increased the non-inactivating current density of six AZG cells from 6.7 ± 2.0 pA/pF to 162.4 ± 28.5 pA/pF (Figure 3C).

AA also markedly increased the non-inactivating K⁺ current in cells where the current had been pre-activated with acidified pipette solution containing 5 mM MgATP. While the non-inactivating K⁺ current reached a maximum density of 22.0 ± 3.8 pA/pF (n=6) in control saline, it grew to 222.0 ± 50.5 pA/pF (n=6) in the presence of 10 µM AA (Figure 3C).

In the presence of standard external solution, bTREK-1 appears as an outwardly rectifying K⁺ current (13,17,53). The voltage-dependent rectification of the background K⁺ current in AZG
cells activated by ATP and acidified pipette solution and by 10 µM AA, was characterized using voltage ramps. In the experiment illustrated in Figure 3B, K⁺ currents were recorded with acidified pipette solution (pH 6.4) containing 5 mM MgATP before and after superfusing the cell with 10 µM AA. Under bath conditions, linear voltage ramps applied between +60 and -140 mV induced similar outwardly rectifying currents that reversed at potentials near the theoretical K⁺ equilibrium potential. Thus, AZG cells express a background K⁺-selective current with properties indistinguishable from bTREK-1.

**Angiotensin II Inhibits bTREK-1 and Depolarizes AZG Cells**

*In situ* hybridization, Northern blot, and patch clamp experiments indicate that bTREK-1 is the predominant background K⁺ channel expressed by bovine AZG cells. If bTREK-1 K⁺ channels set the resting membrane potential, then inhibition of these channels by AngII should be coupled to depolarization.

In whole cell recordings, AngII (10 nM) potently and selectively inhibited the non-inactivating K⁺ current. Inhibition began within 90 s, and typically reached a steady state value in 5-7 minutes (Figure 4A). Overall, AngII (10 nM) inhibited bTREK-1 current by 82.1 ± 6.1 % (n=7) (Figure 4A, C).

In current clamp recordings of AZG cell membrane potential, it was discovered that AngII-mediated inhibition of bTREK-1 current was accompanied by membrane depolarization. In the experiment illustrated in Figure 4B, AngII (10 nM) depolarized this AZG cell by 30 mV from its resting value of -58 mV. Maximum depolarization occurred within 6 min. Overall, in current clamp recordings AZG cells maintained a resting membrane potential of -63.6 ± 2.3 mV (n=5). AngII (10 nM) depolarized these cells by an average of 31.6 ± 2.3 mV with a temporal pattern that
paralleled bTREK-1 inhibition.

**Vasopressin Inhibits bTREK-1 Current in AZG Cells**

If AngII-stimulated secretion is mediated through bTREK-1 inhibition, then other agents that physiologically induce aldosterone secretion might also inhibit this background K⁺ current. Vasopressin stimulates aldosterone secretion through a PLC-coupled receptor (48). Vasopressin (50 nM) inhibited bTREK-1 in AZG cells by an average of 73.8 ± 9.4 % (n=4) (Figure 4C).

**Arachidonic Acid Overwhelms AngII-mediated Inhibition of bTREK-1 and Hyperpolarizes AZG Cells**

The correlation that exists between AngII-mediated inhibition of bTREK-1 and membrane depolarization provides further evidence that these channels act pivotally in setting the membrane potential of AZG cells. If so, then activation of bTREK-1 channels by agents such as AA should oppose membrane depolarization by AngII.

In whole cell patch clamp experiments, it was discovered that AA overwhelmed the inhibition of bTREK-1 by AngII and completely reversed AngII-mediated membrane depolarization. In the experiment illustrated in Figure 5, AngII (2 nM) produced near complete inhibition of bTREK-1 (Figure 5A, trace 2) and depolarized the cell by 28.6 mV from its resting potential of -68.6 mV (Figure 5B). Superfusion of the cell with saline containing 2 nM AngII and 10 µM AA dramatically increased bTREK-1 to an amplitude nearly six times the control value (Figure 5A, trace 3). This increase in bTREK-1 was accompanied by a rapid hyperpolarization from -40.6 mV to -72.7 mV within 2 min (Figure 5B).
CDC Activates bTREK-1, Reverses AngII-stimulated Membrane Depolarization, and Inhibits Aldosterone Secretion

Recently, we demonstrated that CDC and other selected caffeic acid esters markedly enhance the activity of native AZF cell and cloned bTREK-1 channels (12). CDC also significantly increased the non-inactivating K⁺ current in AZG cells. In the experiment illustrated in Figure 6A, CDC (20 µM) increased the non-inactivating K⁺ current more than 17 fold in 8 min before the gigohm seal was lost. Overall, CDC (10 µM or 20 µM) increased this current density in 6 AZG cells from a control value of 15.6 ± 3.1 pA/pF to 192.6 ± 65.5 pA/pF (n=6).

CDC resembles AA in effectively opening bTREK-1 K⁺ channels in AZG cells. Within the framework of our model, CDC would also be expected to reverse AngII-stimulated depolarization. In the experiment illustrated in Figure 6B, AngII depolarized an AZG cell by 30.0 mV from its resting potential of -63.2 mV. Superfusing the cell with CDC (20 µM) repolarized the cell within 9 min from -33.2 mV to -58.0 mV. Similar results were obtained in each of 3 cells.

Since CDC reverses AngII-stimulated membrane depolarization, it should also inhibit depolarization-dependent aldosterone secretion. In the experiment illustrated in Figure 7, CDC (20 µM) inhibited AngII-stimulated aldosterone secretion measured at 1.5 and 16 h by 83 % and 95.4%, respectively. CDC also inhibited unstimulated aldosterone secretion at 1.5 and 16 h by 32% and 56.8%, respectively, in the same experiment. Overall, at 1.5 h in three separate experiments, CDC inhibited AngII-stimulated and unstimulated aldosterone secretion by 70.5 ± 7.6% and 39.7 ± 4.4 %. The inhibitory effects of CDC on aldosterone secretion were reversible, and this drug did not affect cell viability as determined by trypan blue exclusion (data not shown).

It has been reported that AngII-stimulated aldosterone secretion is mediated, in part, by 12-lipoxygenase products of AA (20,36). Since CDC is a potent 12-lipoxygenase antagonist (IC₅₀ =
0.06 µM), the possibility that CDC-mediated inhibition of aldosterone secretion occurs through inhibition of this enzyme, rather than bTREK-1 activation, had not been eliminated (8).

Baicalein inhibits 12-lipoxygenase with an IC₅₀ of 0.015 µM, but does not activate bTREK-1 (8,12). At a concentration of 10 µM, baicalein failed to inhibit AngII-stimulated aldosterone secretion at either 1.5 or 16 h (Figure 7).

**Angiotensin Has No Effect on T-type Ca²⁺ Current (Iₜ-Ca) in AZG Cells**

AngII inhibited bTREK-1 currents in AZG cells with pipette solutions containing 5 mM MgATP and [Ca²⁺]ᵢ buffered to 22 nM using 11 mM BAPTA. Experiments were done to determine whether AngII modulated voltage-gated Ca²⁺ currents through the same signaling pathway.

In whole cell recordings of Ca²⁺ current, the majority of AZG cells express only low voltage-activated T-type Ca²⁺ currents that are distinguished by their rapid inactivation and slow deactivation kinetics (Figure 8A). The slow rate of T channel closing is observed in whole cell recordings as a prominent decaying “tail” current upon repolarization after a brief depolarizing step (Figure 8A, right traces).

The modulation of Iₜ-Ca in AZG cells by AngII was monitored in whole cell recordings with pipette solutions containing nucleotides and 11 mM BAPTA to buffer Ca²⁺, as described above for recording K⁺ currents. Under these conditions, AngII (2 or 10 nM) failed to alter Iₜ-Ca amplitudes measured in response to short (10 ms) or long (300 ms) voltage steps to -5 mV or -10 mV, from a holding potential of -80 mV (Figure 8A). Overall, at concentration of 2 nM and 10 nM, AngII reduced Iₜ-Ca insignificantly to 0.96 ±0.02 (n=6) and 0.97 ± 0.01 (n=3) of its control amplitude (Figure 8A).

AngII also failed to alter the amplitude of $I_{T-Ca}$ measured over a wide range of test voltages. In the experiments illustrated in Figure 8B, $I_{T-Ca}$ was activated by voltage steps between -60 and +50 mV before and after superfusing the cell with AngII (2 nM) for 7-10 min. Plotting the averaged current densities from 3 cells against membrane voltage showed that AngII did not significantly change $I_{T-Ca}$ at any of the 12 test potentials.
DISCUSSION

It was discovered that bovine AZG cells express bTREK-1 background K⁺ channels that set the resting membrane potential and couple AngII receptor activation to membrane depolarization. These results suggest a model for aldosterone secretion wherein inhibition of bTREK-1 K⁺ channels by AngII leads to depolarization and the activation of voltage-gated Ca²⁺ channels. Accordingly, TREK-1 K⁺ channel openers reverse AngII-stimulated depolarization and inhibit aldosterone secretion. The pivotal role assigned to TREK-1 K⁺ channels in this model contrasts with previous studies that focused on a direct effect of AngII on T-type Ca²⁺ channels. In our experiments, AngII failed to produce a measurable change in the activity of T-type Ca²⁺ channels under conditions where bTREK-1 currents were nearly completely inhibited by this peptide hormone.

TREK-1 is the Major Background K⁺ Channel of Bovine AZG Cells

The combination of Northern blot, in situ hybridization, and patch clamp studies revealed that TREK-1 rather than TASK-3 is the major K⁺ channel expressed by bovine AZG cells. Northern blot analysis showed that AZF and subcapsular AZG cells both expressed the same three bTREK-1 mRNA transcripts, each of which were similarly induced by forskolin. Thus, bTREK-1 expression in AZG cells is likely regulated at the transcriptional level by ACTH through a cAMP-dependent mechanism as it is in AZF cells (17). In situ hybridization on bovine adrenal gland sections corroborated the Northern blot results and showed that bTREK-1 mRNA was uniformly distributed in AZF and AZG cells, but was virtually undetectable in the adrenal medulla.

Results from patch clamp experiments were in agreement with those measuring TREK-1 mRNA distribution in the bovine adrenal gland. Bovine AZG cells expressed two K⁺ currents that were indistinguishable from those of AZF cells. Most importantly, these cells expressed hundreds
of background K$^+$ channels that are either dormant or have a low open probability and display a composite profile that matches that of bTREK-1. These outwardly rectifying channels were activated by intracellular acidification, ATP, AA, and CDC and inhibited by AngII. Of the 4TMS/2P channels identified thus far, only bTREK-1 channels possess all of these properties (16,17,53). However, our results do not formally exclude the unlikely possibility that a background K$^+$ channel in addition to bTREK-1 is expressed by bovine AZG cells.

Although we were extremely careful in our dissection to obtain only subcapsular glomerulosa tissue, it is possible that isolated glomerulosa cells were contaminated with a small fraction of AZF cells. By choosing smaller cells, we further reduced the possibility that patch clamp recordings mistakenly included AZF cells. In this regard, it is important to note that each of the more than 25 AZG cells exposed to the bTREK-1 openers responded with large increases in the non-inactivating K$^+$ current. Thus, bTREK-1 appears to be uniformly expressed in both AZF and AZG cells.

Although TASK-3 may be the major background K$^+$ current found in rat AZG cells (11), we found no evidence that this channel is expressed in bovine AZG. TASK-3 was undetectable in Northern blots of bovine AZG mRNA. When whole cell K$^+$ currents were recorded with pipette solutions that minimized the expression of TREK-1, no background K$^+$ current was activated. These is little doubt that bTREK-1 is the major background channel that sets the resting potential of bovine AZG cells.

In contrast to its expression in the adrenal cortex, no evidence of TREK-1 expression in neural crest-derived chromaffin cells was found in in situ hybridization or whole cell patch clamp experiments. When K$^+$ currents were recorded from chromaffin cells with acidified pipette solution containing 5 mM MgATP, no bTREK-1 current was detected. Further, neither CDC nor AA
activated such a current in chromaffin cells (unpublished observations). Although these neural crest-derived cells express multiple K\(^+\) channel subtypes, including voltage- and Ca\(^{2+}\)-activated K\(^+\) channels, the background K\(^+\) channel that sets their resting membrane potential remains to be identified (28).

AngII Regulates AZG Membrane Potential Through TREK-1 Inhibition: A Model for Depolarization-dependent Secretion

A requirement for Ca\(^{2+}\) in AngII-stimulated aldosterone secretion is well established (7,24,25,41,47). In exploring the cellular mechanism, a number of studies have focused on AngII modulation of T-type Ca\(^{2+}\) channels (6,9,26,31). However, none of these have provided a satisfactory explanation whereby AngII could trigger large increases in Ca\(^{2+}\) entry through voltage-gated channels.

The results of the present study suggest a specific model for AngII-stimulated secretion that assigns a critical role to bTREK-1 K\(^+\) channels. In this model, AngII-mediated inhibition of bTREK-1 is coupled to membrane depolarization, Ca\(^{2+}\) channel activation, and aldosterone secretion. This model allows for the indirect activation of T- or L-type Ca\(^{2+}\) channels through TREK-1 inhibition. Several possibilities exist that could produce efficient Ca\(^{2+}\) entry through either channel. If AngII-mediated inhibition of bTREK-1 produces a sustained depolarization under physiological condition, it would produce a continuous influx through non-inactivating Ca\(^{2+}\) channels. The effects of AngII on L-type Ca\(^{2+}\) channels in AZG cells are complex and both enhancements and inhibition of Ca\(^{2+}\) entry have been reported (23-25,30,46). However, no L-type Ca\(^{2+}\) current was present in the majority of freshly cultured bovine AZG cells.

Alternatively, bTREK-1 may serve as a brake on the electrical activity of AZG cells.
Inhibition of bTREK-1 by AngII could remove this brake, triggering Ca\(^{2+}\)-dependent action potentials driven by opposing T-type Ca\(^{2+}\) currents and A-type K\(^{+}\) currents. Regenerative Ca\(^{2+}\)-dependent action potentials in AZG cells have been observed (42). It is unlikely that bovine AZG cells generate action potentials when membrane potential is recorded at 21-23 °C with a whole cell patch electrode. Since membrane potential was sampled at 100 ms intervals in these experiments, fast action potentials would not have been readily detected. Action potentials would most likely be recorded from AZG cells in an adrenal slice using a sharp intracellular electrode at physiological temperatures. Regardless, the combined direct effects of AngII on both T-type Ca\(^{2+}\) channels and bTREK-1 K\(^{+}\) channels produce the ionic effects that mediate aldosterone secretion.

Similar to AngII, vasopressin stimulates aldosterone secretion through a PLC-coupled receptor requiring Ca\(^{2+}\) influx (21,44,50). The inhibition of bTREK-1 K\(^{+}\) channels in AZG cells by vasopressin suggests that it also may stimulate aldosterone secretion through depolarization-dependent Ca\(^{2+}\) entry.

**Signaling Pathways for AngII Modulation of Ion Channels in AZG Cells**

The signaling pathways by which AngII modulates the activity of ion channels in AZG cells are incompletely understood. In particular, the modulation of T-type Ca\(^{2+}\) channels by AngII has produced conflicting results. In several studies, it has been reported that AngII increased I\(_{\text{T-Ca}}\) by a mechanism that involved a hyperpolarizing shift in the voltage dependence of channel activation (6,9,27,31). These effects may occur through Ca\(^{2+}\)-dependent activation of Ca\(^{2+}\)/CaM Kinase II (1,6,27).

However, AngII has also been reported to inhibit T-type Ca\(^{2+}\) currents in bovine AZG cells through activation of protein kinase C (45). Notably, the activity of protein kinase C by
diacylglycerol is also enhanced by \([Ca^{2+}]_i\) (37). Finally, in perforated patch whole cell recordings, AngII failed to alter the activity of \(I_{T-Ca}\) in rat AZG cells (25).

Although our results do not explain the conflicting findings described above, they do suggest that AngII modulates ion channels in AZG cells by multiple signaling pathways, not all of which are \(Ca^{2+}\)-dependent. In our experiments, including those measuring the activity of \(b\)TREK-1 and T-type \(Ca^{2+}\) channels, \([Ca^{2+}]_i\) was strongly buffered to 22 nM using 11 mM BAPTA. If AngII modulation of \(I_{T-Ca}\), including activation or inhibition, requires activation of \(Ca^{2+}\)-dependent enzymes, this response would likely be blunted or eliminated in our experiments (2, 54).

Regardless, our results do show that AngII potently and nearly completely inhibits \(b\)TREK-1 current, and depolarizes AZG cells through a signaling pathway that does not modulate T-type \(Ca^{2+}\) channels. It appears that AngII-mediated modulation of \(Ca^{2+}\) and \(K^+\) channels in bovine AZG cells occurs through multiple \(Ca^{2+}\)-dependent and independent signaling pathways.

**TREK-1 Channel Activation and Inhibition of Aldosterone Secretion**

The ability of AA and CDC to activate \(b\)TREK-1 channels and restore membrane potential to AZG cells depolarized by AngII provides convincing evidence that these channels set the resting potential of AZG cells. The effectiveness of CDC in reversing AngII-stimulated depolarization and inhibiting AngII-stimulated aldosterone secretion is consistent with a model in which TREK-1 couples AngII receptor activation to depolarization-dependent \(Ca^{2+}\) entry. Within this framework, CDC would negate the membrane depolarization that leads to \(Ca^{2+}\) channel activation.

These results also illustrate the utility of this new type of \(K^+\) channel activator in exploring
the function of 2P/4TMS channels in cell physiology. However, additional studies will be needed to determine its specificity as an ion channel modulator. AA and other cis polyunsaturated fatty acids that activate the same subgroup of 2P/4TMS background channels also modulate several types of voltage-gated channels limiting their value as pharmacological tools (38,49).

Summary

The results of the current and previous studies clearly demonstrate that bTREK-1 is the predominant background K⁺ channel that sets the resting membrane potential of both bovine AZF and AZG cells, and couples ACTH and AngII receptors to depolarization-dependent Ca²⁺ entry and the secretion of cortisol and aldosterone. In contrast, TASK-type K⁺ channels may function similarly in the rat adrenal cortex, at least in the adrenal glomerulosa (10,11).

Thus, significant species differences that exist between rat and bovine adrenal cortical background K⁺ channels highlight the importance of identifying the background K⁺ channels in the human adrenal cortex. PCR using human cDNA as the template showed that TREK-1 is strongly expressed in the human adrenal (data not shown). Additional experiments will be required to determine if TREK-1 functions in the human adrenal cortex as it does in bovine AZF and AZG. If so, then TREK-1 channel activators might serve as therapeutic agents in endocrine diseases marked by excessive cortisol or aldosterone secretion.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Distribution of bTREK-1 and TASK-3 mRNA expression in bovine adrenal gland. The distribution of bTREK-1 and TASK-3 mRNA in the bovine adrenal gland was determined by Northern blot and in situ hybridization. A) Northern blot analysis. Bovine AZF (F) and AZG (G) cells were cultured as described in the Methods for 8 h before media was replaced with the same media with or without forskolin (5 µM) as indicated for 20 h before isolating RNA. For Northern blots, 10 µg of total AZG or AZF RNA isolated from control or forskolin-treated cells was loaded in duplicate lanes. The membrane was divided into similar lanes, and hybridized as described in the Methods with either a 370 bp EcoRI fragment from the 5’ end of bTREK-1 or the full-length cDNA for rTASK-3. B) In situ hybridization: mRNA for bTREK-1 and 11 ß hydroxylase (CYP11B) was detected in bovine adrenal cross sections by hybridization with 35S-labeled oligonucleotide probes as described in the Methods. Upper panel, #1: Specific binding of the labeled oligonucleotide probe (antisense) to the bTREK-1 mRNA transcript. Upper panel, #2: 3X magnification of highlighted area in #1. Upper panel, #3: Hybridization with bTREK-1 sense oligonucleotide probe to show non-specific binding. Lower panel, #1: For morphological identification, binding of an oligonucleotide probe specific to CYP11B mRNA known to be present in both glomerulosa and fasciculata is shown. Lower panel, #2: 3X magnification of highlighted area in #1. Lower panel, #3: hybridization with CYP11B sense oligonucleotide probe to show non-specific binding. Scale bar (5 mm) applies to columns 1 and 3.

Figure 2. Activation of a non-inactivating K⁺ current in AZG cells by ATP and acidification of the pipette solution. Whole cell K⁺ currents were recorded from AZG cells with pipette solutions containing 5 mM ATP at pH 6.4 or 1 mM MgATP at pH 7.1. Currents were activated by voltage
steps to +20 mV with or without depolarizing prepulses as illustrated and described in the Methods. Current traces show K⁺ currents recorded after the non-inactivating current had reached a maximum value. K⁺ current amplitudes are plotted against time at right for recordings made with (open circles) and without (closed circles) depolarizing prepulses. Numbers on traces correspond to those on graph.

Figure 3. Arachidonic acid activates the non-inactivating outwardly rectifying K⁺ current of AZG cells. Whole-cell K⁺ currents were recorded from AZG cells while superfusing control saline or saline containing 10 µM AA. A) Temporal pattern and reversibility. K⁺ currents were recorded from an AZG cell at 30 s intervals in response to voltage steps to +20 mV from a holding potential of -80 mV with (open circles) or without (closed circles) a depolarizing prepulse as indicated. After recording currents in standard saline, the cell was superfused with 10 µM AA for 10 min. Numbers on traces correspond to those on plot of current amplitudes at right. B) Voltage-dependent rectification of K⁺ current activated by acidified pipette solution containing 5 mM MgATP and externally applied AA. Linear voltage ramps of 100 mV/s were applied from a holding potential of 0 mV to potentials between +60 mV and -140 mV with pipette solutions containing 5 mM MgATP at pH 6.4 (control). A second voltage ramp was applied after AA (10 µM) produced a maximum increase in the non-inactivating current. C) Bar graphs summarize data from experiments as in Figure 1 and Figure 2A. Values are mean ± SEM of the indicated number of determinations.

Figure 4. AngII inhibits the acid and ATP-activated background K⁺ current and depolarizes AZG cells. A) Whole cell K⁺ currents were activated by voltage steps to +20 mV applied at 30 s intervals from a holding potential of -80 mV with (open circles) or without (closed circles)
depolarizing prepulses to -20 mV, as indicated. After the noninactivating K⁺ current reached a stable maximum value, the cell was superfused with 10 nM AngII. bTREK-1 amplitude is plotted against time at right. Numbers on traces correspond to those on graph. B) AngII depolarization of AZG cells. Membrane potential (V_m) was recorded in current clamp with pipette solution containing 5 mM MgATP at pH 6.4. After 4 min, the cell was superfused with saline containing 10 nM AngII, as indicated. V_m was sampled at 100 ms intervals. Each data point is the average of 100 values sampled over a 10 s interval. C) Inhibition of bTREK-1 by AngII and vasopressin. Summary of experiments in which AZG cells were superfused with either AngII (2 nM) or vasopressin (50 nM) and inhibition of bTREK-1 was measured. Values are mean ± SEM of indicated number of determinations.

**Figure 5.** Arachidonic acid reverses AngII-mediated inhibition of bTREK-1 and membrane depolarization. AZG cell was voltage clamped in whole cell mode with pipette solution containing 5 mM MgATP at pH 6.4. Whole cell K⁺ current were recorded in response to voltage steps to +20 mV from a holding potential of -80 mV. After bTREK-1 reached a maximum value Vm was recorded under current clamp. While recording V_m, the cell was superfused sequentially with saline containing AngII (2 nM), then with AngII plus AA (10 µM). bTREK-1 current was recorded intermittently by switching to voltage clamp. A) bTREK-1 currents recorded at times indicated by numbers in (B) below. B) AZG membrane potential was sampled at 100 ms intervals and plotted as averaged values obtained over 10 s intervals. Numbers on plot correspond to those on current traces above.

**Figure 6.** CDC activates bTREK-1 and reverses AngII-mediated depolarization of AZG cells. A)
CDC activates bTREK-1 in AZG cells. Whole cell K+ current were recorded in response to voltage steps to +20 mV applied at 30 s intervals from a holding potential of -80 mV using standard pipette solution (1 mM MgATP, pH 7.1). The cell was superfused with 20 µM CDC as indicated. Current amplitudes are plotted against times at right. Numbers on plot correspond to current traces. B) CDC reverses AngII depolarization. AZG membrane potential was recorded in current clamp. The cell was sequentially superfused with saline containing AngII (2 nM) followed by AngII + CDC (20 µM). Membrane potential was sampled at 100 ms intervals and plotted as averaged values over 10 s intervals.

**Figure 7.** CDC inhibits AngII-stimulated aldosterone secretion. Cultured AZG cells were incubated in serum-free defined media (see Methods) or the same media containing AngII (10 nM), CDC (20 µM), or AngII (10 nM) and either CDC (20 µM) or baicalein (10 µM). Media samples were collected at 1.5 h and 16 h and assayed for aldosterone as described in the Methods. Values are mean ± SEM of indicated number of determinations.

**Figure 8.** Effect of AngII on I_{T-Ca}. A) T-type Ca^{2+} currents were activated by long (300 ms) test pulses to -5 mV (left traces) or short (10 ms) test pulses to 0 mV (right traces) applied at 30 s intervals from a holding potential of -80 mV. Representative traces from the control (black) and AngII (2nM)-treated cell (red) (left traces) are superimposed. For tail currents (right), traces from control (black) and AngII (10 nM)-treated cell (blue) are superimposed. Peak current amplitudes for both protocols are plotted against time in graph. B) AngII and the IV relationship. T-type Ca^{2+} currents were activated from -80 mV by voltage steps applied at 0.1 Hz to various test potentials between -60 and +50 mV before and after superfusing 2 nM AngII.
Representative current traces recorded at indicated test potentials (-30, -20 and -10 mV) before (black) and after (red) superfusion of 2 nM AngII are superimposed. Current-voltage relationship: Peak current amplitudes from three cells before (open circles, black line) and after (closed circles, red line) addition of 2 nM AngII were averaged and plotted against test potential. Values are mean ± SEM.
**FIGURE 1**

**A**

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**TREK-1**

1 h exposure

**TASK-3**

24 h exposure

**B**

1. bTREK-1

2. CYP11B

3. 

5 mm
Figure 6

A

-80 +20 mV -80 mV

500 pA 50 ms

B

2 nM AngII

20 μM CDC

VM (mV)

0 2 4 6 8 10 12

TIME (min)