Ca\(^{2+}\) and K\(^{+}\) channels of normal human adrenal zona fasciculata cells: Properties and modulation by ACTH and AngII

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In whole cell patch clamp recordings, we found that normal human adrenal zona fasciculata (AZF) cells express voltage-gated, rapidly inactivating Ca\(^{2+}\) and K\(^{+}\) currents and a noninactivating, leak-type K\(^{+}\) current. Characterization of these currents with respect to voltage-dependent gating and kinetic properties, pharmacology, and modulation by the peptide hormones adrenocorticotropic hormone (ACTH) and AngII, in conjunction with Northern blot analysis, identified these channels as Ca,3.2 (encoded by CACNA1H), Kv1.4 (KCNA4), and TREK-1 (KCNK2). In particular, the low voltage–activated, rapidly inactivating and slowly deactivating Ca\(^{2+}\) current (Ca,3.2) was potently blocked by Ni\(^{2+}\) with an IC\(_{50}\) of 5 μM. The voltage-gated, rapidly inactivating K\(^{+}\) current (Kv1.4) was robustly expressed in nearly every cell, with a current density of 95.0 ± 7.2 pA/pF (n = 64). The noninactivating, outwardly rectifying K\(^{+}\) current (TREK-1) grew to a stable maximum over a period of minutes when recording at a holding potential of ~80 mV. This noninactivating K\(^{+}\) current was markedly activated by cinnamyl 1-3,4-dihydroxy-\(\alpha\)-cyanocinnamate (CDC) and arachidonic acid (AA) and inhibited almost completely by forskolin, properties which are specific to TREK-1 among the K2P family of K\(^{+}\) channels. The activation of TREK-1 by AA and inhibition by forskolin were closely linked to membrane hyperpolarization and depolarization, respectively. ACTH and AngII selectively inhibited the noninactivating K\(^{+}\) current in human AZF cells at concentrations that stimulated cortisol secretion. Accordingly, mibefradil and CDC at concentrations that, respectively, blocked Ca,3.2 and activated TREK-1, each inhibited both ACTH- and AngII-stimulated cortisol secretion. These results characterize the major Ca\(^{2+}\) and K\(^{+}\) channels expressed by normal human AZF cells and identify TREK-1 as the primary leak-type channel involved in establishing the membrane potential. These findings also suggest a model for cortisol secretion in human AZF cells wherein ACTH and AngII receptor activation is coupled to membrane depolarization and the activation of Ca,3.2 channels through inhibition of hTREK-1.

INTRODUCTION

In mammals, adrenal zona fasciculata (AZF) cells of the adrenal cortex secrete glucocorticoids in a diurnal pattern in response to stimulation by adrenocorticotropic hormone (ACTH). Superimposed on this basal secretory pattern, physical and psychological stress triggers bursts of ACTH-stimulated cortisol production by activation of the hypothalamic pituitary adrenal axis (Stewart and Krone, 2011). In some species, including bovine and human, AngII may also stimulate cortisol secretion (Glyne et al., 1993; Lebrethon et al., 1994; Mlinar et al., 1995). Cortisol acts pivotally in regulating physiological functions ranging from energy metabolism to long-term memory formation (Stewart and Krone, 2011; Chen et al., 2012). At the cellular level, the biochemical and ionic mechanisms that regulate glucocorticoid production are only partially understood. However, in bovine and rodents, a pivotal role for depolarization-dependent Ca\(^{2+}\) entry is well established (Matthews and Safran, 1973; Lymangrover et al., 1982; Enyeart et al., 1993; Mlinar et al., 1993a,b; Barbara and Takeda, 1995). In this regard, relatively few studies exist describing the specific ion channels of normal mammalian AZF cells, including their modulation by ACTH and AngII.

Early intracellular recordings from cat, rabbit, bovine, rat, and mouse adrenocortical tissue and isolated AZF cells showed that they maintained negative resting potentials, determined primarily by the membrane permeability to K\(^{+}\) (Matthews, 1967; Matthews and Safran, 1968, 1973; Natke and Kabela, 1979; Lymangrover et al., 1982; Quinn et al., 1987). In addition, ACTH was found to depolarize mouse and rabbit AZF cells and, in some cases, to induce action potential–like spikes in these cells (Matthews and Safran, 1968, 1973; Lymangrover et al., 1982). Ca\(^{2+}\)-dependent action potential–like waveforms have also been recorded in cat, rat, and bovine AZF cells in response to application of depolarizing current (Natke and Kabela, 1979; Quinn et al., 1987).
Most of the action potentials observed in AZF cells were obtained in recordings from intact tissue rather than isolated cells. Recently, spontaneous action potential–like oscillations have been observed in a mouse adrenal zona glomerulosa (AZG) slice (Hu et al., 2012). Overall, these studies suggested a critical role for ion channels and voltage-dependent Ca\(^{2+}\) channels in ACTH-stimulated cortisol secretion. Later studies that combined patch clamp and molecular cloning techniques identified each of the ion channels expressed by bovine AZF cells and described their modulation by ACTH and AngII. Specifically, bovine AZF cells were found to express voltage-gated, rapidly inactivating Ca\(_{3.2}\) Ca\(^{2+}\) and Kv1.4 K\(^{+}\) channels and a novel leak-type K\(^{+}\) channel that set the resting membrane potential (Mlinar and Enyeart, 1993b; Mlinar et al., 1993a,b). This leak-type K\(^{+}\) channel, later identified as TREK-1 of the two-pore K\(^{+}\) (K2P) channel family, was potently inhibited by ACTH and AngII, leading directly to membrane depolarization (Mlinar et al., 1993a; Enyeart et al., 2005). These findings led us to propose a model for cortisol secretion in which ACTH or AngII receptor activation was coupled to membrane depolarization and the activation of Ca\(_{3.2}\) channels through the inhibition of TREK-1 channels (Enyeart et al., 1993, 2005; Mlinar et al., 1993a; Liu et al., 2008).

The molecular identities of ion channels expressed in AZF cells, other than bovine, have not been determined. Therefore, it isn’t known to what extent mammalian AZF cells resemble each other with respect to their ion channels and associated electrical properties. In particular, it isn’t known whether these cells all express a similar group of channels, including a K2P channel that sets the resting membrane potential and whose inhibition by ACTH or AngII is tightly linked to membrane depolarization. In one patch clamp study, rat AZF cells were found to express only a relatively Ni\(^{2+}\)-insensitive T-type Ca\(^{2+}\) current and a slowly inactivating A-type K\(^{+}\) current, whereas no leak-type K\(^{+}\) current was detected (Barbara and Takeda, 1995). By comparison, in the mouse adenocortical V1 cell line, only two types of voltage-gated Ca\(^{2+}\) currents and a Ca\(^{2+}\)-activated K\(^{+}\) current were detectable (Tabares et al., 1985).

Recordings from bovine, rat, and mouse adenocortical cells suggest that considerable variability may exist among mammalian AZF cells with respect to the ion channels and electrical events that mediate corticosteroid secretion. To understand the role of specific ion channels in secretion by human AZF cells, it will be necessary to identify these channels, including their modulation by peptide hormones that regulate cortisol production. In the present study, we have identified and characterized the ionic currents of normal human AZF cells with respect to their biophysical properties, pharmacology, and modulation by ACTH and AngII.

### MATERIALS AND METHODS

#### Materials

Tissue culture media, antibiotics, fibronectin, and FBS were obtained from Invitrogen. Coverslips were purchased from Bellco. PBS, 1:2 bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), MgATP, collagenase (C0130), DNase, forskolin, ACTH (1-24), AngII, GdCl\(_3\), NiCl\(_2\), mibefradil, and arachidonic acid (AA) were obtained from Sigma-Aldrich. Cinnamyl 1,3,4-dihydroxy-alpha- cyanocinnamate (CDC) was purchased from Enzo Life Sciences. hTASK-3 (KCNK9 cDNA clone) was purchased from Thermo Fisher Scientific (MGC:103976 IMAGE:50915383). \(^{32}\)P)dCTP was purchased from PerkinElmer.

#### Isolation and culture of AZF cells

Human adrenals were obtained from 11 deceased organ donors (age 10–62 yr, male and female, of either Caucasian or African–American race) through the Ohio State University Department of Transplant Surgery and Lifeline of Ohio within 3 h of organ removal. Institutional Review Board and ethical consent practices for donor tissue were strictly followed. Organs were kept in cold saline, on wet ice until they were available to be collected. Some adrenal cell isolations (3/11) yielded cells that could not be used for our experiments, as indicated by extremely fragile membranes and lack of recordable ion currents. We were not able to ascertain whether this was caused by our cell isolation protocol or the treatment of the organs after harvest before we received them. This characteristic was not related to age, sex, or race of the donor. However, we were able to develop a method for isolating and storing human AZF cells from a majority of the glands received (8/11) wherein they retained their biochemical and electrophysiological properties for a minimum of 18 mo. In brief, glands with some surrounding fat were submerged in cold PBS, kept on ice, and transported within 3 h of removal from the donor to the laboratory. Fat was removed, and thin tissue slices were obtained using a Stadie-Riggs tissue slicer. The first slice containing mainly adrenal capsule and zona glomerulosa was used for the isolation of adrenal glomerulosa cells. Subsequent slices were designated as fasciculata and used to prepare AZF cells. In 7 of 11 isolations, the adrenal medulla could be easily identified and manually dissected away from the cortex. In the remaining cases, the medulla, as observed under a dissecting microscope, appeared to infiltrate the cortex and was thus more difficult to separate from the outer layers of the adrenal cortex. This characteristic was not related to age, sex, or race of the donor. Cortical slices were finely chopped into 1–2-mm fragments, followed by two incubation periods of 60 min in 2 mg/ml DMEM/F12 containing collagenase (C0140; Sigma-Aldrich) and 0.5 mg/ml DNase (DN25; Sigma-Aldrich). Cells were then disrupted by gentle aspiration with a sterile, fire-polished Pasteur pipette before being filtered using a cell dissociation sieve (#60 mesh; CD-1; Sigma-Aldrich) and centrifuged for 5 min at 100 g. The cell pellet was washed twice with DMEM/F12, resuspended in DMEM/F12 (1:1), 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 µM of antioxidant alpha-tocopherol, 20 nM selenite, and 100 µM ascorbic acid (DMEM/F12). Cells were then plated for immediate use or resuspended in FBS/5% DMSO, divided into 1-ml aliquots, and frozen (initially at −80°C for 24 h and then at approximately −196°C in a Thermoloc Locator Plus liquid nitrogen canister) for future use. Frozen cells were found to be viable when thawed, after storage for as long as 18 mo, with no change in measurable K\(^{+}\) or Ca\(^{2+}\) currents. To ensure cell attachment when culturing cells, glass coverslips (9 × 9 mm; Bellco) in 35-mm dishes were treated with 10 µg/ml fibronectin at 37°C for 30 min and then rinsed with warm, sterile PBS immediately before adding cells. Cells were maintained at 37°C in a humidified atmosphere of 95% air–5% CO\(_2\).

138 Ion channels of the human adrenal fasciculata
Measurement of ion channel mRNA

RNase-free DNase (QIAGEN) to remove genomic contamination were used to extract total RNA from hAZF cells that had been isolated and cultured in DMEM/F12 as described above for 24 h. 10 µg of total RNA/lane was separated on an 8% formaldehyde, 1.0% agarose gel and then transferred to a nylon membrane (Gene Screen Plus, NEN). RNA was fixed to the membrane by UV cross-linking. Northern blots were prehybridized for 2 h at 42°C in ULTRAhyb (Ambion), hybridized with each α-[32P]dCTP-labeled probe for 18 h, and washed as previously described (Enyeart et al., 2000). Specific probes were as follows: 800 bp CACNA1H probe, which codes for the Ca.3.2 channel, was as previously described (Li et al., 2010; J.-A. Enyeart et al., 2011); 1.3-kb PVU II fragment for the KCNA4 probe (coding for the Kv1.4 K+ channel) was obtained as previously described (Enyeart et al., 2000); 700 bp KCNK2 probe for the TREK-1 channel was obtained by ECOR1 digest of the full-length KCNK2 cDNA as previously described (Enyeart et al., 2002); and full-length KCNK9 probe for the TASK-3 channel was obtained from EcoRI digest of purchased KCNK9 cDNA clone (MGC:103976 IMAGE:50915383; Thermo Fisher Scientific). Northern autoradiograms were imaged using a Typhoon 9200 variable mode phosphorimager after 4-h exposure to phosphoimaging screen (GE Healthcare).

Patch clamp experiments

Cellular identification for patch clamp experiments. Precautions were taken to ensure that the cells selected for recording were AZF cells. First, within the AZF fraction, we selected larger cells (Cp = 15–30 pF) because AZG cells are typically smaller with capacitance < 15 pF. Second, in whole cell patch voltage clamp recordings, we found that human AZG cells expressed a distinctive inwardly rectifying K+ current that was not present in AZF cells. This current resembled the KCNJ5 K+ current recently reported to be selectively expressed in the zona glomerulosa of the human adrenal gland (Choi et al., 2011). Furthermore, the Ca2+ current that we identified in human AZF cells differs markedly from those previously described in a study of human AZG ion currents (Payet et al., 1994). Presumed AZG cells expressing an inwardly rectifying current were not included in the present study. A few adrenal chromaffin cells were occasionally present in the human AZF fraction. These were easily distinguished by their distinct appearance and the presence of a TTX-sensitive Na+ current.

Ca2+ currents. Patch clamp recordings of Ca2+ channel currents were made in the whole cell configuration from human AZF cells. The standard pipette solution was 120 mM CsCl, 1 mM CaCl2, 2 mM MgCl2, 11 mM BAPTA, 10 mM HEPES, and 1 mM MgATP, with pH titrated to 7.2 with CsOH. The external solution consisted of 117 mM tetraethylammonium chloride, 5 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, and 5 mM glucose, with pH adjusted to 7.4 with tetraethylammonium hydroxide.

K+ currents. Patch clamp recordings of K+ channel currents were made in the whole cell configuration from human AZF cells. The standard external solution consisted of 141.5 mM NaCl, 5 mM KCl, 1 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 120 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 11 mM BAPTA, 10 mM HEPES, 5 mM ATP, and 200 µM GTP, with pH titrated to 6.8–7.2 using KOH. All solutions were filtered through 0.22-µm cellulose acetate filters.

Recording conditions and electronics

AZF cells were used for patch clamp experiments 2–48 h after plating. Typically, cells with diameters of 15–30 µm and capacitances of 15–30 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 Corning 0010 glass (World Precision Instruments). These electrodes routinely yielded access resistances of 1.5–4.0 MΩ and voltage clamp time constants of <100 µs. K+ and Ca2+ currents were recorded at room temperature (22–25°C) according to the procedure of Hamill et al. (1981) using a List EPC-7 patch clamp amplifier. To minimize series resistance errors, smaller cells (15–20 pF) and lower resistance electrodes (<1 MΩ) were chosen for measurement of voltage-dependent gating and kinetic properties. Currents in these cells were not qualitatively different from those in larger cells.

Pulse generation and data acquisition were performed using a personal computer and PCLAMP software with Digidata 1200 interface (Axon Instruments, Inc.). Currents were digitized at 2–50 kHz after filtering with an 8-pole Bessel filter (Frequency Devices). 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 CLAMPFIT 9.2 (Molecular Devices) and SigmaPlot (version 11.0) software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

Cortisol assay

In experiments measuring cortisol secretion, human AZF cells were plated in fibronectin-coated 35-mm dishes at a density of 0.2–0.4 million cells per dish in DMEM/F12. After a recovery period of 4 h, media were changed to defined media consisting of DMEM/F12 with 50 µg/ml, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (DMEM/F12/BSA/pen-strep). Cells were maintained in DMEM/F12/BSA/pen-strep for an additional 48 h before changing to the same media either with or without ACTH and/or AngII for the required times. Media from experiments were either assayed immediately after collection or frozen (−20°C) until all samples were collected. All assay conditions were performed in triplicate. Cortisol secretion by AZF cells was measured using a Cortisol EIA kit (11-CORHU-E01; Alpco Immunoassays) according to the manufacturer’s directions. If necessary, media samples were diluted using DMEM/F12/BSA/pen-strep media.

RESULTS

Voltage-gated Ca2+ currents

In whole cell patch clamp recordings, we found that human AZF cells expressed only a low voltage-activated, rapidly inactivating Ca2+ current resembling T-type Ca2+ currents carried through Ca3.3 channels (Perez-Reyes, 2003). The current-voltage (I-V) relationship for Ca2+ currents was obtained by applying voltage steps to various test potentials from a holding potential of −80 mV. Ca2+ currents were measurable at test voltages positive to −50 mV, reached a maximum amplitude between −30 and −10 mV, and declined thereafter, reversing near 50 mV (Fig. 1 A). At test voltages positive to −40 mV, the Ca2+ current decayed to near zero during a 300-ms depolarization. In 14 similar experiments, in which I-V relationships were obtained, only a low voltage–activated, rapidly inactivating Ca2+ current with mean maximum density of 15.8 ± 1.9 pA/pF (n = 13) was present. A noninactivating Ca2+ current was not detected in any of these cells.
Voltage-dependent activation of Ca\textsuperscript{2+} channels. Voltage-dependent opening at relatively negative potentials is a distinctive feature of all three Ca\textsubscript{3.2} channel subtypes (Perez-Reyes, 2003). The voltage-dependent activation of Ca\textsuperscript{2+} channels in human AZF cells was studied by applying voltage steps to various test potentials and measuring the peak Ca\textsuperscript{2+} tail current upon repolarization to \(-80\) mV (Fig. 1 B). Current amplitudes were normalized, plotted as a fraction of maximal conductance against test voltage, and fit by a Boltzmann function of the form \(G = \frac{1}{1 + \exp (v_{1/2} - v) / k}\), where \(G\) is the relative conductance, \(v_{1/2}\) is the voltage of half-maximal conductance, and \(k\) is the slope factor. Averaged data from eight cells were well fit by a Boltzmann function with a \(v_{1/2}\) of \(-20.19 \pm 0.66\) mV and a slope factor of \(6.67 \pm 0.59\) (Fig. 1 B).

Voltage-dependent steady-state inactivation. The voltage dependence of steady-state inactivation of the Ca\textsuperscript{2+} current was measured by applying 10-s depolarizing pulses to various potentials between \(-80\) and \(-25\) mV, followed by short activating steps to 10 mV after 10-s conditioning steps to potentials between \(-80\) and \(-25\) mV. Peak tail current amplitudes, normalized to the maximum current recorded at \(-80\) mV, were plotted against conditioning voltage. Mean values \(\pm\) SEM \((n = 4)\) were fitted with a Boltzmann relationship: \(I / I_{\text{MAX}} = \frac{1}{1 + \exp (v - v_{1/2}) / k}\), where \(v_{1/2}\) is the voltage at which half of the channels were inactivated and \(k\) is the slope factor.

Voltage-dependent gating kinetics. Although the voltage-dependent gating, including activation and steady-state inactivation, is similar among the three Ca\textsubscript{3.2} subtypes, their
gating kinetics display significant variability (Klöckner et al., 1999; Perez-Reyes, 2003). The expression of only voltage-gated T-type Ca\(^{2+}\) channels by the great majority of human AZF cells allowed their voltage-dependent kinetic properties to be studied in isolation over a wide range of test potentials.

The activation kinetics of the human Ca\(^{2+}\) current was voltage dependent, accelerated at positive potentials, and marked by a clear delay in onset. To describe the sigmoidal current, traces were fit with an equation of the form

\[
I_{Ca} = I_{n} \left[ 1 - \exp \left( \frac{T}{\tau_a} \right) \right]^n \exp \left( -T / \tau_i \right),
\]

where \(\tau_a\) and \(\tau_i\) are activation and inactivation time constants and \(n\) is an integer between 1 and 4. Best fits were obtained with \(N\) fixed at 4. The activation time constant (\(\tau_a\)) varied from 2.39 ± 0.12 ms to 0.50 ± 0.08 ms (\(n = 21\)) at test voltages of −30 mV and 40 mV, respectively. The relationship between \(\tau_a\) and voltage could be fit with a single exponential that included an \(e\) fold change per 42.4 ± 3.41 mV (Fig. 2 A).

Human AZF cell T current inactivation kinetics was also voltage dependent, accelerated by stronger depolarizations, and reached a clear voltage-independent minimum. The inactivating component of T-type currents, recorded as described in the legend of Fig. 1 A at test voltages between −40 and 40 mV, was fit with a single exponential function. For test potentials ranging from −40 to −50 mV, \(\tau_i\) decreased smoothly with voltage until, at potentials between 0 and 40 mV, \(\tau_i\) approached a distinct voltage-independent minimum value (Fig. 2 B). The relationship expressing \(\tau_i\) as a function of voltage could be fit by a single exponential with an \(e\) fold change per 9.47 ± 1.15 mV (\(n = 11\)) and a voltage-independent offset of 18.31 ± 1.68 ms.

Among all Ca\(^{2+}\) channel subtypes, Ca\(^{3}\) channels are distinctive in their slow rate of closing upon repolarization (Armstrong and Matteson, 1985; Perez-Reyes, 2003). The voltage-dependent kinetics of Ca\(^{2+}\) channel closing in human AZF cells was determined after an activating voltage step by measuring the rate of tail current decay at repolarization potentials ranging from −40 to −130 mV. Decaying currents (Fig. 2 C) could be fit with a single exponential time constant (\(\tau_d\)) that decreased monotonically with repolarization voltage. \(\tau_d\) ranged from 6.55 ± 0.60 ms at −40 mV to 0.48 ± 0.06 ms (\(n = 6\)) at −130 mV. The function relating \(\tau_d\) to repolarization potential could be fit with a single exponential that included an \(e\) fold change in \(\tau_d\) per 36.4 mV (Fig. 2 C).

Ca\(^{2+}\) channel pharmacology. Among the three Ca\(^{3}\) channel subtypes, Ca\(^{3.1}\) is distinctive in its relatively slow kinetics of voltage-dependent activation and inactivation (Klöckner et al., 1999). The rapid activation and inactivation kinetics of the Ca\(^{3}\) current in human AZF cells suggest that it is either Ca\(^{3.2}\) or Ca\(^{3.3}\). In this regard, Ca\(^{3.2}\) channels are unique in their extreme sensitivity to block by Ni\(^{2+}\). Ni\(^{2+}\) inhibits Ca\(^{3.2}\) channels with reported IC\(_{50}\) values between 2 and 13 \(\mu\)M, whereas Ca\(^{3.1}\) and Ca\(^{3.3}\) channels are 15- to 20-fold less sensitive (Lee et al., 1999; Perez-Reyes, 2003). We found that Ni\(^{2+}\) reversibly inhibited the T-type Ca\(^{2+}\) current in human AZF cells with an IC\(_{50}\) of 3.0 \(\mu\)M, identifying it as Ca\(^{3.2}\) (Fig. 3 A).

Trivalent lanthanide elements are among the most potent inorganic inhibitors of T-type Ca\(^{2+}\) channels (Biagi and Enyeart, 1990; Lansman, 1990; Milnar and Enyeart, 1993a). Accordingly, gadolinium (Gd\(^{3+}\)) was ~10-fold more potent than Ni\(^{2+}\) as an inhibitor of Ca\(^{3}\) channels in human AZF cells, with an estimated IC\(_{50}\) of 245 nM (Fig. 3 B).

The combined results strongly suggest that Ca\(^{3.2}\) is the predominant Ca\(^{2+}\) channel expressed by human AZF cells. Although ACTH stimulates cortisol synthesis in bovine AZF cells through mechanisms that require Ca\(^{2+}\) entry through Ca\(^{3.2}\) Ca\(^{2+}\) channels, this peptide does not directly modulate the activity of T-type Ca\(^{2+}\) channels in these cells (Milnar et al., 1993b). At concentrations between 500 pM and 2 nM, ACTH failed to significantly alter the amplitude of the T-type Ca\(^{2+}\) current in human AZF cells (\(n = 7\); Fig. 3 C).

K\(^{+}\) currents: I\(_{k}\) current

In whole cell patch clamp recordings, a voltage-gated, rapidly inactivating K\(^{+}\) current was expressed by each of 64 human AZF cells examined. This robust I\(_{k}\) current was activated at potentials positive to −50 mV and averaged 2.397 ± 153 pA in amplitude at a test potential of 20 mV, with a mean current density of 95.0 ± 7.2 pA/pF (Fig. 4 A). Experiments were performed to characterize the voltage-dependent gating and kinetic properties of these channels.

I\(_{k}\): Voltage-dependent gating. The voltage dependence of I\(_{k}\) activation was measured by dividing the peak current amplitudes taken from the steady-state I-V relationship by corresponding amplitudes from the instantaneous I-V (II-V) relationship, as previously described (Milnar and Enyeart, 1993b). The II-V or open channel I-V provides a measure of membrane conductance at different potentials after all available channels have been activated by a short, strong depolarization. The II-V for I\(_{k}\) was obtained by activating channels with brief (5 ms) depolarizing steps to 50 mV, after which the membrane potential was stepped to new levels between 40 and −70 mV. K\(^{+}\) current was then measured after 1 ms, before a significant change in the number of open channels occurred (Fig. 4 B).

The values obtained upon dividing peak current amplitudes from the steady-state IV by corresponding amplitudes from the II-V were plotted as the fraction of open channels against membrane potential and fit by a Boltzmann function of the form

\[
\text{Fraction open} = 1 / \left[ 1 + \exp \left( \frac{v_{1/2} - v}{k} \right) \right],
\]

where \(v_{1/2}\) is the voltage at which half of the channels are in the open conformation and \(k\) is the slope factor (Fig. 4 C).
Curves were fit to data points acquired at test potentials between $-60$ and $0$ mV. The activation function had a midpoint of $-37.8$ mV and a $k$ of $7.56 \pm 0.02$ mV per $e$-fold change in the fraction open (Fig. 4 C).

The voltage-dependent steady-state inactivation of the A-type $K^+$ current was studied by applying 10-s conditioning pulses to potentials between $-90$ and $-25$ mV in 5-mV increments, followed by activating voltage steps to $20$ mV. The normalized current was plotted as a function of the conditioning voltage and fitted with the equation $I / I_{\text{MAX}} = 1 / \left[ 1 + \exp\left( v - v_{1/2} \right) / k \right]$, where $I_{\text{MAX}}$ was the current activated from a holding potential of $-90$ mV. $I_A$ inactivation varied as a steep function of voltage with $v_{1/2}$ equal to $-51.9 \pm 0.36$ mV ($n = 5$) and a slope factor of $3.66 \pm 0.31$ mV ($n = 5$) per $e$-fold change in the fraction inactivated (Fig. 4 D).

$I_A$: Voltage-dependent activation and inactivation kinetics. The voltage-dependent activation and inactivation kinetics of the human AZF cell $I_A$ current were characterized in whole cell recordings. For activation, current traces from I-V protocols were fit with an equation of the form $I = I_{\text{MAX}} \left[ 1 - \exp\left( -v / \tau_a \right) \right] / \left[ 1 + \exp\left( v - v_{1/2} \right) / k \right]$, where $\tau_a$, $\tau_i$, and $\tau_d$ are the activation and inactivation time constants, and $N$ is an integer between 1 and 4. Activation time constants (mean $\pm$ SEM) obtained at various potentials for 21 different cells were plotted as a function of test voltage and fit with a single exponential function. For inactivation, single exponential fits were made to data points acquired at test potentials between $-60$ and $0$ mV. The function relating $\tau_i$ and membrane voltage could be expressed as a single exponential with a $k$ of $7.56 \pm 0.62$ mV per $e$-fold change in the fraction inactivated (Fig. 4 C).
that the decaying component of scaled current traces recorded from a single cell at test potentials between \(-20\) and \(40\) mV was almost super-imposable, with nearly identical \(\tau_i\) values of \(34\) ms. In Fig. 5 C, averaged time constants determined from 15 separate cells are plotted against test voltages. Least squares linear regression analysis of these data yielded a line with a slope not significantly different from zero and an intercept of \(34.2 \pm 0.72\) ms.

**Noninactivating K⁺ current**

In addition to the prominent A-type K⁺ current that could be recorded in virtually every healthy human AZF cell, approximately half of these cells also expressed a significant (i.e., >50 pA) noninactivating K⁺ current that displayed properties of the K2P leak current TREK-1. Specifically, upon voltage clamping the cell at a holding potential of \(-80\) mV and applying voltage steps to \(20\) mV at regular intervals, the noninactivating current typically grew to a stable maximum amplitude over a period of 10–20 min (Fig. 6 A). The noninactivating current could be measured at the end of a 300-ms test pulse when \(I_A\) had completely inactivated. Alternatively, the noninactivating current could be observed in isolation by inactivating \(I_A\) with a 10-s depolarizing prepulse (Fig. 6 A, right traces). By itself, the leak-type current appeared to be largely instantaneous with little time- or voltage-dependent activation. This current was present at its highest density on the first day in culture and declined over a period of several days. Overall, this K⁺ current reached a maximum density of \(16.6 \pm 3.1\) pA/pF (\(n = 31\)) under our recording conditions.

Although the current in human AZF cells resembled TREK-1 expressed in other cells, including bovine AZF and AZG cells, it was important to distinguish it from other members of the K2P family, especially TASK channels, which appear to be the predominant background channel expressed in mouse and rat adrenal cortical cells (Czirják et al., 2000; Czirják and Enyedi, 2002; Enyeart et al., 2002; Enyeart et al., 2004). In this regard, of the
Forskolin effectively inhibited the leak-type $K^+$ current in human AZF cells, whereas the rapidly inactivating A-type current was unaffected (Fig. 6A). In seven cells, 5 µM forskolin inhibited the noninactivating $K^+$ current by 84.2 ± 4.2% when measured at test potentials 15 members of the K2P $K^+$ channels family, only TREK-1 and TREK-2 are inhibited by cAMP (Enyedi and Czirják, 2010). We used the diterpene adenylyl cyclase activator forskolin to determine whether the noninactivating $K^+$ current in human AZF cells was TREK-1.

Forskolin effectively inhibited the leak-type $K^+$ current in human AZF cells, whereas the rapidly inactivating A-type current was unaffected (Fig. 6A). In seven cells, 5 µM forskolin inhibited the noninactivating $K^+$ current by 84.2 ± 4.2% when measured at test potentials.

Figure 4. Voltage-dependent gating of A-type $K^+$ current in human AZF cells. (A) I-V relationship: Whole cell $K^+$ currents were activated by voltage steps to various test potentials applied at 30-s intervals from a holding potential of −80 mV. Peak current amplitudes are plotted against test potential from corresponding current traces shown at left. (B) Open channel I-V relationship: After activating $I_A$ with a 3-ms voltage step to 50 mV from a holding potential of −80 mV, membrane potential was stepped to various values between 40 and −70 mV where decaying currents were recorded. (C) Activation: The voltage dependence of $I_A$ channel activation was determined by dividing peak current amplitudes derived from the steady-state I-V by corresponding values from the II-V. These values were plotted as the fraction of open channels against voltage and fit by a Boltzmann function of the form $\text{Fraction open} = 1 / [1 + \exp(v_{1/2} - v) / k]$, 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. Curve was fit to data points at test potentials between −60 and 0 mV. (D) Inactivation: The voltage dependence of steady-state inactivation was measured by applying 10-s prepulses to potentials between −90 and −25 mV, followed by activating voltage steps to 20 mV. Normalized current (mean ± SEM) for six cells was plotted against conditioning voltage and fitted with the equation $I / I_{\text{MAX}} = 1 / [1 + \exp(v - v_{1/2}) / k]$, where $I_{\text{MAX}}$ is the current activated from a holding potential of −90 mV and $v_{1/2}$ is the potential at which half of the channels are inactivated.
of 20 mV. In other experiments, I-V relationships were obtained and showed that the noninactivating K⁺ current was outwardly rectifying and blocked by forskolin, although less effectively at test potentials positive to 20 mV (Fig. 6 B). Previously, it had been shown that TREK-1 inhibition by cAMP was voltage dependent and less effective at positive potentials (Bockenhauer et al., 2001). Collectively, these results strongly suggest that the noninactivating current expressed by human AZF cells is TREK-1.

**Activation of the noninactivating current by AA and CDC.** The noninactivating K⁺ current was not detectable in approximately one third of freshly plated human AZF cells. This current also spontaneously decreased when human AZF cells were maintained in culture over a period of several days, as previously reported for bTREK-1 channels in bovine AZF cells (Enyeart et al., 2003, 2010). In this regard, of the 15 K2P channels, TREK and TRAAK are distinctive in their activation by AA (Enyedi and Czirják, 2010). We found that AA markedly increased the noninactivating K⁺ current in human AZF cells even when this current was undetectable under control conditions. In the experiment illustrated in Fig. 7 A, the cell was in culture for 48 h before recording K⁺ currents. Only a large A-type K⁺ current was detectible during the first 15 min of recording. However, superfusing the cell with 20 µM AA rapidly inhibited the A-type current and triggered a slower increase in the noninactivating K⁺ current, which reached 2 nA after a 15-min exposure to AA. Overall, 20 µM AA increased the noninactivating K⁺ current density in human AZF cells from near zero to 125.6 ± 44.2 pA/pF (n = 5).

The caffeic acid derivative CDC has also been shown to activate native TREK-1 channels in bovine adrenocortical cells (Danthi et al., 2004). We found that 10–20 µM CDC markedly enhanced the activity of the noninactivating K⁺ current in human AZF cells, while also suppressing the voltage-gated A-type current. In the experiment illustrated in Fig. 7 B, when CDC was applied to a cell, it increased the current amplitude from an undetectable level to >3,000 pA within 5 min. Overall, 10 or 20 µM CDC...
CDC increased the noninactivating current density from nearly zero to 40.9 ± 9.7 pA/pF \((n = 7)\).

The results of experiments with AA and CDC suggested that these agents activated TREK-1 channels, which remained present in the cells but had become inactive after several days in culture. If so, then the K2P current activated by either of these agents could be inhibited by forskolin only if it were TREK. The experiment illustrated in Fig. 7 C shows that forskolin effectively inhibited the current activated by 10 µM CDC. Similar results were obtained in three experiments.

**TREK-1 and membrane potential.** The activation of the noninactivating K⁺ current by AA and its inhibition by forskolin were tightly coupled to the membrane potential measured under current clamp. In the experiment illustrated in Fig. 7 D, the inhibition of the putative TREK-1 current by forskolin depolarized the cell by 34 mV, whereas subsequent activation of this current by AA produced a 42-mV hyperpolarization. In a total of five AZF cells, the activation of the noninactivating K⁺ current by AA increased the membrane potential by \(-37.0 ± 4.2\) mV \((n = 5)\). In contrast, inhibition of this current by forskolin depolarized AZF cells by 30.3 ± 4.2 mV \((n = 3)\).

**Inhibition of the nonactivating current by ACTH.** ACTH stimulates cortisol secretion by activation of a Gs-coupled MC2R melanocortin receptor, leading to the activation of adenylate cyclase (Mountjoy et al., 1992). If the noninactivating K⁺ current in human AZF cells is caused by TREK, then it should be inhibited by ACTH. The putative TREK-1 current expressed by human AZF cells was potently and effectively inhibited by ACTH (Fig. 8 A). At concentrations of 500 pM and 1 nM, ACTH inhibited the nonactivating current by 90.2 ± 4.9% \((n = 6)\) and 86.0 ± 4.8% \((n = 5)\), respectively. In other experiments, ACTH produced near complete inhibition of the nonactivating current at concentrations as low as 20 pM. Inhibition of this current by ACTH was voltage independent over a wide range of test potentials.
the cell with 1 nM ACTH resulted in near complete inhibition of the remaining noninactivating current. Overall, in nine cells, 5 or 10 nM AngII inhibited the noninactivating K+ current by 72.4 ± 6.6%, without any measurable effect on the A-type K+ current. These results indicate that a large fraction of the ACTH-sensitive K+ current expressed by human AZF cells is also inhibited by AngII and further suggest that this current is TREK-1.

In the experiment illustrated in Fig. 8 B, 1 nM ACTH selectively and completely inhibited the nonactivating K+ current at potentials from −50 to 40 mV.

Inhibition of the noninactivating K+ current by AngII. Although ACTH is the principal peptide hormone regulating cortisol secretion, AngII may also stimulate secretion in some species, including humans (Lebrethon et al., 1994). AngII inhibits multiple K2P channels, including TREK-1, through activation of a Gq-coupled AT1 receptor (Enyeart et al., 2005; Enyedi and Czirják, 2010). Accordingly, we found that AngII also inhibited the nonactivating K+ current in human AZF cells, although it was less effective than ACTH. In the experiment shown in Fig. 9, 10 nM AngII selectively inhibited the nonactivating K+ current by 57%, without altering the rapidly inactivating A-type current. Subsequent superfusion of the cell with 1 nM ACTH resulted in near complete inhibition of the remaining nonactivating current. Overall, in nine cells, 5 or 10 nM AngII inhibited the nonactivating K+ current by 72.4 ± 6.6%, without any measurable effect on the A-type K+ current. These results indicate that a large fraction of the ACTH-sensitive K+ current expressed by human AZF cells is also inhibited by AngII and further suggest that this current is TREK-1.

Northern blot analysis of human AZF ion channel mRNAs. Whole cell patch clamp experiments indicated that human AZF cells express at least three specific ion channels that we tentatively identified as Ca3.2, Kv1.4, and TREK-1. Northern blot analysis showed that mRNA transcripts coding for each of these channels were robustly expressed by human adrenocortical cells (Fig. 10).

Figure 7. AA and CDC activate the nonactivating K+ current in human AZF cells. Whole cell recordings of K+ currents were made from human AZF cells that had been in culture for 48–72 h. After recording K+ currents in standard saline, cells were superfused with AA or CDC. (A) Effect of AA. K+ currents were activated by voltage steps to 20 mV applied at 30-s intervals from a holding potential of −80 mV. After 15 min, the cell was superfused with 20 µM AA. Numbers on current traces correspond to those in the plot of current amplitudes against time at right. (B) Effect of CDC. K+ currents were activated as described in A. After 15 min, the cell was superfused with 20 µM CDC. Traces show currents at times indicated after the superfusion of CDC. Nonactivating current amplitudes are plotted against time in the graph at right. (C) Forskolin inhibits CDC-activated current. K+ currents were activated as described in A. Currents were initially recorded in saline, then in 10 µM CDC, followed by CDC and 5 µM forskolin, as shown. Numbers on traces correspond to those on the plot of nonactivating current amplitudes at right. (D) K+ current expression and membrane potential. K+ currents and membrane potential were measured at 30-s intervals by switching between voltage clamp and current clamp. The cell was superfused with 5 µM forskolin and 20 µM AA at the indicated times. Numbers on traces in the left panel correspond to those in the plot of current amplitudes at right.
AngII, as well as ACTH, stimulate cortisol secretion from human AZF cells. However, in contrast to ACTH, which produced both rapid and delayed increases, AngII significantly increased the quantity of cortisol produced only after delays exceeding 2 h (Fig. 11, A and B). Overall, the effectiveness of the two peptides in stimulating cortisol secretion paralleled their effectiveness as inhibitors of the noninactivating K⁺ current in these cells. In

Figure 9. AngII selectively inhibits the noninactivating K⁺ current in human AZF cells. K⁺ currents were activated by voltage steps to 20 mV applied at 30-s intervals from a holding potential of −80 mV with (right traces) or without (left traces) 10-s prepulses to −20 mV. When the noninactivating current reached a stable maximum, the cell was superfused with 1 nM ACTH. Current amplitudes are plotted against time in the absence (closed circles) and presence (open circles) of ACTH in the graph at right.

**Figure 8.** ACTH inhibits the noninactivating K⁺ current in human AZF cells. (A) ACTH selectively inhibits noninactivating current. K⁺ currents were activated by voltage steps to 20 mV applied at 30-s intervals from a holding potential of −80 mV with (right traces) or without (left traces) 10-s prepulses to −20 mV. When the noninactivating current reached a stable maximum, the cell was superfused with 1 nM ACTH. Current amplitudes are plotted against time at right. (B) Voltage-independent inhibition by ACTH. After the noninactivating K⁺ current reach a maximum, K⁺ currents were activated by voltage steps applied at 30-s intervals from a holding potential of −80 mV to test potentials between −60 and 40 mV. I-V relationships were recorded before (left traces) and after (right traces) superfusing the cell with 1 nM ACTH. Noninactivating current amplitudes are plotted against voltage in the absence (closed circles) and presence (open circles) of ACTH in the graph at right.

**Effect of ACTH, AngII, and CDC on cortisol secretion.** The inhibition of the noninactivating current in human AZF cells by ACTH and AngII suggests that both of these peptides could stimulate cortisol secretion by human AZF cells through a mechanism requiring depolarization-dependent Ca²⁺ entry. We compared the effect of ACTH and AngII on cortisol secretion by human AZF cells in culture at times ranging from 2 to 24 h. We found that
At a concentration of 2.5 µM, mibefradil inhibited Ca\textsuperscript{2+} channels (Mishra and Hermsmeyer, 1994). At a concentration of 2.5 µM, mibefradil inhibited the Ca\textsuperscript{2+} channel present in both bovine and human AZF cells. In both cells, TREK-1 channels are potently and selectively inhibited by ACTH at concentrations that stimulate cortisol secretion. AngII also inhibited the noninactivating K\textsuperscript{+} current and stimulated delayed increases in cortisol secretion from human AZF cells. The activation and inhibition of TREK-1 in human AZF cells were shown to be coupled to membrane hyperpolarization and depolarization, respectively. Mibefradil inhibited Ca\textsubscript{3.2} Ca\textsuperscript{2+} currents as well as ACTH- and AngII-stimulated cortisol secretion at identical concentrations. In addition to identifying the major ion channels expressed by human AZF cells and providing the first description of the modulation of one of these channels by the peptide hormones that physiologically regulate cortisol secretion, these results suggest a specific mechanism wherein ACTH and AngII receptor activation is coupled to depolarization-dependent Ca\textsuperscript{2+} entry and secretion by the inhibition of TREK-1 channels.

**Comparison of human, bovine, and rodent AZF cell ion currents**

Bovine, rat, and mouse AZF cells have been used as models for human cortisol secretion (Simpson and Waterman, 1988; Waterman, 1994). Because ion channels act pivotally in the physiology of cortisol secretion, the relevance of the model systems to human AZF cell secretion rests on the similarity of their ion channels. The ion channels of normal bovine and rat AZF cells, and a mouse AZF cell line, have been described in patch clamp studies. Although bovine AZF cell ion channels are remarkably similar to those of humans, channels expressed in rodent AZF cells are quite different. Specifically, rat AZF cells express only voltage-gated T- and L-type Ca\textsuperscript{2+} channels and a slowly inactivating A-type K\textsuperscript{+} current, whereas no leak-type current similar to TREK-1 is detectable (Barbara and Takeda, 1995). Mouse Y1 adrenocortical cells express only a Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel and two types of voltage-gated Ca\textsuperscript{2+} currents (Tabaeres et al., 1985).

In contrast to rat and mouse, bovine AZF cells appear to express the same three ion channels as normal human cells. Importantly, TREK-1 is the primary or sole K2P channel present in both bovine and human AZF cells.
The Ca\(^{2+}\) and K\(^{+}\) currents that we have identified in human AZF cells differ fundamentally from those described in a previous study on human AZG cells (Payet et al., 1994). Specifically, in the AZG study, a large non-inactivating Ca\(^{2+}\) current that inexplicably reached a maximum amplitude at 70 mV was reported in addition to a slowly activating voltage-gated delayed rectifier K\(^{+}\) current. No K\(^{+}\) current resembling TREK-1 was observed. Voltage-gated Ca\(^{2+}\) channels in human AZF cells

Whole cell patch clamp recordings showed that human AZF cells express predominantly or exclusively low voltage-activated Ca\(_{3.2}\) T-type Ca\(^{2+}\) channels. These channels resembled native and cloned Ca\(_{3.2}\) channels with respect to voltage-dependent gating and kinetic properties and pharmacology (Chen and Hess, 1990; Mlinar et al., 1993b; Perez-Reyes et al., 1998; Klöckner et al., 1999). In particular, the potent inhibition of the T-type Ca\(^{2+}\) current by Ni\(^{2+}\) (IC\(_{50}\) = 3.0 µM) clearly identified this current as Ca\(_{3.2}\) among the three Ca\(_{3}\) subtypes. In contrast, Ca\(_{3.1}\) and Ca\(_{3.3}\) are blocked by Ni\(^{2+}\) with IC\(_{50}\) values > 100 µM (Lee et al., 1999). The T-type Ca\(^{2+}\)
observed in bovine cells, inactivation kinetics of the human A-type current was independent of voltage over a wide range of test potentials. Furthermore, the human and bovine A-type currents both inactivate rapidly with similar time constants (Mlinar and Enyeart, 1993b). The A-type K\textsuperscript{+} currents of bovine and human AZF cells are inhibited similarly by CDC and AA (Danthi et al., 2003, 2004). Overall, our patch clamp experiments and Northern blot results indicate that the A-type K\textsuperscript{+} current in human AZF cells is Kv1.4. In contrast, the A-type K\textsuperscript{+} current in rat AZF cells inactivates 10 to 20 times more slowly than that of human and bovine cells and is therefore not likely Kv1.4 (Barbara and Takeda, 1995). The function of these large A-type K\textsuperscript{+} currents in human AZF cells has not been determined. In vertebrates, A-type K\textsuperscript{+} currents are present in excitable cells, ranging from cardiac and smooth muscle cells to peripheral and central neurons where they regulate action potential spacing and duration (Belluzzi et al., 1985; Clark et al., 1988; Cooper and Shrier, 1989; Imaizumi et al., 1990; Ficker and Heinemann, 1992). Although AZF cells are not channels of humans and bovine AZ cells are clearly different from those of rats, which are blocked by Ni\textsuperscript{2+} with an IC\textsubscript{50} of 132 µM (Durroux et al., 1988).

In human AZF cells, Ca\textsubscript{3.2} channels probably provide the major portal for depolarization-dependent Ca\textsuperscript{2+} entry. However, it does not appear that ACTH enhances Ca\textsuperscript{2+} entry through direct modulation of these channels because this peptide had no effect on the Ca\textsubscript{3.2} current. Accordingly, ACTH does not enhance the Ca\textsubscript{3.2} current in either bovine or rat AZF cells (Mlinar et al., 1993b; Barbara and Takeda, 1995).

Voltage-gated A-type K\textsuperscript{+} current in human AZF cells
The voltage-gated, rapidly inactivating A-type current was the largest current present in virtually every human AZF cell examined. This A-type current resembled the Kv1.4 current in bovine AZF cells with respect to voltage-dependent gating and kinetic properties (Mlinar and Enyeart, 1993b). In particular, activation kinetics was voltage dependent but reached a voltage-independent offset at potentials positive to 0 mV. However, as previously observed in bovine cells, inactivation kinetics of the human A-type current was independent of voltage over a wide range of test potentials. Furthermore, the human and bovine A-type currents both inactivate rapidly with similar time constants (Mlinar and Enyeart, 1993b). The A-type K\textsuperscript{+} currents of bovine and human AZF cells are inhibited similarly by CDC and AA (Danthi et al., 2003, 2004). Overall, our patch clamp experiments and Northern blot results indicate that the A-type K\textsuperscript{+} current in human AZF cells is Kv1.4. In contrast, the A-type K\textsuperscript{+} current in rat AZF cells inactivates 10 to 20 times more slowly than that of human and bovine cells and is therefore not likely Kv1.4 (Barbara and Takeda, 1995).

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typically classified as excitable, the presence of action potential–like waveforms from AZF cells of several species suggests that this form of electrical activity could be present in the intact gland under physiological conditions (Matthews and Saffran, 1968, 1973; Natke and Kabela, 1979; Lymangrover et al., 1982; Quinn et al., 1987; Barbara and Takeda, 1995). In the absence of action potentials, this robust current has no obvious purpose in the human AZF.

Background K⁺ channels in human AZF cells
The noninactivating K⁺ current expressed in human AZF cells displayed several properties which, collectively, identify it as TREK-1. First, in whole-cell recordings where the membrane potential was held at −80 mV, the noninactivating current grew to a stable maximum value over a period of minutes. When human or mouse TREK-1 channels are heterologously expressed in *Xenopus laevis* oocytes, TREK-1 activity can be increased or decreased over a period of minutes by, respectively, hyperpolarizing or depolarizing the cell membrane (Segal-Hayoun et al., 2010; Bagriantsev et al., 2012). The molecular mechanism that underlies the slow voltage-dependent modulation of open probability is currently unknown, but the phenomenon appears to be specific to TREK among the K2P channels.

Similar to TREK-1 currents in other cells including bovine AZF, the human noninactivating current included a large instantaneous component that was outwardly rectifying. Furthermore, the selective and near complete inhibition of the noninactivating K⁺ current in human AZF cells by forskolin and ACTH provides additional evidence that the noninactivating current is TREK-1 and that this channel is the predominant K2P channel expressed by these cells. In this regard, the effective inhibition of the noninactivating K⁺ current by forskolin provides convincing evidence that this current is either TREK-1 or TREK-2 because these are the only K2P channels inhibited by cAMP-dependent pathways (Enyedi and Czirják, 2010). Because TREK-2 does not appear to be expressed in the adrenal cortex (its EST profile on Unigene is reported as 0/32935 in the human adrenal), it is highly likely that the human AZF channel is TREK-1 (Lesage et al., 2000).

It was also observed that forskolin was less effective at inhibiting the noninactivating K⁺ current at potentials positive to 20 mV. Accordingly, it was previously reported that TREK-1 inhibition by cAMP is voltage dependent and less effective at positive potentials (Bockenhauer et al., 2001). In contrast to forskolin, the inhibition of TREK-1 by ACTH was voltage independent. In this regard, ACTH may produce effects in bovine AZF cells through multiple cAMP-dependent and -independent mechanisms (Omura et al., 2007; Liu et al., 2010).

The remarkable increase in the amplitude of the noninactivating K⁺ current stimulated by AA and CDC in human AZF cells provided further evidence that this current is TREK-1 and indicates that these cells express thousands of these channels. Of the K2P family, AA activates only the mechano-gated subgroup comprised of TREK-1, TREK-2, and TRAAK channels, and CDC has only been shown to activate TREK-1 (Danthi et al., 2004; Enyedi and Czirják, 2010). Accordingly, the inhibition of the CDC-activated K⁺ current in human cells by forskolin argues that it is TREK-1.

It is not clear why human AZF cells express leak-type channels at this high density when only a small fraction of these might be sufficient to establish a resting potential near the K⁺ equilibrium potential. Regardless, experiments with CDC and AA indicated that TREK-1 is the predominant K2P channel expressed in human AZF cells. They further showed that after AZF cells are maintained in culture for several days, their TREK-1 channels can exist in a dormant form that can still be activated by CDC or AA.

Inhibition of the noninactivating K⁺ current by ACTH and AngII
The inhibition of the noninactivating K⁺ current by ACTH and AngII is the first demonstration of the modulation of a specific ion current by either of these two peptide hormones in normal human AZF cells and provides additional proof that this current is TREK-1. TREK channels are the only K2P channels inhibited by the activation of both Gₛ and Gₛ₇-coupled receptors. In bovine AZF cells, ACTH activates a Gₛ-coupled MC²R receptor, leading to TREK-1 inhibition through PKA-dependent phosphorylation and perhaps other cAMP-dependent pathways (Mountjoy et al., 1992; Liu et al., 2008). The near complete inhibition of the noninactivating K⁺ current further argues that TREK-1 is the predominant leak-type K⁺ channel in human AZF cells.

In our experiments, the inhibition of noninactivating K⁺ current by ACTH and AngII was measured under identical conditions, with the pipette solutions containing 2 mM MgATP and [Ca²⁺]₁, strongly buffered by 11 mM BAPTA. Therefore, inhibition by either peptide was likely independent of any increases in [Ca²⁺]₁ (Augustine et al., 2003; Fakler and Adelman, 2008). In this regard, in bovine AZF cells, AngII inhibits TREK-1 currents by separate Ca²⁺- and ATP hydrolysis–dependent pathways (J.J. Enyeart et al., 2005, 2011; Liu et al., 2007). It will be interesting to determine whether AngII inhibits the noninactivating K⁺ current in human cells by dual signaling pathways.

TREK-1 K⁺ channels, membrane potential, and cortisol secretion
Experiments that showed a close correlation between the expression of TREK-1 current and membrane potential suggested a model for ACTH- and AngII-stimulated cortisol secretion by human AZF cells that relies on
depolarization-dependent Ca\textsuperscript{2+} entry. Accordingly, in secretion experiments, we showed that the selective T-type Ca\textsuperscript{2+} channel antagonist mibefradil effectively inhibited Ca\textsubscript{3.2} channels and cortisol secretion stimulated by either ACTH or AngII. Furthermore, CDC inhibited cortisol secretion stimulated by these two peptide hormones at the same concentration that markedly increased TREK-1 activity. In previous studies, we found similar effects of these two agents on Ca\textsubscript{3.2}, TREK-1, and cortisol secretion in bovine AZF cells (Gomora et al., 2000; Danthi et al., 2004).

Overall, the results of patch clamp and secretion studies suggest a model for cortisol secretion by human AZF cells in which ACTH and AngII receptor activation is coupled to depolarization and the activation of Ca\textsubscript{3.2} channels through TREK-1 inhibition. In this regard, the action potential-like waveforms that have been recorded from AZF cells of several species suggest that, under physiological conditions in the intact adrenal gland, these cells are excitable. It is possible that TREK-1 inhibition induces Ca\textsuperscript{2+}-dependent action potentials in human AZF cells, driven by opposing Kv1.4 and slowly deactivating Ca\textsubscript{3.2} currents. However, in current clamp recordings from dissociated human AZF cells in culture, we have not detected the presence of action potentials.

If human AZF cells do not generate action potentials, it is not clear how the strong depolarization produced by ACTH and AngII through TREK-1 inhibition leads to sustained Ca\textsuperscript{2+} entry via the rapidly inactivating Ca\textsubscript{3.2} channels and subsequent prolonged increases in cortisol secretion. It is possible that, at concentrations sufficient to only partially inhibit TREK-1, ACTH and AngII depolarize cells to a potential where a sustained “window” current through Ca\textsubscript{3.2} persists.

The function of the large Kv1.4 current expressed by nearly every human AZF cell remains to be clarified. This K’ current, averaging one to several nanoamperes in nearly every human AZF cell remains to be clarified. This K’ current, averaging one to several nanoamperes in amplitude, by rapidly repolarizing the AZF cells at a rate of 100 mV/\textmu s or more, could provide for the repolarization phase of a Ca\textsuperscript{2+}-dependent action potential. Alternatively, in response to TREK-1 inhibition, the opposing Kv1.4 and Ca\textsubscript{3.2} currents could generate an oscillatory membrane potential resulting in a sustained Ca\textsuperscript{2+} influx. Ultimately, the endogenous electrical activity of human AZF cells could be more reliably determined in recordings from intact tissue, such as in an adrenal slice.

In humans, glucocorticoids regulate physiological processes, ranging from energy metabolism to memory consolidation (Stewart and Krone, 2011; Chen et al., 2012). Chronic excessive cortisol secretion as that which occurs in Cushing’s disease or prolonged stress produces systemic as well as central nervous system toxicity, including hippocampal damage and memory impairment (Lupien et al., 1998; Kaouane et al., 2012). Knowledge of human AZF cell ion channels, including their gating and modulation by ACTH and AngII, will be necessary to better understand the physiology and pathophysiology of cortisol secretion and may be useful in identifying new therapeutic targets to modulate aberrant secretion in disease states.

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