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Corticotropin Induces the Expression of TREK-1 mRNA and K^+ Current in Adrenocortical Cells

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ABSTRACT

Bovine adrenal zona fasciculata (AZF) cells express a two-pore/four-transmembrane segment bTREK-1 K^+ channel that sets the resting potential and couples hormonal signals to depolarization-dependent Ca^{2+} entry and cortisol secretion. It was discovered that corticotropin (1–2000 pM) enhances the expression of bTREK-1 mRNA and membrane current in cultured AZF cells. Forskolin and 8-pcpt-cAMP mimicked corticotropin induction of bTREK-1 mRNA, but angiotensin II (AII) was ineffective. The induction of bTREK-1 mRNA by corticotropin was partially blocked by the A-kinase antagonist H-89. 8-(4-Chloro-phenylthio)-2-*O*-methyladenosine-3′-5′-cyclic monophosphate, a cAMP analog that activates cAMP-regulated guanine nucleotide exchange factors (Epac), failed to increase bTREK-1 mRNA. Corticotropin-stimulated increases in bTREK-1 mRNA were eliminated by inhibitors of protein synthesis or gene transcription. bTREK-1 current disappeared after 24 h in serum-supplemented medium, but in the presence of corticotropin, bTREK-1 expression was maintained for at least 48 h. The enhancement of bTREK-1 mRNA and ionic current contrasts with the corticotropin-induced down-regulation of the Kv1.4 voltage-gated K^+ current and associated mRNA in AZF cells. These results demonstrate that corticotropin rapidly and potently induces the expression of bTREK-1 in AZF cells at the pretranslational level by a cAMP-dependent mechanism that is partially dependent on A-kinase but independent of Epac and Ca^{2+}. They further indicate that prolonged stimulation of AZF cells by corticotropin, as occurs during long-term stress or disease, may produce pronounced changes in the expression of genes encoding ion channels, thereby reshaping the electrical properties of these cells to enhance or limit cortisol secretion.

Although cortisol-secreting cells of the adrenal zona fasciculata (AZF) lack Na^+-dependent action potentials, a hallmark of excitable cells, ion channels play a prominent role in the physiology of cortisol secretion (Enyeart et al., 1993, 1996b; Mlinar et al., 1993a). AZF cells express both voltage-gated Ca^{2+} and K^+ channels and maintain resting membrane potentials near the K^+ equilibrium potential (Mlinar et al., 1993a,b; Mlinar and Enyeart, 1993). Corticotropin- and AII-stimulated cortisol secretion by AZF cells involves depolarization-dependent Ca^{2+} entry through Ca^{2+} channels (Enyeart et al., 1993; Mlinar et al., 1993a,b).

Ion channels in adrenal cortical cells have been identified and characterized using a combination of patch clamp and molecular methods. In addition to low voltage-activated T-type Ca^{2+} channels, bovine AZF cells express two types of K^+-selective channels. These include a voltage-gated, rapidly inactivating Kv1.4 K^+ channel and a noninactivating, background K^+ channel (Mlinar et al., 1993a; Mlinar and Enyeart, 1993; Enyeart et al., 2002). Recently, we identified the background channel as bTREK-1 (or KCNK2), a member of the two-pore, four-transmembrane-segment (2P/4TMS) family of K^+ channels (Fink et al., 1996; Maingret et al., 2000; Goldstein et al., 2001; Enyeart et al., 2002).

This bTREK-1 K^+ channel sets the resting potential of AZF cells and is potently inhibited by corticotropin and AII at concentrations identical to those that trigger membrane depolarization and cortisol secretion (Enyeart et al., 1993, 1996b; Mlinar et al., 1993a, 1995). Unlike cloned TREK-1 channels, bTREK-1 channels in their native environment are activated by intracellular ATP at physiological concentrations (Enyeart et al., 1997; Xu and Enyeart, 2001) and inhibited by Ca^{2+} (Gomora and Enyeart, 1998). The properties of bTREK-1 K^+ channels that allow them to set the resting membrane potential and to couple hormonal and metabolic signals with depolarization-dependent Ca^{2+} entry indicate a

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ABBREVIATIONS: AZF, bovine adrenal zona fasciculata; 2P/4TMS, two-pore/four-transmembrane segment; AII, angiotensin II; PBS, phosphate-buffered saline; 8-pcpt-cAMP, 8-(4-chlorophenylthio)-cAMP; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; 8-CPT-2-O-Me-cAMP, 8-(4-chloro-phenylthio)-2-O-methyl-cAMP; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); DMEM/F12+, Dulbecco's modified Eagle's medium/Ham's F12 medium (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; SSPE, standard saline citrate/phosphate/EDTA; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

pivotal role for these channels in the physiology of cortisol secretion.

In addition to its rapid effects on the activity of AZF cell ion channels and steroidogenic enzymes, corticotropin also produces more enduring effects by altering the expression of selected genes. Specifically, corticotropin and its primary second messenger cAMP induce the transcription of steroidogenic enzymes as well as immediate early genes (John et al., 1986; Zuber et al., 1986; Parker and Schimmer, 1995; Enyeart et al., 1996a).

Recently, we discovered that corticotropin and cAMP rapidly reduce the expression of bKv1.4 K^+ channel mRNA and associated membrane current in bovine AZF cells (Enyeart et al., 2000). This result suggests that corticotropin could potentially produce sustained changes in the electrical and secretory activity of AZF cells by altering the expression of genes that encode K^+ channel proteins. We now report that corticotropin induces the expression of bTREK-1 mRNA and associated K^+ channels in these same cells by a mechanism that requires protein synthesis and gene transcription.

Materials and Methods

Materials

Tissue culture media, antibiotics, fibronectin, fetal bovine sera, formamide, and salmon sperm DNA were obtained from Invitrogen (Carlsbad, CA). Genescreen+ hybridization transfer membrane and [α-^{32}P]dCTP were from PerkinElmer Life Sciences (Boston, MA). Culture dishes were purchased from Corning (Corning, NY) Coverslips were from Bellco (Vineland, NJ). Phosphate-buffered saline (PBS), enzymes, corticotropin (1–24), AII, 8-pcpt-cAMP, forskolin, H-89, chloroform, dextran sulfate, and formaldehyde were from Sigma (St. Louis, MO). 8-CPT-2-O-Me-cAMP was purchased from Alexis Biochemicals (San Diego, CA). Cycloheximide was obtained from Calbiochem (La Jolla, CA). 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was from Biomol (Plymouth Meeting, PA). Mibefradil was a gift from Dr. George Billman (Physiology and Cell Biology, College of Medicine and Public Health, The Ohio State University, Columbus, OH). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, ULTRAhyb, RNA*later*, and Poly(A)pure were from Ambion (Austin, TX). RNeasy columns for total RNA isolation were obtained from QIAGEN (Valencia, CA). Probes were labeled with [^{32}P]dCTP by random priming (Prime-It II kit; Stratagene, La Jolla, CA). A full-length bTREK-1 cDNA (1414 base pairs) was obtained from the *Bst*X1 and *Hin*dIII digest of a bTREK-1-pCR3.1-Uni plasmid that was constructed as described previously (Enyeart et al., 2002). A 1.3-kb cDNA for bKv1.4 was obtained as described previously (Enyeart et al., 2000) and included transmembrane segments S1 through S6 of the bovine Kv1.4 K^+ channel.

Methods

Isolation and Culture of AZF Cells. Bovine adrenal glands were obtained from steers (age range, 2 to 3 years) within 1 h 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 AZF cells were obtained and prepared as described previously (Enyeart et al., 1997). After isolation, cells were either resuspended in DMEM/Ham's F12 medium (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% dimethyl sulfoxide, divided into 1-ml aliquots, each containing about 4×10^6 cells, and stored in liquid nitrogen for future use. Approximately 120×10^6 cells were obtained by enzymatic dissociation of six bovine adrenal glands. Cells were plated in either 60-mm dishes or 35-mm dishes containing 9-mm^2 glass coverslips. Dishes or coverslips were treated with fibronectin (10 μg/ml) at 37°C for 30 min 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 and 5% CO_2.

Measurement of bTREK-1 mRNA. AZF cells were plated on 60-mm fibronectin-treated dishes in DMEM/F12+ at a density of 6 to 8×10^6 cells/dish. After 24 h, the serum-supplemented media were removed and replaced with either control media (DMEM/F12+) or the same media containing corticotropin (1–24), or other agents, as required. At the end of the incubation period, total RNA was extracted using RNAeasy columns (QIAGEN). RNA concentrations were determined by absorbance at 260 nm. For Northern blot assays, RNA (12 μg) was separated by agarose (1%) gel electrophoresis in the presence of 5% formaldehyde and transferred to nylon transfer membrane (GeneScreen Plus; PerkinElmer Life Sciences). Full-length bTREK-1 cDNA was used to detect bTREK-1 mRNA expression. cDNA probes were labeled with [α-^{32}P]dCTP by random primer labeling (Prime-It II). Blots were prehybridized in heat-sealable plastic bags for 2 h at 42°C in either ULTRAhyb (Ambion) for bTREK-1 or bKv1.4 probing or 50% formamide, 5× SSPE, 5× Denhardt's solution, 10% dextran sulfate, 1% SDS, and 100 μg/ml salmon sperm DNA (Invitrogen) for hybridization with GAPDH probe. Blots were hybridized overnight in a minimal volume of hybridization solution. After 15 to 18 h, they were washed twice at room temperature in 2× SSPE for 15 min, then twice at 40°C in 1× SSPE for 30 min. Membranes probed with bTREK-1 or bKv1.4 were further washed with 0.1× SSPE and 1% SDS at 65°C for 30 min. Autoradiograms were obtained by exposing the blots for 5 to 24 h to Kodak X-O-Mat AR film (Eastman Kodak, Rochester, NY) at −70°C. RNA was quantitated using ImageQuant 4.0 software (Amersham Biosciences, Piscataway, NJ). Value for bTREK-1 mRNA was obtained by averaging 3.6- and 2.8-kb transcript area values. Values for bTREK-1 and bKv1.4 mRNA were normalized to GAPDH mRNA value for the same gel lane.

Patch-Clamp Experiments. Patch-clamp recordings of K^+ channel currents were made in the whole-cell configuration. The standard pipette solution was 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, 11 mM BAPTA, 200 μM GTP, and 5 mM MgATP with pH buffered to 7.2 using KOH. The external solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4 buffered to using NaOH. All solutions were filtered through 0.22-μm cellulose acetate filters.

AZF cells were used for patch-clamp experiments 1 to 48 h after plating. Typically, cells with diameters of <15 μm and capacitances of 8 to 15 pF were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml), which was continuously perfused by gravity at a rate of 3 to 5 ml/min. Patch electrodes with resistances of 2 to 3 MΩ were fabricated from Corning 0010 glass (World Precision Instruments, Sarasota, FL). These routinely yielded access resistances of 4 to 6 MΩ and voltage-clamp time constants of less than 100 μs. K^+ currents were recorded at room temperature (22–25°C) following the procedure of Hamill et al. (1981) using an List EPC-7 patch-clamp amplifier. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

Pulse generation and data acquisition were done using a personal computer and pCLAMP software with a TL-1 interface (Axon Instruments, Inc., Union City, CA). Currents were digitized at 5 to 20 kHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using scaled hyperpolarizing steps of 0.25 to 0.33 amplitude. Data were analyzed and plotted using pCLAMP 5.5 and 6.04 (Clampan and Clampfit) and SigmaPlot 8.0 (SPSS Science, Chicago, IL).

Results

Effect of Corticotropin on AZF Cell K^+ Channel mRNA. In Northern blots using RNA isolated from AZF cells, a 1414-base pair cDNA probe that included the entire coding sequence of bTREK-1 hybridized to separate mRNA transcripts of ~4.9, 3.6, and 2.8 kb. Corticotropin was found to markedly increase the expression of bTREK-1 mRNA by cultured AZF cells. In the experiment illustrated in Fig. 1, AZF cells were cultured overnight in serum-supplemented media before exposing them to corticotropin (2 nM) for periods of 1 to 24 h. In unstimulated cells, bTREK-1 mRNA was weakly expressed at each of four time points. In the presence of 2 nM corticotropin, bTREK-1 mRNA had increased 3.4-, 7.4-, and 21.2-fold by 5, 10, and 24 h in comparison to time-matched control values. Whereas each of the three transcripts was induced by corticotropin, the smaller 3.6- and 2.8-kb transcripts were preferentially increased (Fig. 1). Dramatic corticotropin-induced increases in bTREK-1 expression were observed in three separate experiments, with the average increase after 5-h exposure measuring 5.6 ± 1.5-fold.

The corticotropin-induced increase in bTREK-1 mRNA occurred nearly simultaneously in the same cells with the rapid reciprocal time-dependent decrease in bKv1.4 mRNA described previously (Enyeart et al., 2000). The experiment illustrated in Fig. 2 shows that when AZF cells are maintained in culture for 24 h in serum-supplemented media before isolating RNA, mRNA coding for bKv1.4 is prominently expressed, whereas bTREK-1 mRNA is nearly undetectable. However, after a 3-h exposure to 1 nM corticotropin, bTREK-1 mRNA has increased 5-fold, whereas bKv1.4 has decreased to 30% of the control value. By 5 h, the relative expression of bTREK-1 and bKv1.4 transcripts has reversed, compared with their initial values. bTREK-1 mRNA has increased 7.3-fold in this experiment, whereas bKv1.4 mRNA has decreased to an almost undetectable value (Fig. 2, A and B).

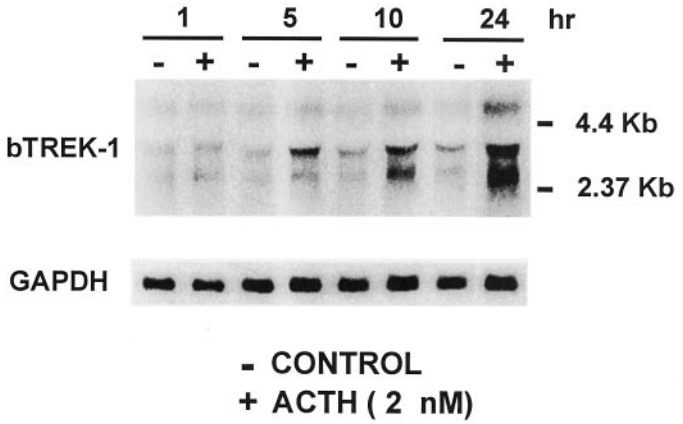


Fig. 1. Time-dependent effect of corticotropin on bTREK-1 mRNA expression. The temporal pattern of corticotropin-stimulated increase in bTREK-1 mRNA expression was monitored at times ranging from 1 to 24 h. Bovine AZF cells were cultured as described under *Materials and Methods* for 24 h after which time the media was replaced by the same media with (+) or without (−) the addition of corticotropin (ACTH, 2 nM). Total RNA was isolated after incubations of 1, 5, 10, and 24 h as indicated. Each lane was loaded with 12 μg of total RNA. Membrane was hybridized with full-length bTREK-1 cDNA probe as described under *Materials and Methods*. Image was obtained from film exposed to hybridized membrane for ~3 h. After stripping, membrane was reprobed with GAPDH cDNA as a control.

AZF cells express a single population of corticotropin receptors with K_d values of 1 to 2 nM (Mountjoy et al., 1992; Raikhinstein et al., 1994). However, corticotropin inhibits bTREK-1 K^+ channel activity, depolarizes AZF cells, stimulates cortisol secretion, and inhibits the expression of bKv1.4 mRNA at 100- to 1000-fold lower concentrations (Mlinar et al., 1993a; Enyeart et al., 2000). We found that corticotropin also potently induced the expression of bTREK-1 mRNA at picomolar concentrations. In the experiment illustrated in Fig. 3, AZF cells in serum-free media were exposed to corticotropin for 4 h at concentrations ranging from 0.1 to 1000 pM. Corticotropin induced a concentration-dependent increase in bTREK-1 transcripts with an EC_{50} of approximately 10 pM. By comparison, bKv1.4 mRNA expression was inhibited in the same experiment with similar potency.

Role of cAMP, A-Kinase, and Ca^{2+} in Induction of bTREK-1. cAMP is the primary intracellular messenger for corticotropin and mediates many of the rapid and delayed effects of this peptide in AZF cells (Simpson and Waterman, 1988; Enyeart et al., 1996b). Forskolin, a diterpene that directly activates adenylate cyclase, induced an increase in bTREK-1 mRNA with an effectiveness and temporal pattern similar to that observed with corticotropin. In the experiment illustrated in Fig. 4A, forskolin (5 μM) produced a time-dependent increase in bTREK-1 mRNA measured over a 22-h period. After exposure to forskolin for 14 and 22 h, bTREK-1 mRNA had increased to approximately 8- and 20 times its initial value. As observed with corticotropin, the two smaller transcripts were preferentially induced by forskolin. By comparison, the time-matched control value increased slightly to 1.4 times its initial value after 22 h. bTREK-1 mRNA was also effectively induced by the membrane-permeable cAMP analog 8-pcpt-cAMP (see Fig. 5).

Many of the actions of corticotropin and cAMP in adrenocortical cells are mediated through activation of A-kinase (Simpson and Waterman, 1988). However, other responses, including induction of immediate early genes, acute inhibition of bTREK-1 current, and inhibition of bKv1.4 mRNA expression are insensitive to, or only partially inhibited by, selective A-kinase antagonists, suggesting the involvement of additional signaling pathways (Enyeart et al., 1996a,b, 2000).

In particular, the cAMP-activated guanine nucleotide exchange factors Epac1 and Epac2 (cAMP-GEFI and cAMP-GEFII) activate RAP1 guanine nucleotide binding proteins that in turn activate A-kinase–independent signaling pathways (de Rooij et al., 1998; Kawasaki et al., 1998). In the present study, we found that the potent A-kinase inhibitor H-89 (IC_{50} < 50 nM) (Hidaka et al., 1991) only partially suppressed corticotropin-induced bTREK-1 mRNA expression. In the experiment illustrated in Fig. 4B, a 16-h exposure to corticotropin increased bTREK-1 mRNA 6.2-fold over its control value. H-89 (5 μM) reduced the corticotropin-induced increase in bTREK-1 by 25%. Overall, in three similar experiments, the A-kinase antagonist inhibited the corticotropin-induced increase in bTREK-1 by 28.1 ± 4.5%.

To determine whether corticotropin-induced increases in bTREK-1 mRNA could be mediated in part through activation of Epac, AZF cells were exposed to 8-CPT-2-O-Me-cAMP, a novel cAMP analog that activates Epac but not A kinase (Enserink et al., 2002). As illustrated in Fig. 4B, 8-CPT-2-O-Me-cAMP (100 μM) failed to significantly increase bTREK-1

mRNA. Similar results were obtained in each of three experiments at concentrations ranging from 30 to 200 μM.

In addition to cAMP, Ca^{2+} acts as a second messenger for corticotropin in AZF cells. Specifically, the acute inhibition of bTREK-1 K^+ channel activity by corticotropin leads to membrane depolarization and Ca^{2+} entry through T-type Ca^{2+} channels. Corticotropin-stimulated cortisol secretion is inhibited by T-type Ca^{2+} channel antagonists (Enyeart et al., 1993; Mlinar et al., 1993b).

If corticotropin-mediated increases in bTREK-1 are mediated by Ca^{2+}, then other agents that increase $[Ca^{2+}]_i$ in AZF cells should also enhance expression of this gene. AII activates PLC-coupled AT_1 receptors on adrenocortical cells, leading to the release of intracellular Ca^{2+} as well as depolarization-dependent Ca^{2+} entry (Elliot et al., 1985; Kojima et al., 1985; Sasaki et al., 1991; Ambroz and Catt, 1992; Mlinar et al., 1995). Although AII does not increase cAMP, it elicits several responses that are characteristic of corticotropin, including acute inhibition of bTREK-1 K^+ channels, stimulation of cortisol secretion, and induction of immediate early genes (Enyeart et al., 1993, 1996; Mlinar et al., 1995). To determine whether an increase in internal Ca^{2+} concentration might be sufficient to increase bTREK-1 expression, AZF cells were exposed to AII.

In the experiment illustrated in Fig. 4C, left, AZF cells were exposed to AII (20 nM), corticotropin (2 nM), or corti-

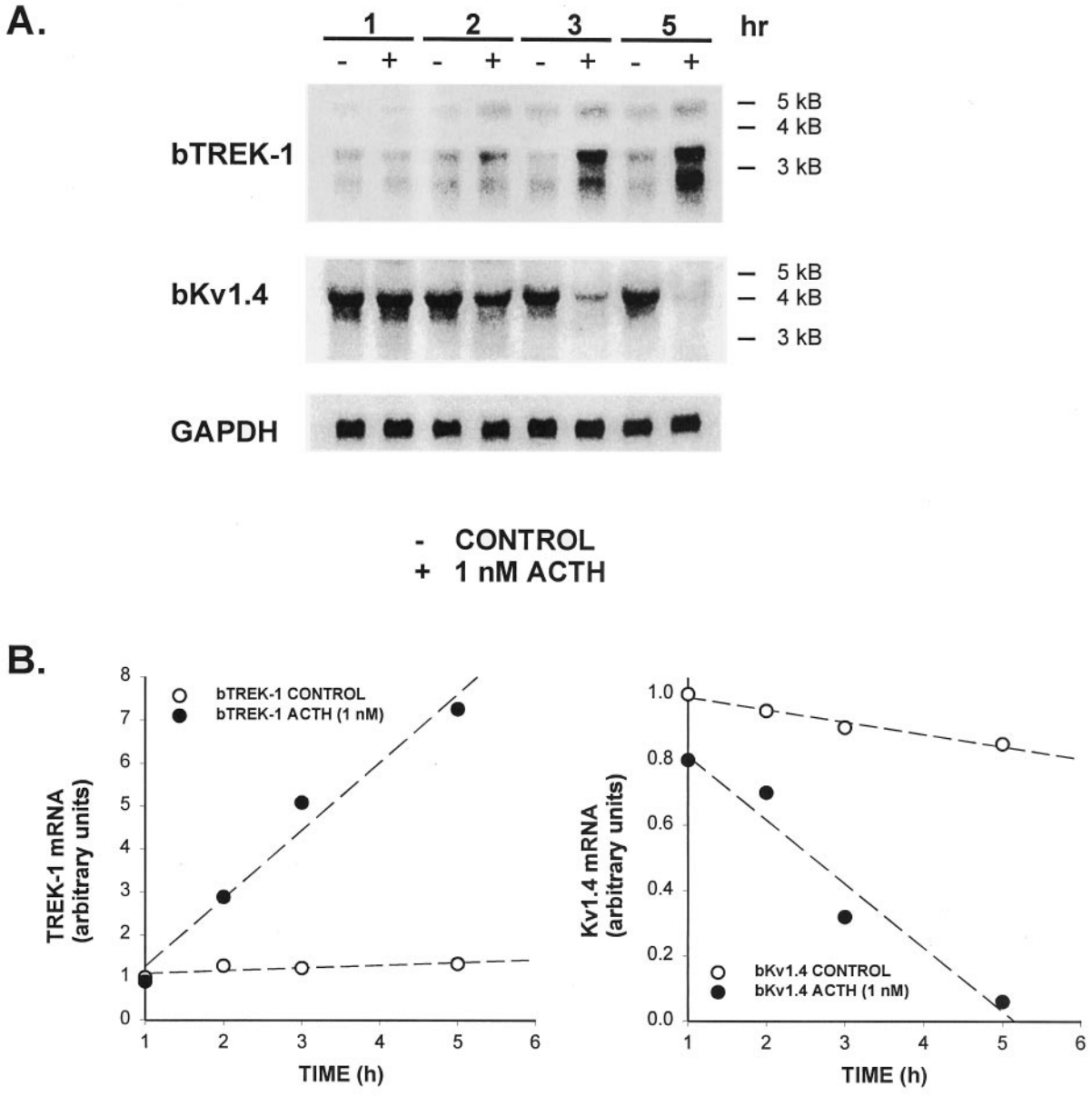


Fig. 2. Reciprocal time-dependent effects of corticotropin on bTREK-1 and bKv1.4 mRNA. The temporal pattern of corticotropin-stimulated changes in bTREK-1 and bKv1.4 mRNA expression was determined by Northern blot at times ranging from 1 to 5 h. Bovine AZF cells were cultured as described under *Materials and Methods* for 24 h, after which time the media was replaced by the same media with (+) or without (−) the addition of 1 nM corticotropin (ACTH). Total RNA was isolated after incubations of 1, 2, 3, and 5 h as indicated. Each lane was loaded with 12 μg of total RNA and the membrane was hybridized sequentially with a full-length bTREK-1 cDNA probe, a 2295-nucleotide fragment of bKV1.4 cDNA probe and GAPDH cDNA probe as a control. A, images were obtained from film exposed to hybridized membrane for ~24 h. B, bTREK-1 and bKv1.4 mRNAs from experiment in A were quantitated based on optical density measurements using ImageQuant software and plotted against time.

cotropin in combination with AII for 8 h before isolating total RNA for Northern analysis. Corticotropin alone induced an 8-fold increase in bTREK-1 mRNA. In contrast, AII failed to significantly increase bTREK-1 mRNA and did not alter induction of this message by corticotropin. This result was obtained in each of three separate experiments.

Although the failure of AII to enhance bTREK-1 mRNA expression indicates that an increase in $[Ca^{2+}]_i$ alone is insufficient to induce this response, it does not exclude the possibility that cAMP and Ca^{2+} function as duel messengers in this respect. In this regard, cAMP synthesized in response to corticotropin receptor activation inhibits bTREK-1 K^+ channels leading to depolarization-dependent Ca^{2+} entry through T-type Ca^{2+} channels (Enyeart et al., 1993). Mibefradil blocks T-type Ca^{2+} channels in bovine AZF cells with an IC_{50} of 1 μM (Gomora et al., 2000). Mibefradil, however, failed to reduce corticotropin-stimulated increases in bTREK-1 mRNA, indicating that depolarization-dependent Ca^{2+} entry does not contribute to this response (Fig. 4, right).

Effect of Inhibition of Translation and Transcription on Corticotropin Response. The lag of several hours that preceded corticotropin-induced increases in bTREK-1 expression suggests that the response may require synthesis of a transcription factor that enhances expression of the bTREK-1 gene. To determine whether corticotropin- and cAMP-induced increases in bTREK-1 mRNA are dependent on protein synthesis, the effect of these two agents was studied in the presence of the protein synthesis inhibitor cycloheximide. The experiment illustrated in Fig. 5A shows that cycloheximide (5 μg/ml) effectively inhibited bTREK-1 mRNA expression induced by corticotropin or the membrane-permeable cAMP analog 8-pcpt-cAMP. In this experiment, AZF cells were incubated for 8 h with media containing corticotropin (2 nM) or 8-pcpt-cAMP (250 μM) in the presence or absence of cycloheximide (5 μg/ml), as indicated, before isolating total RNA for Northern blot analysis. Cycloheximide inhibited corticotropin and 8-pcpt-cAMP induced bTREK-1 mRNA almost completely.

Experiments with cycloheximide were consistent with a model in which corticotropin and cAMP induce the synthesis of a *trans*-acting factor that increases the rate of transcription of the bTREK-1 gene. Accordingly DRB, an effective transcriptional inhibitor (Zandomeni et al., 1986), was found to inhibit both corticotropin- and 8-pcpt-cAMP–induced increases in bTREK-1 mRNA. In the experiment illustrated in Fig. 5B, DRB (150 μM) completely inhibited increases in bTREK-1 mRNA induced during an 8-h incubation with corticotropin or 8-pcpt-cAMP. Similar results were obtained in two other experiments.

Prolonged Exposure to Corticotropin Enhances bTREK-1 K^+ Current. Experiments with cultured AZF cells showed that after 24 h in serum-supplemented media,

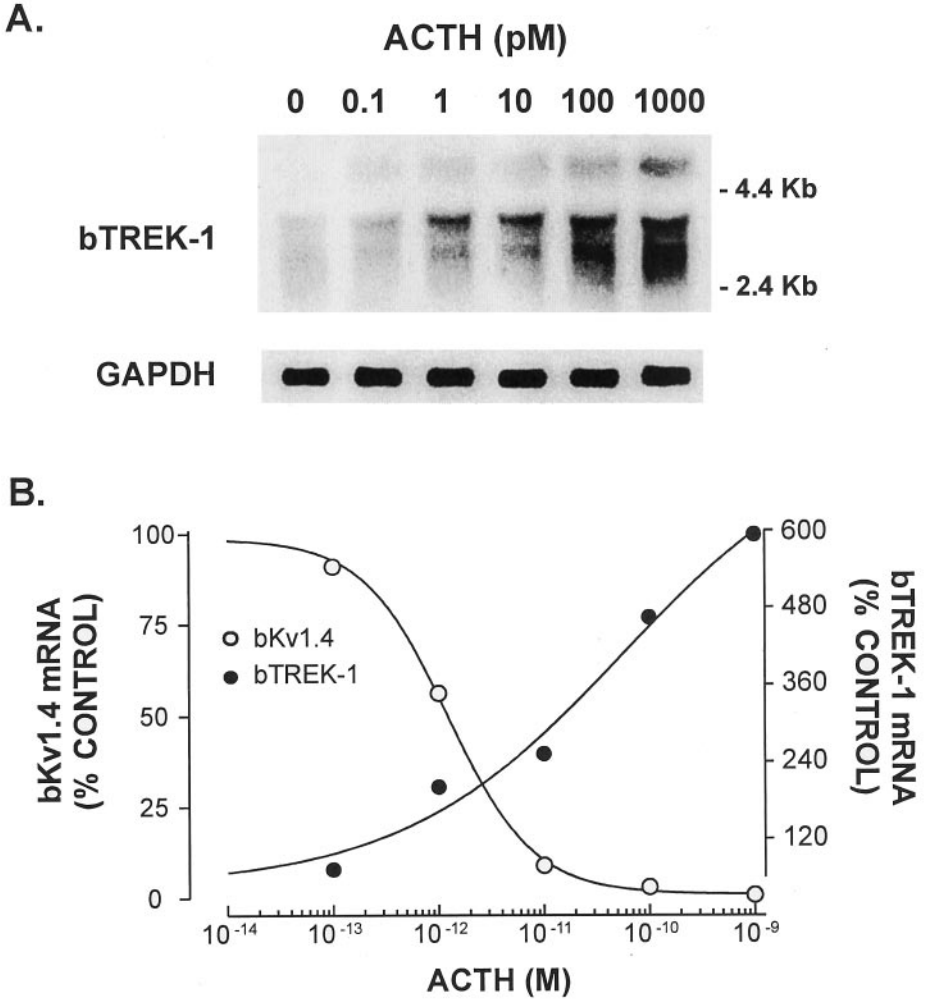
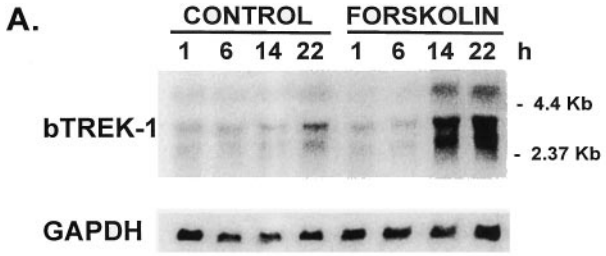
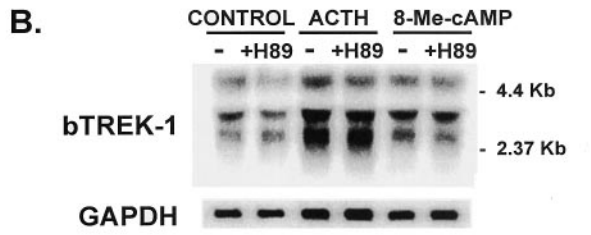


Fig. 3. Concentration-dependent induction of bTREK-1 mRNA expression by corticotropin. Bovine AZF cells were cultured as described under *Materials and Methods* for 24 h, after which time the media was replaced with serum-free media (DMEM/Ham's F12, supplemented with 50 μg/ml BSA, 100 μM ascorbic acid, 1 μM tocopherol, 100 nM insulin, and 10 μg/ml transferrin) or this same media containing corticotropin (ACTH) at concentrations ranging from 0.1 to 1000 pM. Total RNA was isolated after 4 h. Each lane was loaded with 12 μg total RNA. Membrane was hybridized with full-length bTREK-1 cDNA probe as described under *Materials and Methods*. After stripping, membrane was reprobed with GAPDH as a control. A, Northern blot shows concentration-dependent induction of bTREK-1 mRNA by corticotropin. B, concentration-response curve obtained from optical density measurements of the autoradiogram from the experiment shown in A, normalized to GAPDH measurements for the same lane. bTREK-1 mRNA is expressed as percentage control and plotted against corticotropin concentration (●). Data were fit with a curve of the form $y = \min + (\max/\min)/[1 + ([x]/[A])^{n_H}]$, where y is the bTREK-1 mRNA value, $[x]$ is the corticotropin concentration, and $[A]$ is the corticotropin concentration that induces half-maximal RNA expression. The inhibition curve for corticotropin reduction of Kv1.4 mRNA is plotted for comparison (○) (Enyeart et al., 2000).

bTREK-1 mRNA was poorly expressed but was markedly increased within hours in response to exposure to corticotropin. These results suggest that prolonged exposure of AZF cells to corticotropin could sustain or enhance the expression of bTREK-1 K^+ channels and associated current.





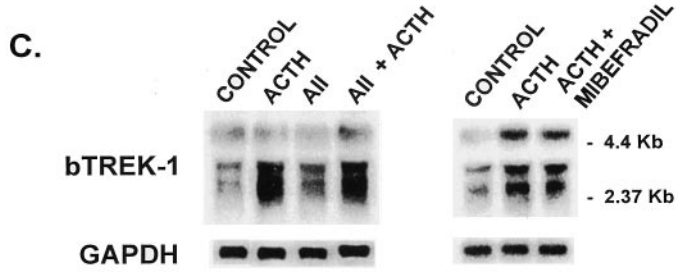
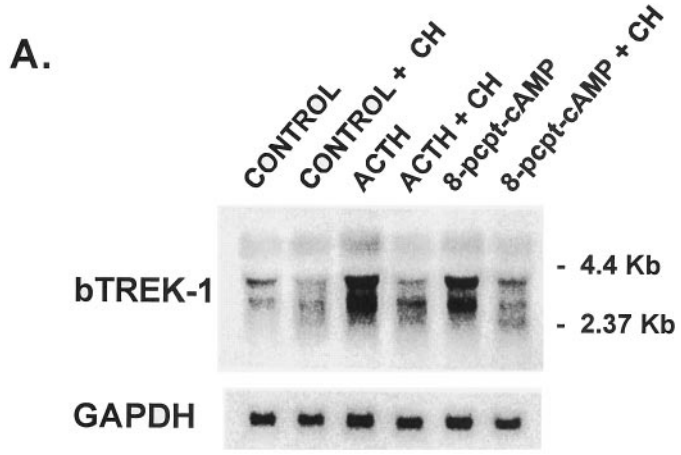


Fig. 4. Signaling pathway for corticotropin induction of bTREK-1 mRNA. A, time-dependent induction of bTREK-1 mRNA expression by forskolin. Bovine AZF cells were cultured as described under *Materials and Methods* for 24 h, after which time media was replaced with the same media with or without forskolin (5 μM) as indicated. AZF cells were incubated with forskolin for periods of 1 to 22 h before isolating total RNA. For Northern blots, each lane contained 12 μg of total RNA. Membranes were probed as described under *Materials and Methods*. After stripping, membranes were reprobed with GAPDH. B, role of A-kinase in the induction of bTREK-1 mRNA by corticotropin. Bovine AZF cells were plated as described under *Materials and Methods*. After 24 h, media was aspirated and replaced with the same media, or one supplemented with H-89 (5 μM), corticotropin (ACTH, 2 nM), corticotropin (2 nM) + H-89 (5 μM), 8-CPT-2-O-Me-cAMP (100 μM), or 8-CPT-2-O-Me-cAMP + H-89 (5 μM), as indicated. AZF cells were preincubated for 30 min in the presence of H-89 (5 μM) before the addition of corticotropin or 8-CPT-2-O-Me-cAMP. Total RNA was isolated after 16 h. For Northern blots, each lane contained 12 μg of total RNA. Membranes were probed with full-length bTREK-1 cDNA as described under *Materials and Methods*. After stripping, membranes were reprobed with GAPDH. C, corticotropin but not AII induces the expression of bTREK-1 mRNA. Bovine AZF cells were cultured as described under *Materials and Methods*. After 24 h, media was aspirated and replaced with the same media with or without the addition of corticotropin (ACTH, 2 nM), AII (20 nM), corticotropin (2 nM) + AII (20 nM; left), or corticotropin (2 nM) + mibefradil (5 μM) (right). Total RNA was isolated after 8 h. Each lane contained 12 μg of total RNA. Membranes were probed with full-length bTREK-1 cDNA as described under *Materials and Methods*. After stripping, membranes were reprobed with GAPDH as a control.

Patch-clamp and molecular cloning studies have shown that bovine AZF cells express only two types of K^+ channels: the rapidly inactivating, voltage-gated Kv1.4 current and the noninactivating bTREK-1 channels (Mlinar and Enyeart, 1993; Mlinar et al., 1993a; Enyeart et al., 2000, 2002). In whole-cell recordings, bTREK-1 often grows dramatically over a period of minutes provided that ATP is present at millimolar concentrations in the pipette solution (Mlinar et al., 1993a; Enyeart et al., 1997).

The absence of time- and voltage-dependent inactivation of the bTREK-1 current allows it to be isolated and measured in whole-cell recordings, using either of two voltage-clamp protocols. When voltage steps of 300-ms duration are applied from a holding potential of −80 mV, bTREK-1 can be measured near the end of a voltage step when the transient Kv1.4 current has inactivated (Fig. 6A, top traces). Alternatively, bTREK-1 can be



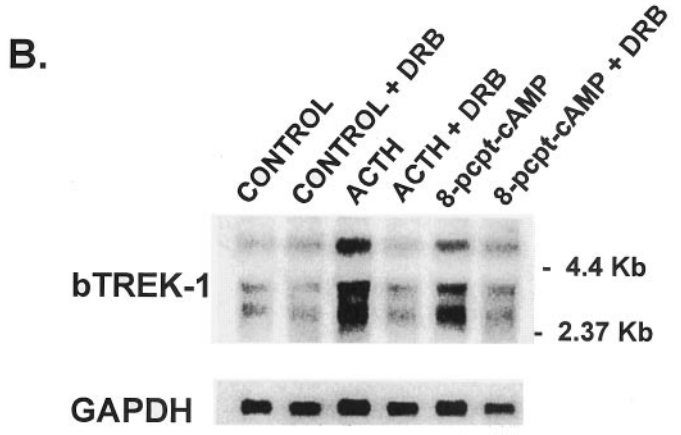


Fig. 5. Effect of transcription and translation inhibitors on bTREK-1 induction. Bovine AZF cells were cultured as described under *Materials and Methods*. After 24 h, media was aspirated and replaced with the same media supplemented with corticotropin, 8-pcpt cAMP and/or cycloheximide (CH) or DRB as indicated. Cells were pretreated with CH or DRB for 30 min before adding corticotropin or 8-pcpt-cAMP. For Northern blots, each lane contained 12 μg of total RNA. Membranes were probed with full-length bTREK-1 cDNA as described under *Materials and Methods*. After stripping, membranes were reprobed with GAPDH. A, effect of cycloheximide on induction of bTREK-1 mRNA by corticotropin and 8-pcpt cAMP. AZF cells were incubated with corticotropin (ACTH, 2 nM) or 8-pcpt cAMP (250 μM) either alone or with CH (5 μg/ml) for 8 h before isolating total RNA. B, effect of DRB on induction of bTREK-1 expression by corticotropin or 8-pcpt-cAMP. AZF cells were incubated with corticotropin (ACTH, 2 nM) or 8-pcpt (250 μM) either alone or with DRB (150 μM) for 8 h before isolating total RNA.

selectively activated after a 10-s prepulse to −20 mV has fully inactivated bKv1.4 (Fig. 6A, bottom traces).

In the experiments illustrated in Fig. 6, K^+ currents were recorded from cultured AZF cells beginning 1 h after plating in serum-supplemented medium or after 24 h in culture in the same medium with or without corticotropin (2 nM). bTREK-1 is prominently expressed in newly cultured AZF cells, but this current nearly disappears after 24 h in serum-supplemented media (Fig. 6). Treatment of cells with corticotropin largely prevented the time-dependent down-regulation observed in its absence (Fig. 6, A and B). Overall, in the absence of corticotropin, bTREK-1 current density decreased from a control value of 43.8 ± 24.4 pA/pF to 4.2 ± 1.7 pA/pF (n = 7) after 24 h in culture. In the presence of 2 mM corticotropin, bTREK-1 current density declined to only 31.8 ± 12.2 pA/pF (n = 11) (Fig. 6C).

The corticotropin-induced enhancement of bTREK-1K^+ current expression persisted for at least 48 h. By this time, the reciprocal effects of corticotropin on the expression of the voltage-gated Kv1.4 and TREK-1 K^+ currents were clearly evident. In these experiments, the volatile anesthetic chloroform ($CHCl_3$) also established the identity of the noninactivating current as bTREK-1. Although many of the more than one dozen 2P/4TM background K^+ channels are activated by various volatile anesthetics, $CHCl_3$ activates only TREK-1 channels (Patel et al., 1999).

In the experiment illustrated in Fig. 7, cells were cultured for 48 h in the absence (control) or presence of corticotropin (2 nM). When K^+ currents were recorded from the control cell, a prominent, rapidly inactivating Kv1.4 current was present, whereas little or no noninactivating bTREK-1 current was detectable (Fig. 7A, left, trace 1). Superfusion of this cell with 5 mM $CHCl_3$ inhibited Kv1.4 current by 52% (trace 2), but failed to elicit any increase in the noninactivating component of K^+ current (Fig. 7, A and B).

In the corticotropin-treated cell, a noninactivating K^+ current was present that reached an amplitude of nearly 500 pA after 5 min of recording (Fig. 7A, right, trace 3). In contrast, the rapidly inactivating Kv1.4 K^+ current was almost undetectable. In this cell, superfusion of 5 mM $CHCl_3$ induced a 4-fold increase in the noninactivating K^+ current (Fig. 7, A, right, trace 4, and B), confirming its identity as TREK-1. Similar results were obtained in each of six corticotropin-treated cells.

Discussion

In this study, it was discovered that corticotropin induces the expression of bTREK-1 K^+ channel mRNA and associated membrane current in cultured bovine AZF cells. 8-pcpt-cAMP and forskolin also induced bTREK-1 mRNA, but 8-CPT-2-O-Me-cAMP and AII were ineffective. Induction of bTREK-1 mRNA by corticotropin was blocked only partially

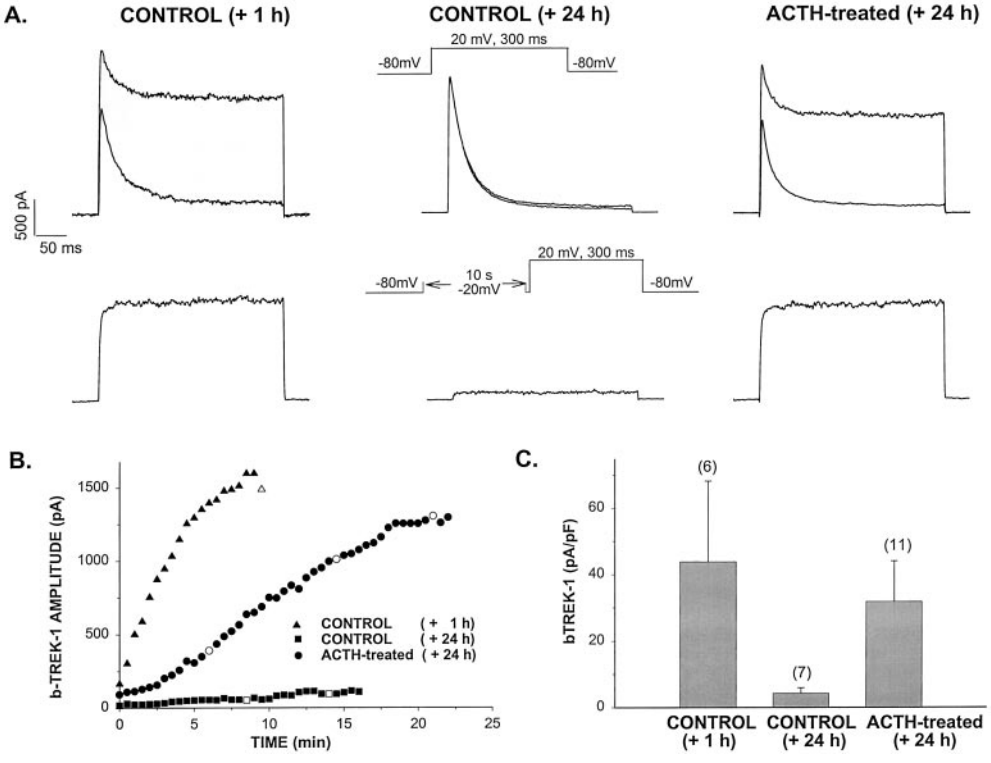


Fig. 6. Corticotropin promotes expression of bTREK-1 K^+ current. Bovine AZF cells were cultured on coverslips in serum-supplemented media with or without the addition of corticotropin (2 nM). K^+ currents were recorded in the whole-cell configuration from untreated (control) cells beginning at 1 h after plating and from untreated and corticotropin-treated cells beginning at 24 h after plating. Media containing corticotropin was replaced with control media 1 h before initiating whole cell recording. Voltage steps were applied from a holding potential of −80 to a test potential of +20 mV at 30-s intervals with or without 10-s prepulses to −20 mV. Corticotropin was replenished after 12 h. A, K^+ current traces. Currents were recorded with (bottom traces) and without (top traces) depolarizing prepulses from untreated cells at 1 h (left traces) and at 24 h (middle traces) and corticotropin (ACTH)-treated cells at 24 h (right traces). B, bTREK-1 amplitudes are plotted against time for the same three cells illustrated in A. Open symbols and closed symbols illustrate currents recorded with and without depolarizing prepulses, respectively. C, effect of corticotropin on bTREK-1 current density. Pooled data from experiments as in A and B were used to determine effect of corticotropin on bTREK-1 current density. Maximum bTREK-1 current amplitudes were divided by cell capacitance to determine individual current densities. Values are mean ± S.E.M. of indicated number of determinations.

by inhibition of A-kinase by H-89 and nearly completely inhibited by blocking protein synthesis or gene transcription. The enhancement of bTREK-1 mRNA and ionic current induced by corticotropin stands in stark contrast to the rapid down-regulation of Kv1.4 K^+ channel mRNA and membrane current induced by this peptide in AZF cells (Enyeart et al., 2000).

Previous research has shown that corticotropin regulates the expression of only a small percentage of AZF cell genes, including those coding for immediate early genes and steroidogenic hydroxylases (Simpson and Waterman, 1988; Parker and Schimmer, 1995; Enyeart et al., 1996a). Ion channels constitute a third group of AZF cell proteins whose expression is tonically regulated by corticotropin.

Signaling Pathways for Corticotropin-Induced bTREK-1 mRNA Expression. In AZF cells, corticotropin and AII function through distinct signaling pathways yet produce several similar responses. Corticotropin receptor activation leads to the synthesis of cAMP and depolarization-dependent Ca^{2+} entry through T-type Ca^{2+} channels (Enyeart et al., 1993, 1996b; Mlinar et al., 1993a). Thus, cAMP and Ca^{2+} function as dual second messengers for corticotropin in these cells. By comparison, AII-mediated activation of AT_1 receptors is coupled to PLC activation leading to Ca^{2+} release from intracellular stores, as well as depolarization-dependent Ca^{2+} entry (Kojima et al., 1984, 1985; Elliot et al., 1985; John et al., 1986; Mlinar et al., 1993a, 1995).

Both of these peptides and exogenously applied cAMP inhibit bTREK-1 K^+ current, depolarize AZF cells, stimulate cortisol secretion, and enhance the transcription of steroidogenic enzymes and several immediate early genes (Simpson and Waterman, 1988; Enyeart et al., 1993, 1996a; Mlinar et al., 1993a, 1995). Thus, Ca^{2+} and cAMP may act independently or in concert as intracellular messengers to mediate all of these responses.

The markedly different actions of corticotropin and AII on the expression of bTREK-1 mRNA and corresponding K^+ current represent a distinct difference in the action of the two peptides on AZF cells and point to a divergence in the underlying signaling pathways. Specifically, the pronounced increases in bTREK-1 mRNA produced by corticotropin and cAMP combined with the failure of AII to produce any measurable increase indicate that cAMP, but not Ca^{2+}, regulates the expression of the bTREK-1 gene. The failure of mibefradil to inhibit the corticotropin-stimulated increase in bTREK-1 mRNA indicates that this response is not caused by the combined action of Ca^{2+} and cAMP.

With respect to signaling pathways involved, the cortico-

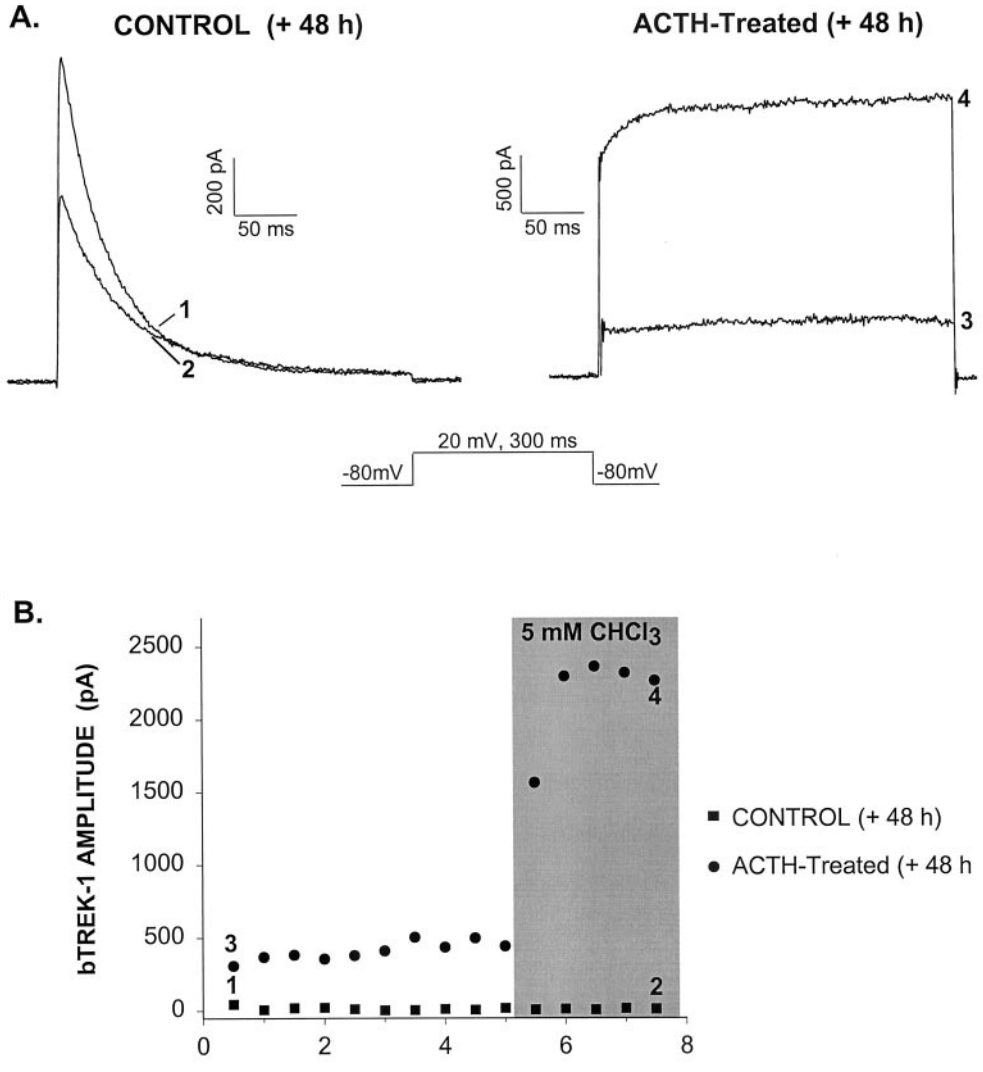


Fig. 7. Effect of long-term corticotropin treatment and chloroform on AZF cell K^+ currents. AZF cells were cultured on coverslips in serum-supplemented media with or without the addition of corticotropin (2 nM). K^+ currents were recorded from cells after 48 h in culture. Corticotropin was replenished at 12-h intervals. Media containing corticotropin were replaced with control media 1 h before initiating whole cell recording. Voltage steps were applied from a holding potential of −80 mV to test potential of +20 mV at 30-s intervals. Cells were superfused with 5 mM chloroform as indicated. A, effect of chloroform on untreated (control) and corticotropin (ACTH)-treated cells. Left, K^+ current traces recorded from an untreated cell after 48 h in culture in standard saline (trace 1) and after superfusion of 5 mM $CHCl_3$ (trace 2). Right, K^+ current records from a corticotropin-treated cell after 48 h in culture in standard saline (trace 3) and after superfusion of 5 mM $CHCl_3$ (trace 4). B, amplitude of noninactivating K^+ current is plotted against time for two cells shown in A. Numbers on graphs correspond to numbers on traces in A. $CHCl_3$ (5 mM) superfusion is indicated by shaded area.

tropin-induced increase in bTREK-1 mRNA resembles the corticotropin- and cAMP-induced reductions in bKv1.4 mRNA and corresponding K^+ current. Specifically, the failure of AII to inhibit the expression of Kv1.4 mRNA or K^+ current indicates that cAMP, but not Ca^{2+}, regulates the expression of this voltage-gated K^+ channel (Enyeart et al., 2000). Thus, corticotropin seems to regulate the expression of the only two K^+ channels expressed by bovine AZF cells in reciprocal fashion through a cAMP-dependent mechanism.

Involvement of A-Kinase in Corticotropin Response. Until recently, all of the cAMP-dependent actions of corticotropin in AZF cells were believed to require the activation of A-kinase. However, corticotropin and cAMP have been shown to acutely inhibit the activity of bTREK-1 K^+ channels, increase the expression of T-type Ca^{2+} channels in rat AZF cells, and inhibit the expression of bKv1.4 K^+ channels by mechanisms that are wholly or partially independent of A-kinase (Barbara and Takeda, 1995; Enyeart et al., 1996b, 2000). Accordingly, cAMP-dependent induction of bTREK-1 mRNA also seems to be partly independent of A-kinase. However, we cannot exclude the possibility that H-89, even though used at concentrations 200-fold higher than the reported IC_{50} of 50 nM, failed to completely inhibit protein kinase A in our study (Hidaka et al., 1991). In this regard, we have previously shown that H-89 (5 μM) inhibited protein kinase A by 97 to 100% in cytoplasmic extracts from AZF cells (Enyeart et al., 1996b).

A specific A-kinase–independent pathway mediating effects of corticotropin and cAMP in AZF cells has not yet been identified. Although Epac guanine nucleotide exchange factor mRNAs are expressed in rat adrenal (Kawasaki et al., 1998) and bovine AZF cells (J. A. Enyeart, unpublished observations), the associated signaling pathways do not seem to mediate the induction of bTREK-1 mRNA and associated K^+ current. However, 8-CPT-2-O-Me-cAMP has not yet been shown to activate EPAC2 in bovine AZF cells.

Molecular Mechanism for Corticotropin-Mediated Increase in bTREK-1 mRNA Expression. A number of recent reports have appeared describing the short-term modulation of 2P/4TMS background K^+ channels activity by peptide hormones and neurotransmitters (Aimond et al., 2000; Goldstein et al., 2001; Patel and Honore, 2001; Terrenoire et al., 2001). The corticotropin-induced increase in bTREK-1 mRNA and corresponding K^+ current described herein is the first demonstration of hormonal regulation of the expression of a background K^+ channel at a pretranslational level in a physiological pathway.

The delayed increase in bTREK-1 mRNA induced by corticotropin and cAMP could be caused by an increase in the rate of bTREK-1 gene transcription or an increase in bTREK-1 mRNA stability. Several studies have explored the regulation of voltage-gated K^+ channel gene expression by cAMP. cAMP increased the transcription rate of a rat cardiac Kv1.5 K^+ channel gene, but inhibited the transcription of a similar K^+ channel gene in the rat pituitary GH_3 cell line (Mori et al., 1993). cAMP accelerated the degradation of Kv1.1 K^+ channel mRNA in a glioma cell line (Allen et al., 1998). The rapid corticotropin and cAMP-induced decrease in AZF cell Kv1.4 K^+ channel mRNA previously reported also probably occurs through an effect on mRNA stability (Enyeart et al., 2000). Thus, cAMP has been shown to alter K^+ channel gene expression by modulating transcription or mRNA stability.

The delay of $\geq$1 h that precedes corticotropin- or cAMP-induced increases in bTREK-1 mRNA and the effectiveness of cycloheximide in inhibiting these increases are consistent with a model in which corticotropin induces the synthesis of a specific transcription factor that then specifically enhances bTREK-1 gene transcription. Corticotropin-mediated increases in the transcriptional activity of genes coding for steroidogenic enzymes follow a similar temporal pattern. Increases in the levels of specific mRNAs coding for these enzymes can be detected after several hours, whereas maximum increases in the rates of synthesis of steroidogenic enzymes reach a maximum only after 24 to 36 h (Zuber et al., 1986; Simpson and Waterman, 1988; Parker and Schimmer, 1995). Furthermore, the transcriptional activation of steroid hydroxylases by corticotropin is inhibited by cycloheximide, suggesting that corticotropin induces the synthesis of one or more protein factors with rapid turnover that enhance transcription of steroid hydroxylase genes (John et al., 1986; Zuber et al., 1986; Parker and Schimmer, 1995).

The identity of the transcription factors that mediate corticotropin effects on transcription of bTREK-1 or steroid hydroxylase genes is unknown. In this regard, corticotropin and cAMP both induce a number of immediate early genes that code for transcription factors in AZF cells. These include *cFos*, *NGFI-A*, and *NGFI-B* (Wilson et al., 1993; Davis and Lau, 1994; Enyeart et al., 1996a). However, it is unlikely that any of these mediates induction of bTREK-1 transcription because each is also induced by AII.

cAMP regulates the transcription of many genes through an A-kinase–dependent activation of CREB transcription factors (Shaywitz and Greenberg, 1999). However, CREB transcription factors are phosphorylated by both cAMP and Ca^{2+}/calmodulin-dependent protein kinases (Sheng et al., 1991; Shaywitz and Greenberg, 1999). Because AII-stimulated activation of AT_1 receptors leads to an increase in intracellular Ca^{2+}, the failure of this peptide to increase bTREK-1 mRNA argues against CREB activation as a mechanism for corticotropin induction of this K^+ channel gene.

The near complete inhibition of corticotropin- and cAMP-induced increases in bTREK-1 mRNA by the transcription inhibitor DRB is consistent with a model in which these two agents act by enhancing the rate of transcription of the bTREK-1 gene. Although this result demonstrates a requirement for the ongoing transcription of the bTREK-1 gene in this response, it does not rule out the possibility of an increase in the stability of bTREK-1 transcripts as well.

Regulation of bTREK-1 mRNA and K^+ Channel Expression: Physiological Significance. In the absence of corticotropin, bTREK-1 mRNA and associated K^+ current are poorly expressed when AZF cells are maintained in culture for 24 h. When corticotropin is included in the culture media, bTREK-1 mRNA is induced and TREK-1 current is sustained. These results indicate that the control of bTREK-1 expression is tightly controlled at the pretranslational level by this peptide hormone and dependent on its continued presence.

When combined with our previous results showing that corticotropin rapidly down-regulates bKv1.4 mRNA and associated K^+ current, the present findings demonstrate that within 1 day, corticotropin can produce significant changes in

the electrical properties of AZF cells by altering the synthesis of these two types of K^+ channels. In this regard, prolonged exposure to corticotropin has been reported to markedly enhance T-type Ca^{2+} current in rat AZF cells (Barbara and Takeda, 1995). Thus, it seems that corticotropin may dramatically alter the electrical properties of AZF cells through regulation of the expression of each of three ion channels expressed by these cells. The impact of these corticotropin-induced changes in ion channel expression on AZF cell function and cortisol secretion has not yet been determined. However, because cortisol production in AZF cells depends on depolarization-dependent Ca^{2+} entry through voltage-gated channels (Enyeart et al., 1993), the implications are significant.

The inhibition by corticotropin of bTREK-1 K^+ channel activity that occurs within 1 to 3 min contrasts with the corticotropin-mediated increases in bTREK-1 mRNA and K^+ channels measured over a period of hours. This delayed increase in the synthesis of bTREK-1 K^+ channels could serve as a negative feedback mechanism under conditions of overstimulation by corticotropin. Specifically, the increased number of bTREK-1 channels could render AZF cells less sensitive to corticotropin-stimulated depolarization, thereby limiting Ca^{2+} entry and cortisol secretion. The pretranslational control of ion channel expression by corticotropin in AZF cells suggests a novel mechanism for long-term control of electrical activity and corticosteroid secretion in adrenal cortical cells.

The extremely potent induction of bTREK-1 mRNA by corticotropin suggests that, under physiological conditions, the expression of these K^+ channels would be tightly coupled to the secretion of this peptide from the pituitary. Because corticotropin is secreted episodically in a circadian rhythm, bTREK-1 mRNA and K^+ channels could display a similar diurnal oscillation (Bondy, 1985). In contrast, the corticotropin-inhibited Kv1.4 channels would oscillate in a reciprocal pattern.

Under conditions of prolonged stress or adrenal disease, in which corticotropin secretion increases dramatically to pathophysiological levels, long-term overstimulation of the adrenal cortex could engender sustained changes in the electrical and secretory properties of AZF cells (Bondy, 1985). It remains to be seen whether the physiological patterns of cortisol secretion that occur in endocrine conditions, such as pituitary adenoma-induced Cushing's disease, may be caused in part by corticotropin-induced changes in ion channel gene expression.

Modulation of 2P/4TMS K^+ channels by neurotransmitters and peptide hormones through G-proteins is an important mechanism regulating membrane potential and excitability in neurons and endocrine cells (Millar et al., 2000; Talley et al., 2000). Recent reports, including the present one, suggest that G-protein-coupled receptors may also exert long-term control over the electrical activity of these cells by controlling expression of background K^+ channels (Brickley et al., 2001).

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