We have cloned a bovine adrenal cortical (bKv1.4) K⁺ channel cDNA whose expression is rapidly inhibited by adrenocorticotropic hormone (ACTH). The 4386-nucleotide cDNA is homologous to other voltage-gated, rapidly inactivating Kv1.4 channels, and includes a 1986-nucleotide coding region and large 5’- and 3’-untranslated regions. Bovine Kv1.4-specific mRNA from adrenal zona fasciculata (AZF) cells was rapidly and potently reduced by ACTH, with a t½ of approximately 1 h and an IC₅₀ of 1.2 pm. The membrane-permeable cAMP analog 8-pcpt-cAMP also reduced bKv1.4 mRNA expression with kinetics similar to that observed with ACTH. Reduction of bKv1.4 mRNA expression by ACTH and 8-pcpt-cAMP was only partially inhibited by the selective protein kinase A antagonist H-89. Consistent with their effect on bKv1.4 mRNA, ACTH and 8-pcpt-cAMP both dramatically reduced the expression of bKv1.4-associated A-type current measured over 72 h. These results demonstrate that bovine AZF cells synthesize a Kv1.4-type channel whose expression is inhibited at the translational level by ACTH and 8-pcpt-cAMP by a mechanism that is partially dependent on the activation of protein kinase A. The rapid, potently reduced of bKv1.4 mRNA produced by ACTH and 8-pcpt-cAMP indicates that the expression of this K⁺ channel is under tonic inhibitory control of the hypothalamic-pituitary-adrenal axis. The basic electrical properties of AZF cells might be tightly regulated at the transcriptional level by the normal diurnal pattern of ACTH secretion, and altered during bouts of stress by the enhanced release of this pituitary peptide. Under conditions of prolonged stress or adrenal insufficiency, persistent ACTH-induced changes in the electrical properties of AZF cells could be coupled to parallel changes in cortisol secretion.

Voltage-gated K⁺ channels are prominently expressed in a variety of secretory cells. In excitable secretory cells, these K⁺ channels regulate action potential frequency and waveform, and therefore function critically in regulating depolarization-dependent Ca²⁺ entry and secretion of neurotransmitters and peptide hormones (1).

Some secretory cells such as the cortisol-producing cells of the adrenal zona fasciculata (AZF) lack voltage-gated Na⁺ channels and Na⁺-dependent action potentials, the hallmark of excitable cells (2–4). Despite the absence of action potentials, ion channels function pivotally in the regulation of cortisol secretion. AZF cells express both voltage-gated Ca²⁺ and K⁺ channels, and maintain membrane potentials near the K⁺ equilibrium potential (2, 3, 5). Cortisol production by these cells is coupled to depolarization-dependent Ca²⁺ entry (6).

Bovine AZF cells express two types of K⁺-selective channels, including a voltage-gated rapidly inactivating A-type K⁺ channel (Iₐ) and a non-inactivating, ATP-activated channel (IₐCₚ) that is only weakly voltage-dependent (3, 5, 7). Of these two, IₐCₚ sets the resting potential of AZF cells, while its rapid inhibition by the pituitary peptide ACTH is linked to membrane depolarization, Ca²⁺ entry through T-type Ca²⁺ channels and cortisol secretion (5, 6, 8). Although the Iₐ, K⁺ current is expressed at high density in nearly every AZF cell (3), its role in AZF cell function and the physiology of cortisol secretion has not been determined. Unlike IₐCₚ channels, Iₐ channels are not acutely inhibited by ACTH in whole-cell patch clamp recordings from AZF cells (3, 5, 8).

In addition to its rapid effects on the electrical and secretory properties of AZF cells, which involve the modulation of existing proteins including K⁺ channels and steroidogenic enzymes, ACTH also induces more prolonged effects by enhancing the transcription of selected genes. Specifically, ACTH and its primary second messenger cAMP induce the transcription of steroidogenic enzymes, as well as immediate early genes that code for transcription factors, which may in turn regulate AZF cell secretion, growth, or differentiation (9–12).

Besides acutely regulating the activity of K⁺ channels involved in cortisol secretion, ACTH could potentially produce enduring changes in the electrical and secretory activity of AZF cells by altering the expression of the genes that encode K⁺ channel proteins. However, the cloning of genes encoding ion channels in steroid-secreting cells has not been reported. We have cloned cDNAs that code for a rapidly inactivating Kv1.4-type K⁺ channel expressed by bovine AZF cells (bKv1.4). The expression of this gene is potently and effectively reduced by ACTH.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tissue culture medium, antibiotics, fibronectin, fetal bovine sera, formamide, and salmon sperm DNA were obtained from Life Technologies, Inc. Genescreen Plus hybridization transfer membrane and [α-³²P]dCTP were from NEN Life Science Products. Culture dishes

*This work was supported by National Institutes of Health Grant R01-DK47875. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank℠/EBI Data Bank with accession number(s) AF286622.

‡ To whom correspondence and reprint requests should be addressed: Dept. of Neuroscience, Ohio State University, College of Medicine, 5190 Graves Hall, 333 W. 10th Ave., Columbus, OH 43210-1239. Tel.: 614-292-3511; Fax: 614-688-8742; E-mail: enyeart.1@osu.edu.

The abbreviations used are: AZF, adrenal zona fasciculata; ACTH, adrenocorticotropic hormone; 8-pcpt-cAMP, 8-[(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate cAMP; AII, angiotensin II; nt, nucleotide(s); kb, kilobase pair(s); DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

1 The abbreviations used are: AZF, adrenal zona fasciculata; ACTH, adrenocorticotropic hormone; 8-pcpt-cAMP, 8-[(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate cAMP; AII, angiotensin II; nt, nucleotide(s); kb, kilobase pair(s); DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.
Isolation and Culture of AZF Cells—Bovine adrenal glands were obtained from steers (age range, 1–3 years) within 1 h of slaughter at a meat-processing facility. Bovine adrenal glands were transported to the laboratory in ice-cold phosphate-buffered saline containing 0.2% dextrose. In a sterile tissue culture hood, the adrenal glands were cut in half lengthwise and the lighter medulla tissue trimmed away from the cortex, immersed in five volumes of RNAlater solution, stored at −20 °C, and subsequently used for poly(A)⁺ mRNA isolation. The capsule with attached glomerulosa and thicker fasciculata-reticularis layer were then dissected into pieces approximately 1 × 1 × 0.5 cm³. A Stadie-Riggs tissue slicer (Thomas Scientific) was used to slice fasciculata-reticularis tissues from the glomerulosa layers by slicing 0.3–0.5-mm slices from the larger pieces. Cortical tissue was either immersed immediately in DMEM/F12 containing 200 μM cAMP, 20 μM HEPES, 11 μM BAPTA, and 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 CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 5 mM glucose, pH 7.4, using NaOH. The development of the non-inactivating I_ABK₄ K⁺ current that is present in these cells was completely eliminated by including 200 μM cAMP in the pipette solution. cAMP selectively inhibits I_ABK₄ K⁺ current, but does not alter I_A under these conditions (8, 11). All solutions were filtered through 0.22-μm cellulose acetate filters.

AFZ cells were used for patch clamp experiments 1–72 h after plating. Typically, cells with diameters of <15 μm and capacitances of 8–15 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 3–5 ml/min. Patch electrodes with resistances of 2–3 MΩ were fabricated from Corning 0010 glass (World Precision Instruments, Sarasota, FL). These routinely yielded access resistances of 4–6 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. (16) using an Axopatch 1D patch clamp amplifier (Axon Instruments).

Pulse generation and data acquisition were done using a personal computer and PCLAMP software with a TL-1 interface (Axon Instruments). Currents were digitized at 5–20 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 1/3 to 1/4 amplitude. Data were analyzed and plotted using PCLAMP 5.5 and 6.04 (CLAMPAN and CLAMPFIT) and SigmaPlot (version 4.0).

RESULTS

Cloning and Sequence of bKv1.4 cDNA—A bovine AZF cDNA library was screened for K⁺ channel clones using a 1.3-kb PVU11 restriction fragment of BAK4 that includes the S1 through S6 transmembrane segments (13). The screening of ~1 × 10⁶ recombinants led to the identification of 64 positives, 13 of which were chosen for sequencing. Sequence analysis of these clones indicated that each coded for the same K⁺ channel that was >99% homologous to BAK4.

The cDNA sequence and predicted amino acid sequence of the largest of the bKv1.4 clones is shown in Fig. 1. This 4386-nucleotide clone is 1231 nucleotides longer than the sequence reported for BAK4, due to large 5’- and 3’-non-coding regions measuring 1157 and 1242 nt, respectively. The large non-coding regions are typical of Kv1.4 K⁺ channels (17). In this regard, the bKv1.4 cDNA contains 5 ATTTA repeats in the 3’-non-coding region, a feature common to other Kv1.4 channels (17).

The coding sequence for the bKv1.4 channel (nt 1158–3143), marked by arrows in Fig. 1, consists of an open reading frame encoding a 661-amino acid protein of molecular weight 73,554. The bKv1.4 translatable sequence codes for a K⁺ channel protein that is highly homologous to other mammalian Kv1.4 proteins, and differs only slightly from that predicted for BAK4 (13, 17). Specifically, one difference (a T to G change at nt 2576) encoded by the bKv1.4 clone is observed between the bKv1.4 and BAK4 mRNAs.
bKv1.4 Is Expressed in the Bovine Adrenal Cortex—Northern blot analysis of poly(A)\(^+\) mRNA isolated from fresh bovine adrenal tissue showed that bKv1.4 mRNA is expressed primarily or solely in the adrenal cortex rather than the adrenal medulla (Fig. 2). A 2295-nt probe (nt 1056–3351 of bKv1.4) that included the full-length coding region of bKv1.4 hybridized to separate >3.4- and >4.4-kb transcripts of poly(A)\(^+\) mRNA isolated from adrenal cortical tissue (Fig. 2). Separate transcripts of 3.5 and 4.5 kb coding for the same mouse Kv1.4 K^+ channel have previously been reported (17). The bKv1.4-derived cDNA probe hybridized much more weakly to mRNA transcripts of similar size that were isolated from adrenal medullary tissue (Fig. 2).

ACTH Inhibits Expression of bKv1.4-specific mRNA—ACTH induces the transcription of genes coding for steroidogenic enzymes and immediate early genes in the adrenal cortex (9, 10). ACTH produced a dramatic time-dependent decrease in the quantity of bKv1.4 mRNA transcripts in cultured bovine AZF cells. In the experiment illustrated in Fig. 3A, AZF cells were plated and maintained in culture for 24 h before treatment with ACTH (1 nM) for periods ranging from 1 to 24 h. bKv1.4 mRNA was strongly expressed in the untreated cells. After a 1-h exposure to ACTH, bKv1.4 mRNA, including both the large and small transcripts, was reduced by 43% compared with time-matched control values. At 5 and 24 h, bKv1.4 mRNA was further reduced by 90.6% and 92.7%, respectively, compared with time-matched controls.

Kv1.4 mRNA decreased much more gradually with time in untreated cells. In Fig. 3A, bKv1.4 mRNA was reduced by only 6% after 5 h in control medium, compared with the 90% reduction observed in the presence of ACTH. At 5 and 24 h, bKv1.4 mRNA was further reduced by 90.6% and 92.7%, respectively, compared with time-matched control values. At 5 and 24 h, bKv1.4 mRNA in untreated cells was reduced by approximately 40% compared with its initial value. bKv1.4 mRNA in ACTH-treated cells was nearly undetectable at this time.

Fig. 1. Nucleotide and predicted amino acid sequence for the bovine adrenal cortical bKv1.4 cDNA. The sequence of bKv1.4 cDNA was deduced from a clone isolated from a bovine adrenal cortical cDNA library. Arrowheads demarcate coding sequence (nt 1158–3143) for bKv1.4 channel protein. Within the 3'-untranslated region, a potential polyadenylation signal is boxed and five ATTTA motifs implicated in destabilization of mRNA are underlined. Within the amino acid sequence (top), predicted transmembrane segments S1–S6 are noted by solid lines. The eight-amino acid signature sequence for the pore region is shaded. Amino acids are represented by their single-letter abbreviations.
eliciting many of the responses that are triggered by ACTH. Specifically, AII inhibits the non-inactivating I_{AC} K⁺ channel, depolarizes AZF cells, stimulates cortisol secretion and triggers the transcription of immediate early genes (5, 10). However, AII failed to induce a time-dependent decrease in bKv1.4 transcripts (Fig. 3B). In the experiment illustrated, AII (10 nM) did not significantly reduce bKv1.4 mRNA compared with time-matched control values measured at 1, 5, and 24 h. Similar results were obtained in each of three experiments.

The ACTH-induced reduction of bKv1.4 mRNA occurred rapidly, with kinetics that lagged only slightly behind those for induction of mRNA for the immediate early gene NGFI-B. In the experiment illustrated in Fig. 4, AZF cells were treated with ACTH (1 nM) for periods ranging from 0.5 to 4 h. At each time point, bKv1.4 mRNA was compared with that for NGFI-B mRNA, which we previously had shown to be induced by ACTH in these cells (10).

At 1, 2, and 4 h, ACTH (1 nM) reduced bKv1.4 mRNA by 54%, 80%, and 91% respectively, relative to time-matched controls. In the same experiment, NGFI-B mRNA was weakly expressed in control medium, but increased 5- and 14-fold after 30- and 60-min exposures to ACTH, returning to near its control value by 4 h. Overall, in a total of five experiments, a 4-h exposure to ACTH reduced bKv1.4 transcripts by 91.8 ± 0.5%.

AZF cells express a single population of ACTH receptors with K_d values of 1–2 nM (18–20). However, ACTH stimulates cortisol secretion, inhibits I_{AC} K⁺ current and depolarizes AZF cells at 100–1000-fold lower concentrations (5, 10). We found that ACTH also potently inhibited bKv1.4 mRNA expression at picomolar concentrations. In the experiment illustrated in Fig. 5, AZF cells were exposed for 4 h to ACTH at concentrations ranging from 0.1 to 1000 pM. ACTH reduced bKv1.4 mRNA with an estimated IC_{50} of 1.2 pM.

ACTH Inhibits Expression of bKv1.4-associated K⁺ Current—The dramatic ACTH-induced reduction in bKv1.4-specific transcripts should eventually lead to a corresponding decrease in the associated K⁺ channel protein, and voltage-gated K⁺ current. In whole-cell patch clamp recordings, the bKv1.4 A-type K⁺ current (I_A) is robustly expressed in nearly every cell as described previously (3). ACTH (1 nM) produced a time-de-
ACTH Potently Inhibits Adrenal Kv1.4 K⁺ Channel Expression

The experiment illustrated in Fig. 6A shows current-voltage relationships recorded from AZF cells that had been cultured for 1 and 48 h in control medium or medium supplemented with ACTH (1 nM). Exposing AZF cells to ACTH for a period of 1 h failed to reduce bKv1.4 peak currents measured at test potentials between -60 mV and +60 mV. In contrast, after 48 h, ACTH dramatically reduced peak current amplitude at every test potential. The maximum peak current measured at +60 mV was reduced by 80%. Overall, ACTH did not alter bKv1.4 K⁺ current after 1 h, but reduced maximum peak currents measured at 24, 48, and 72 h by 36%, 78%, and 79%, respectively (Fig. 6B). Because ACTH reduced Iₐ current to a similar extent over a wide range of test potentials, it is unlikely that this reduction resulted from a shift in the voltage dependence of channel gating.

In contrast to ACTH, AII (10 nM) failed to reduce bKv1.4 currents measured at 24 or 48 h (data not shown). The ineffectiveness of AII in reducing Iₐ K⁺ currents in AZF cells is consistent with its failure to reduce bKv1.4 mRNA.

Role of cAMP and Protein Kinase A in Inhibition of bKv1.4 Expression—cAMP is the primary intracellular messenger for ACTH and mediates rapid and delayed effects of this peptide in AZF cells (8, 9). Accordingly, the membrane-permeable cAMP analog 8-pcpt-cAMP reduced bKv1.4 mRNA with an effectiveness and temporal pattern similar to that observed with ACTH.

In the experiment illustrated in Fig. 7A, 8-pcpt-cAMP produced a time-dependent decrease in bKv1.4 mRNA, measured over a period of 4 h. Exposure of these cells to 250 μM 8-pcpt-cAMP for 1, 2, and 4 h reduced bKv1.4 by 32%, 76%, and 84% relative to the control value measured at 4 h. In a total of four experiments, 8-pcpt-cAMP reduced bKv1.4 mRNA measured at 4 or 5 h by 84.5 ± 2.2%.

Many of the effects of ACTH and cAMP in adrenocortical cells are mediated through the activation of protein kinase A (9). However, induction of immediate early genes and inhibition of Iₐ, K⁺ channels by ACTH and cAMP is not prevented by pretreating AZF cells with selective protein kinase A antagonists, suggesting the involvement of additional signaling pathways (8, 10). In the present study, we found that the potent protein kinase A antagonist H-89 (IC₅₀ < 50 nM) (21) only partially suppresses the inhibition of bKv1.4 expression by ACTH or 8-pcpt-cAMP. In the experiment illustrated in Fig. 7B, a 4-h exposure to ACTH (1 nM) or 8-pcpt-cAMP (250 μM) reduced bKv1.4 mRNA to almost undetectable levels. In the presence of H-89 (25 μM), ACTH and 8-pcpt-cAMP were somewhat less effective reducing bKv1.4 mRNA by 80% and 65%, respectively. Similar results were obtained in three experiments using H-89 at concentrations between 5 and 25 μM.

8-pcpt-cAMP Inhibits Expression of bKv1.4 K⁺ Current—Prolonged exposure of AZF cells to 8-pcpt-cAMP triggered a progressive decrease in bKv1.4-type membrane current, with a temporal pattern similar to that observed for ACTH. The experiment illustrated in Fig. 8A shows that maintaining cells for 1 h in the presence of 250 μM 8-pcpt-cAMP failed to produce a decrease in bKv1.4 K⁺ current measured at test potentials between -60 and +60 mV. However, after AZF cells were cultured for 72 h in the presence of this cyclic nucleotide, bKv1.4 K⁺ peak current amplitude was reduced by >90% over this entire range of voltages.

Overall, 8-pcpt-cAMP did not alter bKv1.4 K⁺ current after 1 h, but reduced maximum peak currents measured at 24, 48, and 72 h by 45%, 57%, and 81% respectively, when measured against time matched controls (Fig. 8B). Over this same 72-h period, control bKv1.4 K⁺ current amplitudes decreased by less than 20% (Fig. 8B).

Comparative Effects of ACTH and 8-pcpt-cAMP on bKv1.4 mRNA and K⁺ Current—Comparison of ACTH- and 8-pcpt-cAMP-mediated effects on bKv1.4 illustrate the similarities between the two agents and the differences in temporal patterns for the reduction of bKv1.4 mRNA and associated K⁺ current. Specifically, as illustrated in Fig. 9, ACTH and 8-pcpt-cAMP reduce the quantity of bKv1.4 mRNA with t₁/₂ values of 1.0 and 1.4 h, respectively. By comparison, the associated membrane currents disappeared with estimated t₁/₂ values of 21 and 27 h. These results indicated that, in the absence of bKv1.4 channel synthesis, AZF cell channels turn over with a t₁/₂ = 1 day.

**DISCUSSION**

A bovine adrenocortical Kv1.4 K⁺ channel cDNA was cloned from an AZF cDNA library. bKv1.4, which codes for a rapidly inactivating voltage-gated channel, is the first K⁺ channel cloned from steroid hormone-secreting cells. The expression of bKv1.4 transcripts is rapidly and potently inhibited by ACTH through a mechanism that may be dependent on cAMP, but which is partially independent of protein kinase A. The dramatic ACTH- and cAMP-induced reduction of bKv1.4 mRNA is followed by a similar reduction in A-type K⁺ current. These
results indicate that the expression of bKv1.4 K\(^+\) channels is tightly coupled to the HPA axis through rhythmic circadian and stress-induced ACTH secretion.

**Comparison of Kv1.4 K\(^+\) Channel cDNAs—** Adrenal cortical bKv1.4 cDNA is typical of other K\(^+\) channel cDNAs of this family (17, 22). With respect to function, each of these code for a voltage-gated, rapidly inactivating, 4-aminopyridine-sensitive K\(^+\) channel, with properties similar to the A-type K\(^+\) current in AZF cells (3).

bKv1.4 also resembles other Kv1.4 channel cDNAs with respect to nucleotide sequence. Specifically, large 5' and 3'-untranslated regions have previously been observed in other Kv1.4 channel cDNAs (17, 22, 23). A complete characterization of a Kv1.4 K\(^+\) channel expressed in mouse heart and brain showed that the transcription unit includes a 1962-nt coding region, a 1337-base pair 5'-non-coding region, and either 1118-nt or 170-nt 3'-non-coding regions (17). The long and shorter 3'-non-coding regions give rise to separate 4.5- and 3.5-kb transcripts, each of which code for an identical K\(^+\) channel.

The 4386-nt AZF cell bKv1.4 cDNA that we have cloned is most homologous to the larger of the two mouse Kv1.4 channel transcripts. Notably, in the 3'-non-coding region of bKv1.4, five ATTTA repeats were identified. An identical number of these repeats are present in the 3'-non-coding region of the mouse large transcript (17).

Further, the mouse Kv1.4 3'-non-coding region contains two polyadenylation signals, one of which is more than 900 base pairs 5' to the other, thereby allowing for the generation of two transcripts that differ by approximately 1 kb in length. Accordingly, we found that bKv1.4 cDNA probes hybridized to two separate AZF cell transcripts that differed in size by about 1 kb. Interestingly, the adrenal cortical bKv1.4 cDNA contains a polyadenylation signal about 1 kb 5' to its 3' end.

**Relationship of bKv1.4 to BAK4 and Specific Expression in AZF Cells—** Although the putative adrenal medullary BAK4 cDNA is 1267 nucleotides shorter than the AZF cell bKv1.4 cDNA due to incomplete characterization of the 5'- and 3'-coding regions, the overall sequence homology between the cDNAs is greater than 99% (13). The near identity of the two

---

**Fig. 6. Inhibition of I\(_A\) K\(^+\) current expression by ACTH in bovine AZF cells.** AZF cells were plated in DMEM/F12 containing no further additions (control) or this same medium supplemented with ACTH (1 nM). I\(_A\) K\(^+\) currents were recorded in the whole-cell configuration at times ranging from 1 to 72 h. K\(^+\) currents were activated by voltage steps from −80 mV applied at 10-s intervals to test potentials between −60 and +60 mV in 10-mV increments. A, current/voltage relationships for control and ACTH-treated cells recorded after 1- or 48-h exposures to ACTH (1 nM) as indicated. Current records and associated current/voltage relationships obtained by plotting peak I\(_A\) currents against membrane potential. B, temporal pattern for ACTH-mediated decrease in I\(_A\) current. Summary of results from experiments as in A is shown. Maximum peak I\(_A\) currents are plotted against time-matched controls for cells incubated for 1, 24, 48, and 72 h in 1 nM ACTH. Values are mean ± S.E. for indicated number of determinations.
ACTH Potently Inhibits Adrenal Kv1.4 K⁺ Channel Expression

**Fig. 7. Regulation of bKv1.4 mRNA expression by cAMP and protein kinase A.** Bovine AZF cells were plated as described above in the legend of Fig. 3. After 24 h, medium was aspirated and replaced with the same medium supplemented with ACTH (1 nM), 8-pcpt-cAMP (250 μM), and/or H-89 (25 μM) as indicated. A. time-dependent inhibition of bKv1.4 mRNA expression by 8-pcpt-cAMP. AZF cells were incubated with 8-pcpt-cAMP for periods of 0.5–4 h before isolating total RNA. B. effect of H-89 on inhibition of bKv1.4 mRNA by ACTH and 8-pcpt-cAMP. AZF cells were preincubated for 30 min in the presence of 25 μM H-89 before the addition of either ACTH or 8-pcpt-cAMP from concentrated stocks. Total RNA was isolated after 4 h. For Northern blots, each lane contained 15 μg of total RNA. Membranes were probed with a 2295-bp fragment of bKv1.4 cDNA as described under "Experimental Procedures." After stripping, membranes were reprobed with GAPDH.

cDNAs suggests that they originate from the same gene, and that the reported differences are due to cloning or sequencing artifacts.

bKv1.4 and BAK4 cDNAs likely originate from a common gene that could conceivably be expressed in both AZF and adrenal chromaffin cells. However, several lines of evidence indicate that this channel is expressed exclusively by adrenocortical cells.

Most importantly, systematic studies of K⁺ channel currents in bovine adrenocortical and chromaffin cells using the patch clamp technique have shown that only cortical cells express a rapidly inactivating A-type K⁺ current (3, 7, 8, 24). In fact, this A-type current is the most prominent K⁺ current expressed in virtually every adrenal fasciculata and glomerulosa cell (3, 5, 8). No similar current is present in chromaffin cells. In a comprehensive study using both whole-cell and single channel patch clamp recording, Marty and Neher (24) identified three distinct K⁺ channels in bovine chromaffin cells, none of which was an A-type channel.

In the absence of functional IₐCa K⁺ channel proteins, it is unlikely that the corresponding mRNA would be expressed by adrenal chromaffin cells. In recognition of this fact, Garcia-Guzman et al., in their study describing the cloning of BAK4 cDNA (13), speculate that the BAK4 channel was expressed in adrenal medullary fibroblasts or endothelial rather than chromaffin cells.

Northern blot analysis of poly(A)⁺ mRNA from freshly dissected adrenal cortex and medulla tissue showed strong expression of the ~3.4- and ~4.4-kb transcripts in the cortex, but much weaker expression of a similar size transcript in the medulla. It is significant that expression by the cortical tissue was still far stronger than that from the medulla.

In view of the fact that it is highly unlikely that bKv1.4 mRNA is expressed by chromaffin cells, the faint signal obtained from medulla tissue could reflect our inability to completely separate cortex from medulla in the dissection, or the expression of a Kv1.4-type channel by medullary fibroblasts or endothelial cells. Alternatively, the bKv1.4 cDNA fragment used as a probe could have hybridized to mRNA coding for other K⁺ channels that are present in the medulla. Among the multiple K⁺ channel subtypes expressed by bovine adrenal chromaffin cells is a Kv1.5 delayed rectifier that is greater than 80% homologous to bKv1.4 in the S1–S4 coding region (24, 25).

It is possible that the bovine adrenal medullary cDNA library that was screened to obtain BAK4 contained contaminating cDNAs from the adrenal cortex. These were preferentially detected upon screening the library with a Kv1.4-type cDNA (13). By comparison, sequencing and polymerase chain reaction-based analysis of 64 “positive” clones obtained after the initial screening of our adrenal cortical cDNA library indicated that all 64 were bKv1.4 cDNAs. It is likely that the adrenal bKv1.4 channel is specifically expressed in the adrenal cortex. Further, the inhibition of bKv1.4 mRNA expression by ACTH proves that it is the corticosteroid-secreting cells in the adrenal cortex that express the gene, since only these cells have ACTH receptors.

Although we cannot formally exclude the possibility that bKv1.4 mRNA is expressed at extremely small concentrations in the adrenal medulla, this has no apparent physiological relevance since functional A-type K⁺ channels have never been detected there. Even if bKv1.4 were expressed in medullary cells, it would not alter the findings of the present study since the HPA axis functions independently to regulate cortisol production by AZF cells.

**Signaling Pathway for ACTH Inhibition of bKv1.4 Expression**—Although ACTH and AII function through different signaling pathways, they produce many similar effects in bovine AZF cells. Each peptide inhibits IₐCa K⁺ current, depolarizes AZF cells, stimulates cortisol secretion, and enhances the transcription of steroidogenic enzymes and immediate early genes (5, 8–10).

The markedly different effects of these two peptides on the expression of bKv1.4 mRNA and corresponding K⁺ current represent a unique difference in their actions in AZF cells and point to a divergence in the underlying signaling pathways. In this regard, the well correlated ACTH- and cAMP-mediated decreases in bKv1.4 mRNA and corresponding K⁺ current imply a cAMP-dependent mechanism. Although cAMP has long been considered to be the primary intracellular messenger for ACTH, a requirement for Ca²⁺ is well established (6, 26). cAMP and Ca²⁺ are dual second messengers in ACTH-stimulated corticosteroid synthesis (8, 26). However, the failure of AII to inhibit the expression of bKv1.4 mRNA or associated K⁺ current argues against the involvement of Ca²⁺ in these responses, since Ca²⁺ is a primary intracellular messenger for AII in AZF cells (27, 28). Thus, ACTH-mediated inhibition of bKv1.4 expression probably requires cAMP but not Ca²⁺.

**Requirement for Protein Kinase A**—Until recently, all of the actions of ACTH in AZF cells were believed to be mediated by cAMP through activation of protein kinase A. The extraordinary potency of ACTH as an inhibitor of bKv1.4 mRNA expression and the relative insensitivity of the ACTH and 8-pcpt-cAMP reductions in bKv1.4 transcripts to H-89 raise questions regarding the signaling pathways involved.

Specifically, ACTH inhibited bKv1.4 mRNA expression almost completely at 10 pm, a concentration where only about 1% of ACTH receptors are occupied and where only small increases
in cAMP can be measured (18, 20, 29, 30). However, ACTH also inhibits IAC $K^+$ current, depolarizes AZF cells, induces steroidogenic enzymes and immediate early genes, and stimulates cortisol secretion at these same picomolar concentrations (5, 8, 10, 31).

The partial inhibition of ACTH- and 8-pcpt-cAMP-mediated reduction of $bKv1.4$ mRNA expression by H-89 indicates a requirement for protein kinase A, but suggests the involvement of additional mechanisms. In this regard, ACTH and cAMP can inhibit IAC $K^+$ current in bovine AZF cells and increase the number of functional T-type Ca$^{2+}$ channels in rat AZF cells by mechanisms that appear to be independent of protein kinase A (8, 32). However, we cannot exclude the possibility that H-89, even though used at concentrations 100- and 500-fold higher than the reported IC$_{50}$ of 50 nM (21), failed to completely inhibit protein kinase A in our study. However, in a previous study, we showed that H-89 (5 mM) inhibited protein kinase A by 97–100% in cytoplasmic extracts from AZF cells (7).

**Molecular Mechanism for ACTH-mediated Reduction of $bKv1.4$ mRNA**—The ACTH-induced reduction of $bKv1.4$ mRNA and subsequent disappearance of the associated $K^+$ current are among the first examples demonstrating the hormonal regulation of $K^+$ channel expression at a pretranslational level in a physiological pathway. Although this response may be mediated through cAMP, the precise mechanism involved has not been determined. The rapid decline in $bKv1.4$ mRNA triggered by ACTH could be due to a reduction in the rate of gene transcription, or a specific decrease in RNA stability.

Several studies reporting the regulation of $K^+$ channel gene expression by cAMP have appeared. In one, 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 tumor GH3 cell line (33). In contrast, elevation of intracellular cAMP accelerates the degradation of $Kv1.1$ $K^+$ channel mRNA in a C6 glioma cell line (34). Thus, cAMP has been shown to alter $K^+$ channel gene expression by modulating transcription or mRNA stability.

If ACTH-mediated reduction of $bKv1.4$ gene expression is mediated through inhibition of transcription, this would likely occur through activation or induction of specific transcription factors. cAMP regulates the transcription of many genes through an protein kinase A-dependent phosphorylation and activation of CREB transcription factors (35). However, CREB transcription factors are phosphorylated by both cAMP- and Ca$^{2+}$/calmodulin-dependent protein kinases (35, 36). Since activation of AT$_1$ receptors by AII leads to an inositol 1,4,5-trisphosphate-induced rise in intracellular Ca$^{2+}$, the failure of this peptide to reduce $bKv1.4$ mRNA argues against CREB activation as a mechanism for ACTH. In addition, CREB pro-

---

**FIG. 8. Inhibition of I$_{A}$ $K^+$ current by 8-pcpt-cAMP in bovine AZF cells.** AZF cells were plated in DMEM/F12 + containing no further additions (control) or this same medium supplemented with 8-pcpt-cAMP (250 μM). $K^+$ currents were recorded in the whole-cell configuration at times ranging from 1 to 72 h. $K^+$ currents were activated by voltage steps applied from −80 mV at 10-s intervals to test potentials between −60 and +80 mV in 10-mV increments. $A$, current/voltage relationships for control and 8-pcpt-cAMP-treated cells recorded after 1- or 72-h exposures as indicated. Current records and associated current/voltage relationships obtained by plotting peak I$_{A}$ currents against membrane potential. $B$, temporal pattern for ACTH-mediated decrease in I$_{A}$ current. Summary of results from experiments as in $A$ is shown. Maximum peak I$_{A}$ current amplitudes are shown with corresponding time-matched controls for 1, 24, 48, and 72 h exposures to 8-pcpt-cAMP. Values are mean ± S.E. for the indicated number of determinations.
ACTH Potently Inhibits Adrenal Kv1.4 K⁺ Channel Expression

Fig. 9. Comparative effects of ACTH and 8-pcpt-cAMP on bKv1.4 and I₂,₅ current amplitudes in bovine AZF cells. The temporal pattern for ACTH and 8-pcpt-cAMP-induced reductions in bKv1.4 mRNA and associated I₂,₅ current are compared in A and B, respectively. In each case, bKv1.4 mRNA was quantitated from optical density measurements taken from autoradiograms shown in Fig. 4 (ACTH) and Fig. 6 (8-pcpt-CAMP). These values were expressed as percentage of maximum and plotted against I₂,₅ current amplitudes expressed as a fraction of the maximum value at t = 0 for ACTH-treated cells (A) or 8-pcpt-cAMP-treated cells (B). Values for I₂,₅ current amplitudes measured at 1, 24, 48, and 72 h are the averaged values displayed in Figs. 5B and 7B.

The extremely potent inhibition of bKv1.4 mRNA expression by ACTH at physiological concentrations suggests that the quantity of this mRNA and the associated channels would be tightly coupled in an inverse relationship to the ACTH concentration in the blood (42). ACTH is secreted episodically in a circadian rhythm and increases dramatically during periods of stress (42, 43). Presumably, bKv1.4 mRNA levels might oscillate in a rhythmic diurnal fashion in parallel with ACTH.

In the absence of stress or diseases, bKv1.4 expression would exist under the tonic inhibitory influence of ACTH. Under stressful conditions where ACTH secretion increases dramatically, the expression of bKv1.4 mRNA and synthesis of the corresponding K⁺ channel could be completely inhibited. Chronic overstimulation of the adrenal cortex by ACTH under conditions of prolonged stress, or diseases such as congenital adrenal hyperplasia, could engender sustained changes in the electrical and secretory properties of AZF cells by altering the expression of specific ion channels.

REFERENCES

1. Hille, B. (1992) Ionic Channels of Excitable Membranes, Sinauer Associates Inc., Sunderland, MA
2. Milnar, B., Biagi, B. A., and Enyeart, J. J. (1993) J. Gen. Physiol. 102, 217–237
3. Milnar, B., and Enyeart, J. J. (1995) J. Gen. Physiol. 102, 239–255
4. Lyngar, C. R., Matthews, E. R., and Saffron, M. (1982) Endocrinology 110, 462–468
5. Milnar, B., Biagi, B. A., and Enyeart, J. J. (1993) J. Biol. Chem. 268, 8640–8644
6. Enyeart, J. J., Milnar, B., and Enyeart, J. A. (1993) Mol. Endocrinol. 7, 1031–1040
7. Enyeart, J. J., Gomora, J. C., Xu, L., and Enyeart, J. A. (1996) J. Gen. Physiol. 108, 251–264
8. Simpson, E. R., and Waterman, M. R. (1988) Annu. Rev. Physiol. 50, 427–440
9. Enyeart, J. J., Boyd, R. T., and Enyeart, J. A. (1996) Mol. Cell. Endocrinol. 124, 97–110
10. Wilson, T. E., Mauw, A. R., Weaver, C. A., Milbrandt, J., and Parker, K. L. (1993) Mol. Biol. Cell. 13, 861–868
11. Davis, I. J., and Lau, L. F. (1994) Mol. Cell. Biol. 14, 3469–3483
12. Garcia-Guzman, M., Calvo, S., Cena, V., and Criado, M. (1992) J. Membr. Biol. 114, 3235–3244
13. Velan, B. (1995) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 6.0.1–6.3.2, Wiley-Interscience, New York
14. Raikhinstein, M., Zohar, M., and Hanukoglu, I. (1994) Biochim. Biophys. Acta 1220, 293–329
15. Catalano R. D., Stuve, L., and Ramachandran, J. (1986) J. Clin. Endocrinol. Metab. 62, 300–304
16. Mountjoy, K. G., Robbins, L. S., Mowrtord, M. T., and Cone, R. D. (1992) Science 257, 1248–1251
17. Hidaka, H., Watanabe, M., and Kobayashi, R. (1991) Methods Enzymol. 201, 328–339
18. Stuhmer, W., Ruppersberg, J. P., Schroter, K. H., Sakhmann, B., Stocker, M., Giese, K. P., Perschke, A., Baumann, A., and Pongs, O. (1989) EMBO J. 8, 3235–3244

The extremely potent inhibition of bKv1.4 mRNA expression by ACTH at physiological concentrations suggests that the quantity of this mRNA and the associated channels would be tightly coupled in an inverse relationship to the ACTH concentration in the blood (42). ACTH is secreted episodically in a circadian rhythm and increases dramatically during periods of stress (42, 43). Presumably, bKv1.4 mRNA levels might oscillate in a rhythmic diurnal fashion in parallel with ACTH.

In the absence of stress or diseases, bKv1.4 expression would exist under the tonic inhibitory influence of ACTH. Under stressful conditions where ACTH secretion increases dramatically, the expression of bKv1.4 mRNA and synthesis of the corresponding K⁺ channel could be completely inhibited. Chronic overstimulation of the adrenal cortex by ACTH under conditions of prolonged stress, or diseases such as congenital adrenal hyperplasia, could engender sustained changes in the electrical and secretory properties of AZF cells by altering the expression of specific ion channels.

REFERENCES

1. Hille, B. (1992) Ionic Channels of Excitable Membranes, Sinauer Associates Inc., Sunderland, MA
2. Milnar, B., Biagi, B. A., and Enyeart, J. J. (1993) J. Gen. Physiol. 102, 217–237
3. Milnar, B., and Enyeart, J. J. (1995) J. Gen. Physiol. 102, 239–255
4. Lyngar, C. R., Matthews, E. R., and Saffron, M. (1982) Endocrinology 110, 462–468
5. Milnar, B., Biagi, B. A., and Enyeart, J. J. (1993) J. Biol. Chem. 268, 8640–8644
6. Enyeart, J. J., Milnar, B., and Enyeart, J. A. (1993) Mol. Endocrinol. 7, 1031–1040
7. Enyeart, J. J., Gomora, J. C., Xu, L., and Enyeart, J. A. (1996) J. Gen. Physiol. 108, 251–264
8. Simpson, E. R., and Waterman, M. R. (1988) Annu. Rev. Physiol. 50, 427–440
9. Enyeart, J. J., Boyd, R. T., and Enyeart, J. A. (1996) Mol. Cell. Endocrinol. 124, 97–110
10. Wilson, T. E., Mauw, A. R., Weaver, C. A., Milbrandt, J., and Parker, K. L. (1993) Mol. Biol. Cell. 13, 861–868
11. Davis, I. J., and Lau, L. F. (1994) Mol. Cell. Biol. 14, 3469–3483
12. Garcia-Guzman, M., Calvo, S., Cena, V., and Criado, M. (1992) J. Membr. Biol. 114, 3235–3244
13. Velan, B. (1995) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 6.0.1–6.3.2, Wiley-Interscience, New York
14. Raikhinstein, M., Zohar, M., and Hanukoglu, I. (1994) Biochim. Biophys. Acta 1220, 293–329
15. Catalano R. D., Stuve, L., and Ramachandran, J. (1986) J. Clin. Endocrinol. Metab. 62, 300–304
16. Mountjoy, K. G., Robbins, L. S., Mowrtord, M. T., and Cone, R. D. (1992) Science 257, 1248–1251
17. Hidaka, H., Watanabe, M., and Kobayashi, R. (1991) Methods Enzymol. 201, 328–339
18. Stuhmer, W., Ruppersberg, J. P., Schroter, K. H., Sakhmann, B., Stocker, M., Giese, K. P., Perschke, A., Baumann, A., and Pongs, O. (1989) EMBO J. 8, 3235–3244
ACTH Potently Inhibits Adrenal Kv1.4 K⁺ Channel Expression

23. Chandy, K. G., and Gutman, G. A. (1994) in Ligand and Voltage-gated Ion Channels (North, R. A., ed) pp. 1–71, CRC Press, Inc., Boca Raton, FL
24. Marty, A., and Neher, E. (1985) J. Physiol. 367, 117–141
25. Garcia-Guzman, M., Sala, F., Criado, M., and Sala, S. (1994) FEBS Lett. 354, 173–176
26. Kojima, I., and Ogata, E. (1986) J. Biol. Chem. 261, 9832–9838
27. Quinn, S. J. (1988) Annu. Rev. Physiol. 50, 409–426
28. Kojima, I., Kojima, K., and Rasmussen, H. (1985) J. Biol. Chem. 260, 9171–9176
29. Yamazaki, T., Kimoto, T., Higuchi, K., Obta, Y., Kawato, S., and Kominami, S. (1986) Endocrinology 119, 478–497
30. Moyle, W. R., Kong, Y. C., and Ramachandran, J. (1973) J. Biol. Chem. 248, 2409–2417
31. Sala, G. B., Hayashi, K., Catt, K. J., and Dufau, M. L. (1979) J. Biol. Chem. 254, 3861–3865
32. Barbara, J.-G., and Takeda, K. (1995) J. Physiol 488, 699–622
33. Meri, Y., Matsubara, H., Pelco, E., Siegel, A., and Koren, G. (1993) J. Biol. Chem. 268, 26482–26493
34. Allen, M., Koh, D.-S., and Tempel, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7693–7698
35. Shaywitz, A. J., and Greenberg, M. E. (1999) Annu. Rev. Biochem. 68, 821–861
36. Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991) Science 252, 1427–1430
37. Zazopoulos, E., Lalli, E., Stocco, D. M., and Sassone-Corsi, P. (1997) Nature 390, 311–315
38. Parker, K. L., and Schimmer, B. P. (1995) Vitam. Horm. 51, 339–370
39. Molina, C. A., Foulkes, N. S., Lalli, E., and Sassone-Corsi, P. (1993) Cell 73, 875–886
40. Stehle, J. H., Foulkes, N. S., Molina, C. A., Simonneau, V., Pevet, P., and Sassone-Corsi, P. (1993) Nature 365, 314–320
41. Zuber, M. X., John, M. E., Okamura, T., Simpson, E. R., and Waterman, M. R. (1986) J. Biol. Chem. 261, 2475–2482
42. Veldhuis, J. D., Iranmanesh, A., Johnson, M. L., and Lizarralde, G. (1990) J. Clin. Endocrinol. Metab. 71, 452–463
43. Bondy, P. K. (1985) in Williams Textbook of Endocrinology, pp. 816–890, W. B. Saunders Co., Philadelphia