Adrenocorticotropic hormone (ACTH) and angiotensin II (AII) are peptides that regulate the production of steroid hormones by cells of the adrenal cortex. The cellular mechanisms linking these peptides to corticosteroid hormone secretion are not understood. In patch clamp recordings from bovine adrenal zona fasciculata (AZF) cells, we have identified a novel choleratoxin-sensitive K⁺ current (I_{AC}), which is potently inhibited by both ACTH and AII with respective EC₅₀ values of 4.5 and 145 pm. These two peptides depolarize AZF cells with a temporal pattern and potency that parallels the inhibition of I_{AC}. With the discovery of I_{AC}, we have identified a common molecular target for both ACTH and AII. The convergent inhibition of I_{AC} by these two peptides suggests a mechanism whereby biochemical signals originating at the cell membrane can be transduced to depolarization-dependent Ca²⁺ entry and steroid hormone secretion.

Transmitter and hormone release by many secretory cells is tightly coupled to depolarization-dependent Ca²⁺ entry. A requirement for Ca²⁺ in steroidogenesis by cells of the adrenal cortex is well established (1, 2). Several lines of evidence indicate that ACTH and AII-stimulated cortisol and aldosterone production involve Ca²⁺ entry through voltage-gated channels (5-8). Although separate signaling pathways and second messengers for these regulatory peptides have been described (9-11), no specific mechanism linking the peptide receptors to membrane depolarization and corticosteroid secretion has been discovered. In particular, ion channels common to steroid secreting cells that control membrane potential and whose modulation by peptides would allow depolarization-dependent Ca²⁺ entry and secretion have not been identified. In this report, we describe a novel K⁺ current I_{AC} in bovine AZF cells. I_{AC} displays properties expected of a K⁺ channel that could set the membrane potential of adrenal cells. In addition, it is potently inhibited by both ACTH and AII.

**MATERIALS AND METHODS**

Tissue culture media, antibiotics, fibronectin, and fetal calf serum were obtained from Gibco. Culture dishes were purchased from Corning (Corning, NY). Coverslips were from Belco (New Jersey). Enzymes and ACTH(1-24), GTP, ATP-γS, GDP-βS, GTP-γS, cholera toxin, pertussis toxin, tetraethylammonium, 4-aminoypyridine, and spamic were obtained from Sigma. α-Dendrotetoxin was obtained from Alomone Laboratories (Jerusalem, Israel).

**Isolation and Culture of AZF Cells**—Bovine adrenal glands were obtained from steers (age 3–9 months) at a local slaughterhouse. Fatty tissue was removed immediately, and the glands were transported to the laboratory in ice-cold phosphate-buffered saline containing 0.2% dextrose. Isolated AZF cells were prepared as previously described (12) with some modifications. In a sterile tissue culture hood, the adrenals were cut in half lengthwise and the lighter medulla tissue trimmed away from the cortex and discarded. The capsule with attached glomerulosa and thicker fasciculata-reticularis layer was then dissected into pieces approximately 1.0 × 1.0 × 0.5 cm. A Stadie-Riggs tissue slicer (Thomas Scientific) was used to slice fasciculata-reticularis tissue from the glomerulosa layers. Fasciculata-reticularis slices were diced into 0.5-mm³ pieces and dissociated with 2 mg/ml (about 200 units/ml) of Type I collagenase, 0.2 mg/ml deoxyribonuclease in MEM plus 100 units/ml penicillin, 0.1 mg/ml streptomycin for approximately 45 min at 37 °C in a shaking water bath, triturating after 15 and 30 min with a sterile, plastic transfer pipette. After incubating, the suspension was filtered through one layer of sterile cheesecloth, centrifuged to pellet cells at 100 x g for 5 min. The cells were then washed twice with MEM, centrifuging as before to pellet. Cells were filtered through 200-μm stainless steel mesh to remove clumps after resuspending in MEM. Isolated cells were again centrifuged and either resuspended in DMEM/Ham's F-12 (1:1) with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and plated for immediate use or resuspended in FBS, 5% MEso, divided into 1 ml aliquots each containing about 1 × 10⁶ cells, and stored in liquid nitrogen for future use. Cells were plated in 35-mm dishes containing 8-mm² glass coverslips, which had been treated with fibronectin (10 μg/ml) at 37 °C for 30 min and then rinsed twice with warm, sterile phosphate-buffered saline immediately before adding cells. Dishes were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

The functional state of isolated AZF cells was determined by measuring cortisol secretion from cultured cells under basal conditions and in response to the pituitary hormone ACTH. Enzymatically dissociated AZF cells were cultured on fibronectin-treated 35-mm plates at a density of about 4 × 10⁶ cells/dish in DMEM/F-12 (1:1) as described above. After 24 h, media was replaced with control media or the same media containing 10⁻⁴ ACTH(1-24). Cells were returned to the incubator for 24 h at which time media was collected and cortisol measured using a solid phase radioimmunoassay kit (Diagnostic Products Corp. Los Angeles, CA). Control cells produced cortisol at a rate of 0.57 ± 0.02 ng/10⁶ cells/24 h. ACTH increased cortisol production 50-fold to 98.30 ± 4.26 ng/10⁶ cells/24 h.

**Solutions and Recording Conditions**—Patch clamp recordings of K⁺ channel currents were made in the whole-cell and outside-out configurations. The standard pipette solution was 120 mM KCl, 2 mM MgCl₂, 7 mM CaCl₂, 10 mM HEPES, 11 mM BaCl₂, 200 μM GTP, 2 mM MgATP with pH buffered to 7.2 using KOH.
solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM HEPES, and 5 mM glucose, pH 7.4, using NaOH. Deviations from these solutions are noted in the text. All solutions were filtered through 0.22-μm cellulose acetate filters.

AZF cells were used for patch clamp experiments 2-48 h after plating. Typically, cells with diameters of 15-30 μm and capacitances which was continuously perfused by gravity at a rate of 4-6 ml/min. Path electrodes with resistances of 1.0-2.0 megohms were fabricated from Corning RC-6 or 0010 glass (Garner Glass Co., Claremont, CA). K\(^+\) currents were recorded at room temperature (22-24 °C) following the procedure of Hamill et al. (13) using a List EPC-7 patch clamp amplifier.

Pulse generation and data acquisition were done using an IBM-AT computer and PCLAMP software with an Axolab interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 1-50 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 1/2 to 1/4 amplitude. Data were analyzed and plotted using PCLAMP (CLAMP-PAN and CLAMPFIT) and GraphPAD InPLOT. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

**Intracellular Voltage Recordings**—Intracellular recordings of membrane potential were made at 35-37 °C using a WPI model FD-223 electrometer and glass electrodes, which when filled with 1 M KCl had resistances of 100-150 M. Cells were continuously superfused with bath solution containing (in mM): 140 NaCl, 5 KCl, 2 MgCl\(_2\), 2 CaCl\(_2\), 10 HEPES, and 5 glucose, pH 7.4.

**RESULTS**

In whole-cell patch clamp recordings, two distinct components of K\(^+\) current were expressed in nearly all of more than 200 AZF cells studied. These included a rapidly inactivating A-type component, described in detail elsewhere, and a novel non-inactivating current, which in view of its location in cells of the adrenal cortex, we have named I\(_{AC}\). During prolonged recordings, I\(_{AC}\) amplitude increased dramatically from 3.73 ± 0.8 pA/picofarads (n = 45) to 34.98 ± 5.2 pA/picofarads (n = 54) approaching the apparent maximum value with a half-time of 8.5 ± 0.5 min (n = 28). The amplitude of the transient K\(^+\) current typically remained nearly constant in these same recordings (Fig. 1A).

The time-dependent growth of macroscopic I\(_{AC}\) was accompanied by a large increase in current noise indicative of a relatively large unitary conductance.

In addition to widely differing inactivation kinetics, I\(_{AC}\) was clearly distinguished from A-type current by its voltage-independent availability. At a holding potential of −40 mV, the transient K\(^+\) current was completely inactivated while I\(_{AC}\) was unaffected. When I\(_{AC}\) was viewed in isolation under these conditions, no delay in current onset was apparent with depolarizing steps to potentials up to +10 mV, suggesting that I\(_{AC}\) channels remain open at the holding potential (Fig. 1B).

Current-voltage relationships obtained from several cells that expressed negligible A-type current indicated that I\(_{AC}\) channels were open at the resting membrane potential of these cells (−71.1 mV) (Fig. 1C). In contrast, the threshold for A current activation was approximately −40 mV.

Although blocked by several K\(^+\) channel antagonists, I\(_{AC}\) was pharmacologically distinguishable from the A-type K\(^+\) current in AZF cells. At a concentration of 2 mM, 4-amino-pyridine inhibited the A-type current activated by voltage steps to +20 mV almost completely, but reduced I\(_{AC}\) by only 19.6 ± 9.5% (n = 6). A second K\(^+\) channel blocker, tetraethylammonium (20 mM), inhibited I\(_{AC}\) by 57.3 ± 4.1% (n = 3). I\(_{AC}\) was insensitive to d-α-dendrotoxin and apamin at concentrations up to 500 nM, but disappeared completely when CsCl replaced KCl in the recording electrode.

The growth of I\(_{AC}\) in whole cell recordings suggested that

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**FIG. 1.** Characteristics of I\(_{AC}\) in bovine AZF cells. A, time-dependent growth of I\(_{AC}\). K\(^+\) currents were activated at 30-s intervals by voltage steps to +20 mV from a holding potential of −80 mV. Current records at indicated times after initiating whole-cell recordings. B and C, current-voltage relationships for I\(_{AC}\). Current records in response to voltage steps of various sizes applied at 30-s intervals from a holding potential of −40 mV. C, I\(_{AC}\) was measured in a cell with no detectable inactivating K\(^+\) current at potentials ranging from −80 to +40 mV. Test pulse was a 2-s ramp applied from a holding potential of 0 mV beginning at −80 mV. Top trace is current plotted against test potential corrected for series resistance error, in control saline. Bottom trace is current from same cell after superfusion of 10 pm ACTH.
The inhibition of IAc by both peptides began after a delay of 0.2 min, and typically, several additional minutes were required before maximum inhibition was achieved (Fig. 2A). The time-dependent amplitude of IAc was measured as the non-inactivating current present at the end of the test pulse. Results are mean ± S.E. of initial (A) and maximal (B) IAc current densities determined for each cell by dividing IAc amplitude by cell capacitance. IAc reached maximum amplitudes after 20–60 min of recording.

Intracellular voltage recordings showed that bovine AZF cells maintained resting potentials of −71.1 ± 1.1 mV (n = 65) and were depolarized by changing external K⁺ concentrations as predicted by the Nernst equation for a membrane selectively permeable to this ion only. ACTH depolarized cells by a maximum of 49 ± 1.8 mV with an EC₅₀ of 10.4 pm (Fig. 3A). All depolarized cells by a similar amount (52.6 ± 0.9 mV), but this peptide was again less potent (EC₅₀ = 283 pm) (Fig. 5A). Onset of depolarization by either peptide occurred after a delay of one to several minutes and was reversed upon superfusion of saline (Fig. 5B and C).

**DISCUSSION**

The discovery of a novel K⁺ channel that sets AZF cell membrane potential while it is potently inhibited by both ACTH and AI1 suggests a specific mechanism for peptide hormone-stimulated corticosteroid secretion, which emphasizes the importance of electrical events and Ca²⁺ entry. In addition to the two types of K⁺ current described above,
**ACTH and AII Inhibit a Novel K⁺ Current in Adrenal Cells**

**Fig. 4.** Effect of ACTH on single channel currents. Outside-out patch recordings were made at +20 mV before and after superfusing the patch with saline containing 1 nM ACTH. A and B, all points amplitude histograms constructed from 48 consecutive 400-ms sweeps (97,152 total points), before (A) and 5 min after (B) exposing the patch to 1 nM ACTH. Cutoff frequency is 1 kHz. C and D, representative 400-ms sweeps before (C) and after (D) ACTH. Scale bars, 2 pA and 20 ms. E, single-channel IV. Single-channel conductance was obtained for a patch containing one channel at various test potentials from −40 to +50 mV. Points are mean ± S.E. of 4−26 measurements. A unitary conductance of 63 picosiemens was determined by least square linear regression between +20 and +50 mV. Pipette and external solutions as in whole-cell recordings.

**Fig. 5.** Depolarization of AZF cells by ACTH and AII. Concentration dependence and temporal pattern are shown. After impaling a cell and obtaining a stable resting potential, cells were superfused with ACTH(1-24) or AII at various concentrations while continuously recording membrane potential. A, concentration dependence; maximum depolarization is plotted against peptide concentration. Values are mean ± S.E. of 5−9 separate determinations. B and C, time course and reversal of depolarization by 50 pM ACTH and 250 pM AII. Cells were superfused with peptides and control solution at the indicated times.

Bovine AZF cells express a low voltage-activated T-type Ca²⁺ current (15). We propose that these channels determine the electrical properties of AZF cells and transduce biochemical signals originating at the cell membrane to depolarization-dependent Ca²⁺ entry and secretion. Specifically, ACTH and AII acting through separate receptors inhibit IAC, triggering the sequence of membrane depolarization, Ca²⁺ channel activation, and corticosteroid production. In this regard, we have found that T-type Ca²⁺ channel antagonists block ACTH- and AII-stimulated cortisol production and Ca²⁺ current at similar concentrations (15). Although ACTH and AII have been reported to modulate both Ca²⁺ and transient K⁺ currents in rat and bovine adrenal glomerulosa cells (3, 5, 17, 18), these peptides did not affect T⁺ or A currents in bovine AZF cells.

ACTH and AII are known to act on adrenal cortical cells through separate signaling pathways. ACTH stimulates the synthesis of cAMP, whereas AII enhances the production of inositol 1,4,5-trisphosphate and diacylglycerol through activation of phospholipase C. Whether the convergent inhibition of IAC by these two peptides indicates a similar convergence of different second messengers is not known. ACTH blocked IAC and depolarized AZF cells in our study at low picomolar concentrations that produce no measureable increase in cAMP (19, 20). Thus it appears unlikely that either of these effects is mediated by this second messenger. In this regard, previous studies on adrenal cells have led to the conclusion that cAMP is the primary intracellular mediator of ACTH-stimulated steroidogenesis (21−23). This notion remains even though ACTH triggers Ca²⁺ uptake and stimulates corticosteroid production at concentrations far lower than those required to produce increases in cAMP (8, 10, 19, 24). The excellent correlation between ACTH-stimulated inhibition of IAC and membrane depolarization with Ca²⁺ influx and corticosteroid secretion suggest that Ca²⁺ may be the primary intracellular messenger, particularly at low ACTH concentrations.

Activation of phospholipase C by AII enhances the synthesis of inositol 1,4,5-trisphosphate and diacylglycerol and triggers the release of Ca²⁺ from intracellular stores. Whether one of these second messengers inhibits IAC is not known. Although Ca²⁺-mediated inhibition of K⁺ channels has been observed in several cell types (14, 25, 26), it is unlikely that Ca²⁺ released from intracellular stores inhibited IAC in our experiments since intracellular Ca²⁺ was highly buffered by BAPTA.

Our results demonstrating inhibition of IAC by GTPγS and CTX indicate that the corresponding channels are under the inhibitory control of a Gs protein. This may be the first outwardly rectifying K⁺ channel to be inhibited by this GTP-binding protein. An outwardly rectifying K⁺ current, which is present at negative potentials and modulated by G-protein activation, has been identified in a leukemic cell line (16). In spite of some resemblance to IAC, the current in leukemia cells differs in several important respects. In particular, the func-

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tional expression of the leukemic cell K* current is blocked by PTx but not CTx indicating the inhibitory control of G* or G* rather than G. The small unitary conductance of the leukemic cell K* channel (8 picosiemens) and its activation by GTP-γ-S also clearly distinguish it from IAC.

It is still unclear whether G* links the activation of ACTH and AII receptors to inhibition of IAC. In this regard, channel modulation that occurs through a G-protein intermediate usually occurs either by a membrane-delimited tight receptor coupling or through a diffusible second messenger. The responses we observed with the two peptides are not consistent with either of these mechanisms. The delay of several minutes required for ACTH or AII to inhibit IAC or to depolarize AZF cells is quite long even for responses requiring synthesis of intracellular second messengers. Furthermore, both peptides inhibit IAC maximally in cells that have been patch-clamped in the whole-cell mode for periods of 1 h. Responses requiring the synthesis of diffusible second messengers would likely have been "washed out" long before this time.

Regardless of the signal transduction pathways involved, the convergent inhibition of IAC by ACTH and AII may represent the major components of a physiological mechanism regulating corticosterone secretion. As in other secretory cells, the physiological regulation of corticosterone production by AZF cells appears tightly coupled to the function and modulation of ion channels.

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