Adrenocorticotropic Hormone and cAMP Inhibit Noninactivating K^+ Current in Adrenocortical Cells by an A-kinase–independent Mechanism Requiring ATP Hydrolysis

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ABSTRACT Bovine adrenal zona fasciculata (AZF) cells express a noninactivating K^+ current (I_{AC}) that is inhibited by adrenocorticotropic hormone (ACTH) at picomolar concentrations. Inhibition of I_{AC} may be a critical step in depolarization-dependent Ca^{2+} entry leading to cortisol secretion. In whole-cell patch clamp recordings from AZF cells, we have characterized properties of I_{AC} and the signalling pathway by which ACTH inhibits this current. I_{AC} was identified as a voltage-gated, outwardly rectifying, K^+-selective current whose inhibition by ACTH required activation of a pertussis toxin–insensitive GTP binding protein. I_{AC} was selectively inhibited by the cAMP analogue 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (8-pcpt-cAMP) with an IC_{50} of 160 μM. The adenylate cyclase activator forskolin (2.5 μM) also reduced I_{AC} by 92 ± 4.7%. Inhibition of I_{AC} by ACTH, 8-pcpt-cAMP and forskolin was not prevented by the cAMP-dependent protein kinase inhibitors H-89 (5 μM), cAMP-dependent protein kinase inhibitor peptide (PKI[5-24]) (2 μM), (Rp)-cAMPS (500 μM), or by the nonspecific protein kinase inhibitor staurosporine (100 nM) applied externally or intracellularly through the patch pipette. At the same concentrations, these kinase inhibitors abolished 8-pcpt-cAMP-stimulated A-kinase activity in AZF cell extracts. In intact AZF cells, 8-pcpt-cAMP activated A-kinase with an EC_{50} of 77 nM, a concentration 2,000-fold lower than that inhibiting I_{AC} half maximally. The active catalytic subunit of A-kinase applied intracellularly through the recording pipette failed to alter functional expression of I_{AC}. The inhibition of I_{AC} by ACTH and 8-pcpt-cAMP was eliminated by substituting the nonhydrolyzable ATP analogue AMP-PNP for ATP in the pipette solution. Penfluridol, an antagonist of T-type Ca^{2+} channels inhibited 8-pcpt-cAMP-induced cortisol secretion with an IC_{50} of 0.33 μM, a concentration that effectively blocks Ca^{2+} channel in these cells. These results demonstrate that I_{AC} is a K^+-selective current whose gating is controlled by an unusual combination of metabolic factors and membrane voltage. I_{AC} may be the first example of an ionic current that is inhibited by cAMP through an A-kinase–independent mechanism. The A-kinase-independent inhibition of I_{AC} by ACTH and cAMP through a mechanism requiring ATP hydrolysis appears to be a unique form of channel modulation. These findings suggest a model for cortisol secretion wherein cAMP combines with two separate effectors to activate parallel steroidogenic signalling pathways. These include the traditional A-kinase–dependent signalling cascade and a novel pathway wherein cAMP binding to I_{AC} K^+ channels leads to membrane depolarization and Ca^{2+} entry. The simultaneous activation of A-kinase- and Ca^{2+}-dependent pathways produces the full steroidogenic response.

KEY WORDS: potassium channel • adrenal cortex • cortisol • protein kinase

INTRODUCTION

Cortisol secretion by zona fasciculata cells of the adrenal cortex occurs in a diurnal pattern under the control of the pituitary peptide adrenocorticotropic hormone (ACTH)[1] (Bondy, 1985; Simpson and Waterman, 1988). The cellular mechanisms that couple ACTH receptor activation to cortisol production are incompletely understood. Early studies have shown that ACTH stimulates the synthesis of cAMP by adrenocortical cells (Lefkowitz, et al., 1971; Moyle et al., 1973). Cyclic AMP, in turn, mimics many of the actions of ACTH: activating steroidogenic enzymes, inducing their synthesis, and stimulating cortisol secretion. Consequently, cAMP has long been considered to be the primary intracellular messenger for ACTH. It has also been widely accepted that the steroidogenic actions of cAMP are mediated by activation of a cAMP-dependent protein kinase signalling cascade (Simpson and Waterman, 1988; Parker and Schimmer, 1995), even though several steroid hydroxylases lack consensus cAMP response elements in their 5′ flanking region (Parker and Schimmer, 1995).

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[1] *Abbreviations used in this paper:* 8-pcpt-cAMP, 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate; ACTH, adrenocorticotropic hormone; A-kinase, cAMP-dependent protein kinase; AMP-PNP, 5′-adenylyl-imidodiphosphate; AII, angiotensin II; AZF, bovine adrenal fasciculata; DMEM, Dulbecco's modified eagle medium; I_{AC}, noninactivating potassium current in bovine adrenal fasciculata cells.; PKI(5-24), cAMP-dependent protein kinase inhibitor peptide; Ptx, pertussis toxin; (Rp)-cAMPS, (Rp)-adenosine-3′,5′-cyclic monophosphorothioate.

In addition to cAMP, a requirement for Ca^{2+} in ACTH-stimulated steroidogenesis by cells of the adrenal cortex is well established (Capponi et al., 1984; Quinn et al., 1991; Enyeart et al., 1993). Transmitter and hormone release by many secretory cells is tightly coupled to depolarization-dependent Ca^{2+} entry. Recently, we identified a novel K^+ current (I_{AC}) in bovine adrenal fasciculata (AZF) cells that may contribute to the resting potential (Mlinar et al., 1993*a*). Importantly, ACTH inhibits I_{AC} with a temporal pattern and potency that parallel membrane depolarization and cortisol secretion. ACTH-stimulated cortisol secretion is inhibited by antagonists of T-type Ca^{2+} channels, the primary Ca^{2+} channel subtype expressed by these cells (Enyeart et al., 1993; Mlinar et al., 1993*b*). These results establish the importance of electrical events and depolarization-dependent Ca^{2+} entry in ACTH-stimulated cortisol secretion. They also suggest a model whereby ACTH receptor activation is coupled to membrane depolarization through inhibition of I_{AC} K^+ channels. The molecular components of the signalling pathway that link ACTH receptors to I_{AC} K^+ channel inhibition have not been identified. However, the suppression of I_{AC} expression by GTP-γ-S as well as cholera toxin suggest that these K^+ channels are under the inhibitory control of G_s, the GTP-binding protein that couples ACTH receptors to adenylate cyclase (Mlinar et al., 1993*a*). In the present study, we have further characterized I_{AC} current with respect to its biophysical properties and inhibition by ACTH and cyclic nucleotides.

MATERIALS AND METHODS

Tissue culture media, antibiotics, fibronectin, and fetal bovine sera were obtained from Gibco BRL (Grand Island, NY). Culture dishes were purchased from Corning (Corning, N.Y.) Coverslips were from Bellco (Vineland, NJ). Enzymes, ACTH (1-24), angiotensin II (AII), 8-pcpt-cAMP (8-[4-chlorophenylthio]-adenosine 3′:5′-cyclic monophosphate), guanosine 5′-*O*-2-(thio)diphosphate (GDP-β-S), cAMP, cGMP, MgATP, 5′-adenylyl-imidodiphosphate (AMP-PNP), and the catalytic subunit of cAMP-dependent protein kinase (bovine heart) were from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin, PKI peptide (5-24), and (Rp)-adenosine-3′,5′-cyclic monophosphorothioate ([Rp]-cAMPS) were from Calbiochem Corp. (San Diego, CA). H-89 and staurosporine were from Biomol (Plymouth Meeting, PA). Cortisol secretion was determined using a solid phase radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA). Protein kinase A (cAMP-dependent protein kinase) assay system was from Gibco BRL. [γ-^{32}P]ATP for use with the kit was from Amersham Life Science (Arlington Heights, IL).

Isolation and Culture of AZF Cells

Bovine adrenal glands were obtained from steers (age range 1–3 yr) within 15 min 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 prepared as previously described (Gospodarowicz et al., 1977) 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 were then dissected into large 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 by slicing 0.3–0.5-mm slices from the larger pieces. The first medulla/fasciculata slices were discarded. One to two subsequent fasciculata slices were saved in cold sterile PBS/0.2% dextrose. The fasciculata/glomerulosa margin (~0.5 mm) was discarded. The remaining glomerulosa tissue with attached capsule was saved for isolation of AZG cells. Fasciculata tissue slices were then diced into 0.5-mm^3 pieces and dissociated with 2 mg/ml (~200–300 U/ml) of Type I collagenase, 0.2 mg/ml deoxyribonuclease in DMEM/F12 for ~1 h at 37°C, triturating after 30 and 45 min with a sterile, plastic transfer pipette. After incubating, the suspension was filtered through 2 layers of sterile cheesecloth, then centrifuged to pellet cells at 100 *g* for 5 min. The cells were washed twice with DMEM/0.2% BSA, centrifuging as before to pellet. Cells were filtered through 200 μm stainless steel mesh to remove clumps after resuspending in DMEM. Dispersed cells were again centrifuged and either resuspended in DMEM/F12 (1:1) with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and plated for immediate use or resuspended in FBS/5% DMSO, divided into 1-ml aliquots each containing ~2 × 10^6 cells and stored in liquid nitrogen for future use. Cells were plated in 35-mm dishes containing 9-mm^2 glass coverslips that had been treated with fibronectin (10 μg/ml) at 37°C for 30 min then rinsed with warm, sterile PBS immediately before adding cells. Dishes were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

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 $CaCl_2$, 10 mM HEPES, 11 mM BAPTA, 200 μM GTP, 2 mM MgATP with pH buffered to 7.2 using KOH. Pipette [Ca^{2+}] was determined using the "Bound and Determined" program (Brooks and Storey, 1992). 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, 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 2–12 h after plating. Typically, cells with diameters of 15–20 μm and capacitances of 10–15 picofarads 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 1.0–2.0 megohms were fabricated from Corning 7052 or 0010 glass (Garner Glass Co., Claremont, CA). These routinely yielded access resistances of 1.5–4 megohms and voltage clamp time constants of <100 μs. K^+ currents were recorded at room temperature (22–25°C) following the procedure of Hamill et al. (1981) using a List EPC-7 patch clamp amplifier. In approximately one-third of AZF cells, I_{AC} reached a maximum amplitude of 200 pA or greater. These cells were used to study modulation of this current.

Pulse generation and data acquisition were done using a personal computer and PCLAMP software with an Axolab interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 1–20 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 1/2 to 1/4 amplitude. Data were analyzed and plotted using PCLAMP (CLAMPAN and CLAMPFIT) and Graph-

PAD InPLOT. Drugs were applied by bath perfusion and were controlled manually by a six-way rotary valve.

Series resistance compensation was not used in most experiments. The mean amplitude of I_{AC} current in AZF cells was <500 pA. A current of this size in combination with a 4 megohm access resistance produces a voltage error of only 2 mV which was not corrected.

Secretion Experiments

AZF cells were cultured on fibronectin-treated 35-mm plates at a density of ~4 × 10^4 per dish in DMEM/F12 (1:1) containing 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+). After 24 h, the media was aspirated and changed to defined media consisting of DMEM/F12 (1:1), 50 μg/ml BSA, 100 μM ascorbic acid, 1 μM tocopherol, 10 nM insulin, and 10 μg/ml transferrin. The Ca^{2+} antagonist penfluridol was added at this time for a 1 h preincubation before switching to media containing this antagonist as well as 8-pcpt-cAMP as required. 200-μl samples of media were collected at selected times and frozen at −20°C and later assayed for cortisol using a solid phase RIA kit (Diagnostic Products Corp., Los Angeles, CA). All experiments were performed on triplicate 35-mm dishes, and hormone assays were performed in duplicate at several dilutions.

Protein Kinase A Assay

AZF cells were cultured on fibronectin-coated 60-mm plates at a density of 3–5 × 10^6 cells per plate in DMEM/F12+. After 24 h, the media was aspirated and changed to defined media (control) or this same media containing 8-pcpt-cAMP. After 15 min, the media was aspirated and cells harvested in 700 μl extraction buffer (5 mM EDTA, 50 mM Tris, pH 7.5). Cells in extract were homogenized using Qiashredder columns (Qiagen, Chatsworth, CA), and 10 μl supernatant from lysate was either assayed directly or incubated with the protein kinase antagonists for 15 min before measuring activity with the Protein Kinase A Assay System from Gibco BRL. All experiments were performed on duplicate 60-mm plates, and enzyme assays were performed in duplicate.

RESULTS

I_{AC}: Kinetics, K^+ Selectivity, and Specific Inhibition by ACTH

In whole-cell patch clamp recordings on bovine AZF cells, two distinct components of K^+ current were expressed by nearly every cell. As previously reported, these include a rapidly inactivating A-type K^+ current (Mlinar and Enyeart, 1993) and a noninactivating K^+ current (I_{AC}) which was present upon initiating whole-cell recording and which grew by an average of ~10-fold over a period of many minutes (Mlinar et al., 1993*a*) (Fig. 1 *A*, *left*).

The I_{AC} K^+ current could be studied in isolation at holding potentials positive to −40 mV where the A-type K^+ current had completely inactivated. I_{AC} recorded in response to a voltage step to +40 mV from a holding potential of −35 mV consisted of an apparent instantaneous component and second time-dependent component (Fig. 1 *A*, *right*). Currents could be fit with a single exponential of the form: $I = I_{max}[1 - \exp(T/\tau_a)] + C$,

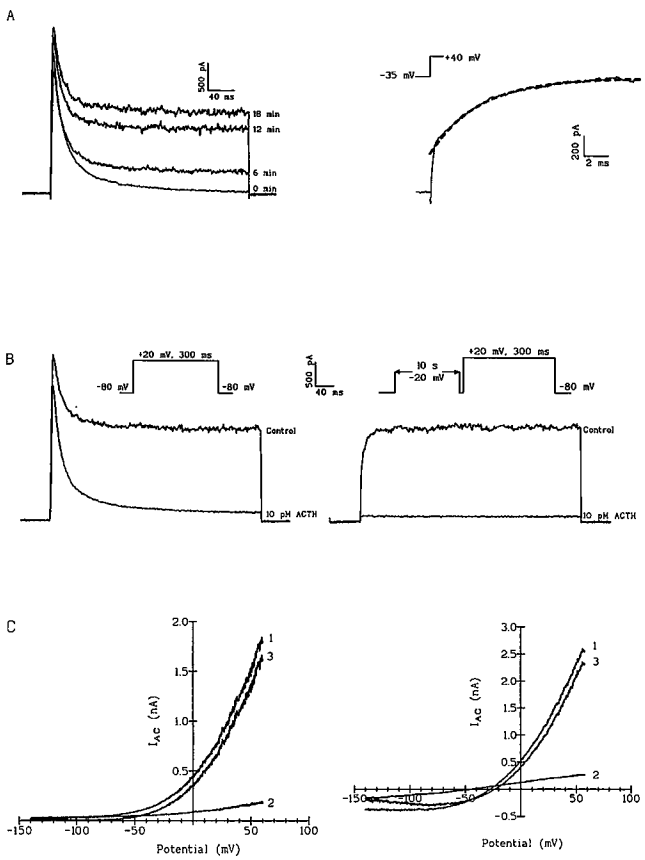

FIGURE 1. Properties of I_{AC} K^+ current. (*A*, *left*) Time-dependent expression. K^+ currents were activated by voltage steps to +20 mV applied at 30-s intervals from a holding potential of −80 mV. Current records recorded at the indicated time after initiating whole-cell recording. (*A*, *right*) I_{AC} activation kinetics. I_{AC} was selectively activated by 20-ms voltage steps to +40 mV from a holding potential of −35 mV. Current record is average of three traces. I_{AC} current was fit with a function of the form: $I = I_{max}[1 - \exp(T/\tau_a)] + C$, where τ_a = 3.30 ms and C = 525 pA. (*B*) Voltage clamp protocols and specific inhibition of I_{AC} by ACTH. K^+ currents were recorded using two different protocols before and after exposing the cell in *A* to 10 pM ACTH. (*left*) K^+ currents were activated by voltage steps to +20 mV applied at 30-s intervals from a holding potential of −80 mV. (*right*) 300-ms voltage clamp steps to +20 mV were preceded by 10-s prepulse to −20 mV which inactivated A-type K^+ current. (*C*) Voltage-independent block and K^+ selectivity of ACTH-inhibited current. (*left*) Voltage ramp of 100 mV/s was applied from a holding potential of 0 mV to voltages between +60 and −140 mV (trace *1*). A second voltage ramp was applied after steady-state block with 100 pM ACTH (trace *2*). Trace *2* was digitally subtracted from trace *1* to give the ACTH-inhibited current (trace *3*). (*right*) Experiment illustrated in left panel repeated in a different cell in saline where external [K^+] was raised to 50 mM by substitution for Na^+. Numbered traces are as in left panel.

where τ_a is the activation time constant and C is the time-independent component. In the example illustrated, the time-dependent component, which comprised 66% of the total current, activated with a time constant of 3.30 ms.

Besides revealing unusual activation kinetics for a voltage-gated channel, recordings such as these indicate that a substantial fraction of I_{AC} channels remain open at membrane potentials negative to −35 mV, as would be anticipated for a channel that contributes to the resting potential in AZF cells. In other experiments, we found that I_{AC} is an outwardly rectifying current that is weakly voltage-dependent at potentials from −30 to +40 mV. Over this range, the relative open probability increased approximately e-fold per 50 mV (data not shown).

The absence of time-dependent inactivation of the slowly developing I_{AC} K^+ current component allowed it to be easily isolated and measured in whole-cell recordings using either of two voltage clamp protocols. When voltage steps of 300-ms duration were applied from a holding potential of −80 mV to a test potential of +20 mV, I_{AC} could be measured in isolation near the end of the test pulse at a point where the A-type K^+ current had inactivated entirely (Fig. 1 *B, left*).

Using the second protocol, I_{AC} was activated with an identical voltage step after a 10-s prepulse to −20 mV had fully inactivated the A-type current (Fig. 1 *B, right*). I_{AC} measured by either method provided nearly identical results. Fig. 1 *B* also illustrates the selective inhibition of I_{AC} by ACTH in the same cell using these two protocols. After a 6-min exposure to ACTH, voltage steps from −80 to +20 mV activate only the residual rapidly inactivating I_A K^+ current (*left*). As expected, when the I_A current had been inactivated by a prepulse, ACTH inhibited the remaining I_{AC} current almost completely (*right*).

Ramp voltage clamp steps applied in 5 and 50 mM external KCl showed that I_{AC} is an outwardly rectifying current with a reversal potential that varies as predicted by the Nernst equation for a channel selectively permeable to K^+ (Fig. 1 *C, left*). Moreover, the K^+ current component inhibited by ACTH also displayed these identical properties. After I_{AC} had reached a stable amplitude, ramp voltage clamp steps were applied from a holding potential of 0 mV over a range from +60 to −140 mV (trace *1*). The cell was then superfused with 100 pM ACTH, and the ramp voltage protocol was repeated after a steady-state block was achieved (trace *2*). Digital subtraction of trace *2* from trace *1* showed that the ACTH-inhibited current (trace *3*) approached zero at potentials negative to −80 mV, almost identical to the control current, and close to the reversal potential predicted by the Nernst equation for a channel selectively permeable to K^+. Repeating this same experiment on a second cell where external KCl had been raised to 50 mM (*right*) showed that the reversal potential for the control and ACTH-inhibited currents shifted to −27 and −25 mV, respectively, values close to −29 mV as predicted by the Nernst equation.

ACTH and GTP-Binding Proteins

ACTH receptors in adrenal cortical cells are coupled to adenylate cyclase through a G-protein intermediate G_S. To determine whether ACTH-mediated inhibition of I_{AC} also involved a G-protein, GTP in the patch pipette was replaced with the inactive guanine nucleotide GDP-β-S. With standard pipette solution (200 μM GTP), 100 pM ACTH inhibited I_{AC} by 95.3 ± 1.3% (n = 12). When 1 mM GDP-β-S replaced GTP in the pipette, ACTH (100 pM) inhibited I_{AC} by only 30.0 ± 12.7% (n = 9). Raising the ACTH concentration to 500 pM enhanced block to only 40.5 ± 16.8% (n = 6) (Table I).

Experiments with GDP-β-S suggested that ACTH-mediated inhibition of I_{AC} occurred through a G-protein intermediate. To determine if either G_i or G_o mediated this inhibition, AZF cells were preincubated before patch clamping with pertussis toxin (Ptx) which suppresses activation of G_i or G_o. Ptx had no significant effect on the time-dependent growth of I_{AC} or its inhibition by ACTH (Table I). In cells pretreated for 9–12 h with Ptx (200 ng/ml), ACTH (100 pM) inhibited I_{AC} by 95.5 ± 1.6% (n = 6). Results with guanine nucleotide and Ptx indicated that ACTH-mediated inhibition of I_{AC} requires activation of a G protein but not G_i or G_o.

Inhibition of I_{AC} by Cyclic Nucleotides

If the inhibitory actions of ACTH on I_{AC} are mediated by cAMP then membrane-permeable cAMP analogues should mimic ACTH in whole-cell patch clamp experiments. 8-pcpt-cAMP inhibited I_{AC} in a concentration-dependent manner. Fig. 2 *A* shows that 8-pcpt-cAMP, like ACTH, selectively inhibited I_{AC}, without reducing the rapidly inactivating A-type current. Typically, 8-pcpt-cAMP–mediated inhibition occurred after a delay of 1 to 2 min, and required several additional minutes to reach a maximum (Fig. 2 *B*). In experiments where 8-pcpt-cAMP was superfused at concentrations ranging from 50 μM to 2 mM, I_{AC} was inhibited with an IC_{50} of

TABLE I

Effect of Guanine Nucleotides and Pertussis Toxin on ACTH-mediated Inhibition of I_{AC}

Treatment	% Inhibition of I_{AC}
100 pM ACTH	95.3 ± 1.3 (n = 12)
100 pM ACTH + PTX	95.5 ± 1.6 (n = 6)
100 pM ACTH + GDPβS	30.0 ± 12.7 (n = 9)
500 pM ACTH + GDPβS	40.4 ± 16.8 (n = 6)

I_{AC} amplitude was measured at 30-s intervals using the two protocols shown in Fig. 1 *B* with pipette solutions containing 200 μM GTP or no added GTP with 1 mM GDP-β-S. Alternatively, cells were pretreated for 9–12 h with 200 ng/ml PTX before recording currents using control pipette solution. Cells were superfused with ACTH at 100 or 500 pM as indicated. Results are mean ± SEM.

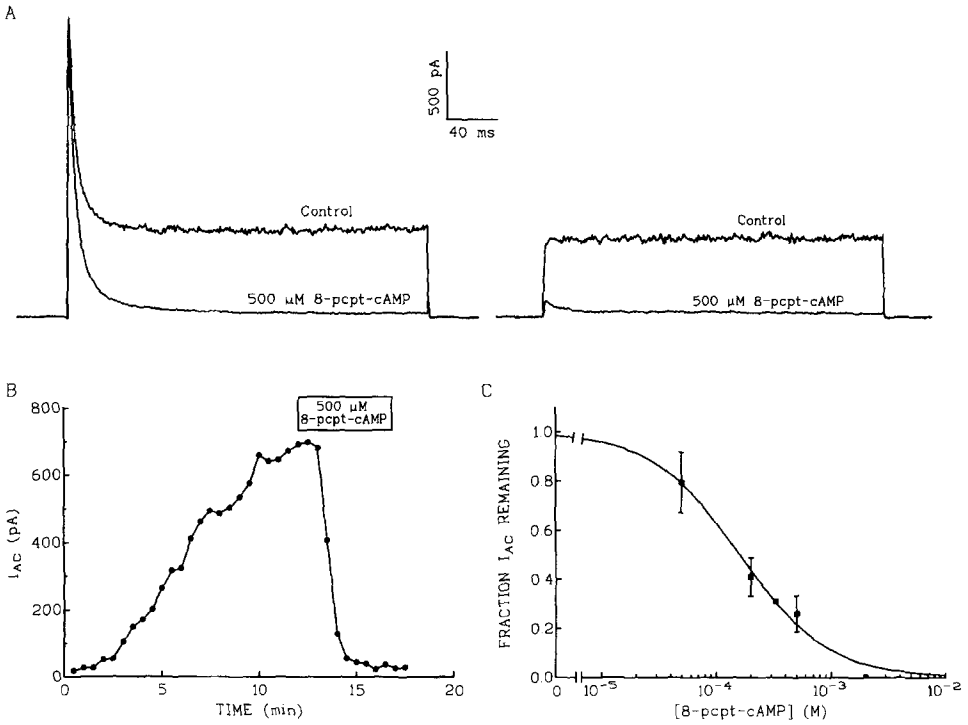

FIGURE 2. Inhibition of I_{AC} by 8-pcpt-cAMP. K^+ currents were activated at 30-s intervals by the two voltage clamp protocols described in the legend of Fig. 1. When I_{AC} had reached a stable amplitude, 8-pcpt-cAMP was superfused at various concentrations between 50 μM and 2 mM. (*A*) Selective inhibition of I_{AC} by 8-pcpt-cAMP. K^+ currents were recorded using two protocols before and after superfusing cell with 500 μM 8-pcpt-cAMP. (*left*) current records in response to voltage steps from −80 to +20 mV in control saline and after block by 8-pcpt-cAMP. (*right*) current records from same cell, recorded with inactivating prepulse. (*B*) Time-dependent block of I_{AC} by 8-pcpt-cAMP. I_{AC} current amplitude, measured as in *A* above, plotted against time before and after superfusion of 8-pcpt-cAMP. (*C*) Inhibition curve. Fraction of unblocked I_{AC} is plotted against 8-pcpt-cAMP concentration. Data was fit with an equation of the form $I/I_{max} = 1/[1 + (B/K_d)]$ where B is 8-pcpt-cAMP concentration and K_d is the equilibrium dissociation constant.

160 μM (Fig. 2 *C*). Although considerably less lipophilic than 8-pcpt-cAMP, cAMP (0.2–2 mM) also inhibited I_{AC} but less potently. At a concentration of 2 mM, cAMP inhibited I_{AC} by 65.9 ± 4.8% (n = 3). In contrast, cGMP had no effect on I_{AC} at this concentration.

The inhibition of I_{AC} by the relatively membrane-impermeant cAMP raised the possibility that cAMP could be acting on I_{AC} through a receptor present on the extracellular surface of AZF cells. To determine whether intracellularly-generated cAMP suppressed I_{AC} K^+ channel activity, we exposed cells to the diterpene adenylate cyclase activator forskolin. Forskolin selectively and reversibly inhibited I_{AC} (Fig. 3, *A* and *B*). At a concentration of 2.5 μM forskolin reduced I_{AC} by 92.0 ± 4.7% (n = 4).

The addition of cAMP to the recording pipette (allowing intracellular cAMP to rapidly increase upon establishment of the whole-cell configuration) was effective in completely suppressing the time-dependent expression of I_{AC} typically observed in whole-cell recordings from AZF cells. The maximum I_{AC} current densities recorded in cells where 0.2 and 2 mM cAMP were added to the pipette were 3.80 ± 0.85 (n = 4) and 2.33 ± 0.95 pa/pF (n = 7), respectively. By comparison, maximum current density recorded with control pipette solution was 11.80 ± 2.06 pA/pF (n = 28) (Fig. 3 *C*). Exposing cells to 100 pM ACTH immediately upon initiating whole-cell recording also eliminated I_{AC} expression completely (data not shown).

Protein Kinases and I_{AC} Modulation

In many cells where cAMP regulates K^+ channel function, modulation proceeds through an A-kinase–dependent phosphorylation (Pedarzani and Storm, 1993; Drain et al., 1994; Levitan, 1994). Two selective A-kinase antagonists were used to determine whether this enzyme was the effector of I_{AC} inhibition by ACTH, cAMP, and forskolin. H-89 is an isoquinoline sulfonamide which has been reported to inhibit cAMP-dependent protein kinase with an IC_{50} of <50 nM (Hidaka et al., 1991). When included in the recording pipette at a concentration of 5 μM, H-89 failed to significantly alter suppression of I_{AC} by any of the three agents (Fig. 4, *A* and *C*). In the presence of the A-kinase antagonist, ACTH (200 pM), 8-pcpt-cAMP (500 μM), and forskolin (2.5 μM) inhibited I_{AC} by 91.5 ± 3.0% (n = 3), 79.8 ± 4.8% (n = 6), and 88.8 ± 1.9% (n = 3), respectively. By comparison, under control conditions, these same three agents inhibited I_{AC} by 96.1 ± 1.7% (n = 6), 73.8 ± 7.4% (n = 8), and 92.0 ± 4.7% (n = 4), respectively.

A synthetic 20 amino acid peptide, PKI(5-24), patterned after a portion of the naturally occurring cAMP-dependent protein kinase inhibitory peptide (PKI) in-

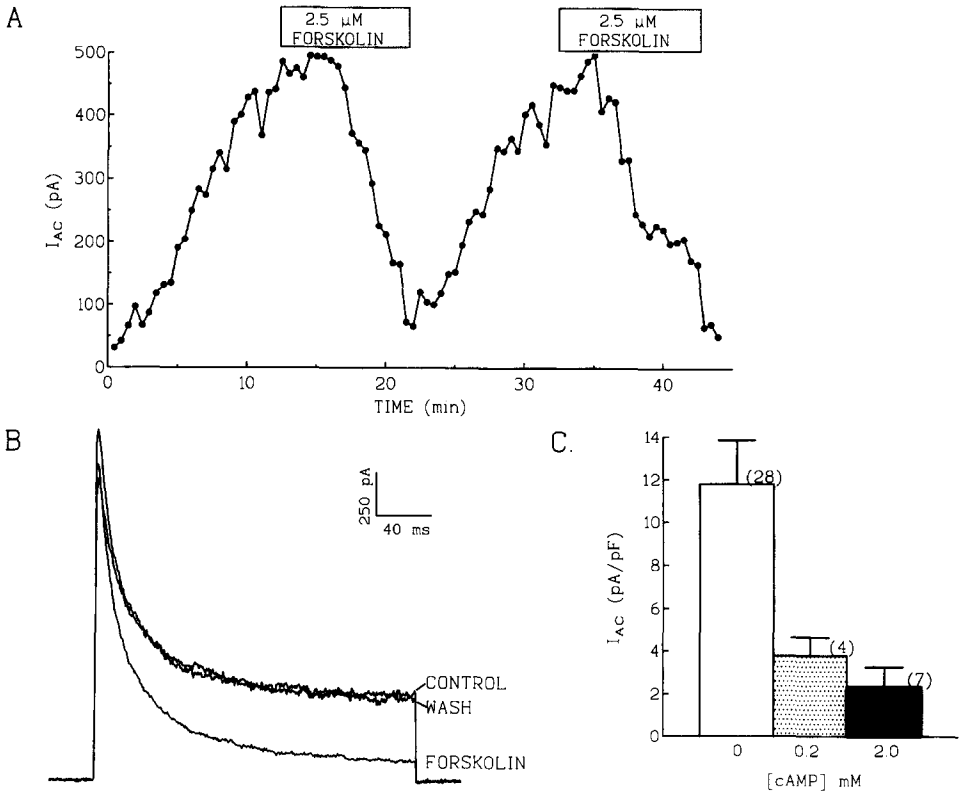

FIGURE 3. Inhibition of I_{AC} by forskolin and cAMP. (*A* and *B*) K^+ current was activated by depolarizing steps to +20 mV from a holding potential of −80 mV. Forskolin (2.5 μM) was superfused for the indicated times. (*A*) I_{AC} amplitude is plotted against time. (*B*) Current records for same cell as in *A*. (*C*) Effect of cAMP on I_{AC} expression. The time-dependent expression of I_{AC} was measured as described in the legend to Fig. 1 with standard pipette solution (n = 28) or pipette solution containing either 0.2 mM cAMP (n = 4), or 2 mM cAMP (n = 7). Results are mean ± SEM of I_{AC} current densities determined for each cell by dividing maximum I_{AC} amplitude by cell capacitance read directly from transient cancellation controls of EPC-7 patch clamp amplifier.

hibits A-kinase with an IC_{50} of 2 nM (Van Haastert et al., 1984; Cheng et al., 1985). Adding PKI(5-24) to the recording pipette at a concentration of 1 or 2 μM (500–1,000 times the IC_{50}) failed to significantly blunt I_{AC} inhibition by ACTH or 8-pcpt-cAMP. In the experiment illustrated in Fig. 4 *B*, the cell was sequentially exposed to 500 μM 8-pcpt-cAMP and 200 μM ACTH. The 65% inhibition of I_{AC} observed upon exposure to 8-pcpt-cAMP was fully reversed upon switching to normal saline. Subsequent infusion of 200 pM ACTH produced near complete inhibition of I_{AC}. Overall, with 2 μM PKI(5-24) in the recording pipette, ACTH (200 pM) and 8-pcpt-cAMP (500 μM) reduced I_{AC} by 85.2 ± 2.5% (n = 8) and 64.5 ± 7.5% (n = 8), respectively. Since none of the three agents that inhibit I_{AC} was superfused until at least 10 min after commencing whole-cell recording in these experiments, it is likely that the intracellular concentrations of PKI(5-24) and H-89 approached that in the pipette solution for many minutes before ACTH, 8-pcpt-cAMP, or forskolin reached the cell (Pusch and Neher, 1988).

Patch clamp experiments with H-89 and PKI(5-24) indicated that inhibition of I_{AC} by either ACTH or 8-pcpt-cAMP did not require the activation of cAMP-dependent protein kinase. To further test this hypothesis and to determine whether other protein kinases function in suppressing this current, I_{AC} inhibition by ACTH and 8-pcpt-cAMP was measured in cells that had been exposed to the potent nonselective protein kinase inhibitor staurosporine. Staurosporine, a microbial alkaloid, inhibits protein kinase A with an IC_{50} of 8.2 nM while C kinase and Ca^{2+}/CaM kinase II are inhibited with IC_{50}s of 2.7 and 20 nM, respectively (Tamaoki, 1991).

Staurosporine (100 nM) was applied to cells through bath perfusion, by including it in the pipette solution, or through these two routes in combination. For external delivery, AZF cells were perfused for 5–20 min with saline containing the membrane-permeable staurosporine before superfusing saline containing this antagonist as well as ACTH (100 or 200 pM) or 8-pcpt-cAMP (500 μM). At concentrations of 100 or 200 pM, ACTH produced nearly identical maximal inhibition of I_{AC} (95.3 ± 1.3% [n = 12] and 96.1 ± 1.7% [n = 6], respectively). Regardless of the route of application, staurosporine only slightly blunted the responses to ACTH. When staurosporine was applied externally, through the pipette or simultaneously through both routes, ACTH (100 or 200 pM) inhibited I_{AC} by 87.6 ± 3.6% (n = 5), 80.4 ± 0.8% (n = 3), and 78.4 ± 10.7% (n = 3) (Fig. 5 *B*).

Staurosporine had no effect on I_{AC} inhibition by 8-pcpt-cAMP. Alone, 500 μM 8-pcpt-cAMP reduced I_{AC} by 73.8 ± 7.4% (n = 8). When cells were exposed to staurosporine by bath perfusion and pipette in combination, 8-pcpt-cAMP inhibited I_{AC} by 78.4 ± 10.7% (n = 3) (Fig. 5, *A* and *B*). In many experiments where cells

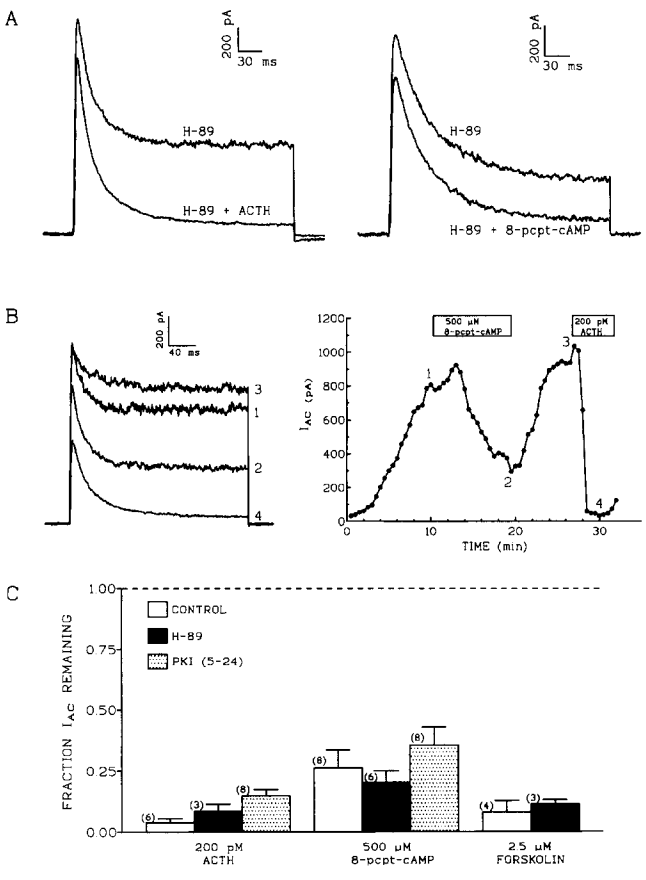

FIGURE 4. Effect of H-89 and PKI (5-24) on I_{AC} inhibition. A-kinase inhibitors H-89 (5 μM) and PKI(5-24) (2 μM) were added to patch electrodes in whole-cell recording. After I_{AC} had developed, cells were superfused with ACTH (200 pM), 8-pcpt cAMP (500 μM), or forskolin (2.5 μM). (*A*) H-89: K^+ currents were activated by voltage steps to +20 mV applied at 30-s intervals from a holding potential of −80 mV, using pipettes containing 5 μM H-89. Cells were superfused with either 200 pM ACTH (*left*) or 500 μM 8-pcpt (*right*). Current records show control and steady-state block by ACTH and 8-pcpt-cAMP. (*B*) PKI (5-24): I_{AC} was monitored for 10 min before superfusing cell with 500 μM 8-pcpt-cAMP, then 200 pM ACTH as indicated (*right*). Numbers on graph correspond to numbered traces on left. Pipette contained 2 μM PKI (5-24). (*C*) Summary of results from experiments such as those in *A*. Bars indicate fraction of I_{AC} remaining after steady-state block by ACTH, 8-pcpt-cAMP, and forskolin in the absence or presence of either kinase inhibitor as indicated. Values are mean ± SEM.

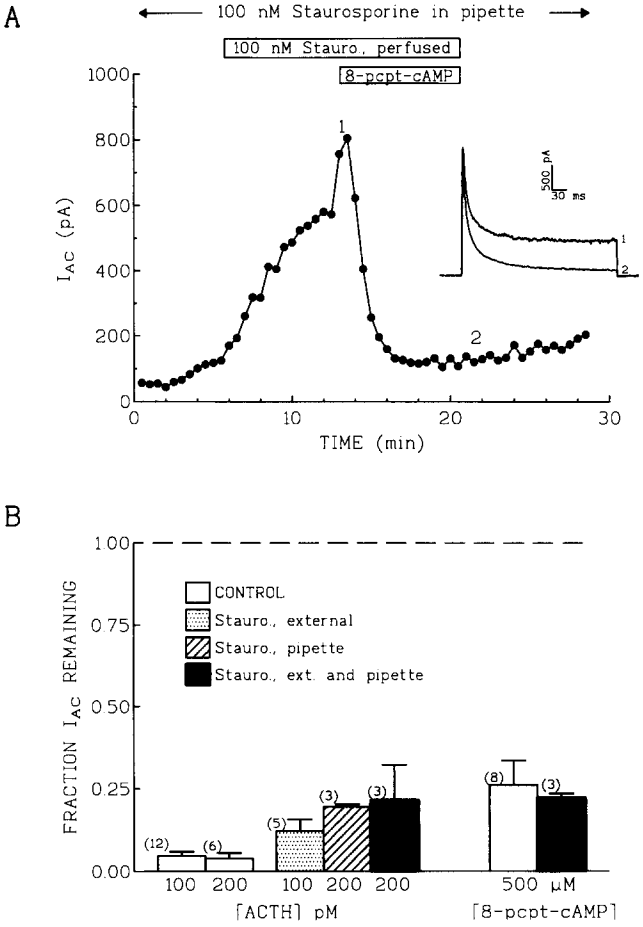

FIGURE 5. Effect of staurosporine on I_{AC} inhibition by ACTH and 8-pcpt-cAMP. Inhibition of I_{AC} by ACTH and 8-pcpt-cAMP was measured in the presence or absence of staurosporine (100 nM). Staurosporine was applied intracellularly through the patch pipette, externally by bath perfusion, or by these two routes in combination. K^+ currents were activated by voltage steps to +20 mV applied at 30-s intervals from a holding potential of −80 mV. (*A*) Effect of 500 μM 8-pcpt-cAMP on I_{AC} in cell exposed to staurosporine by patch pipette and bath perfusion. I_{AC} current amplitude is plotted against time. Current records show control current (*1*) and current after steady-state block by 8-pcpt-cAMP (*2*). (*B*) Summary of results from experiments such as in *A*. Bars indicate fraction of I_{AC} remaining after steady-state block by ACTH (100 or 200 pM) or 8-pcpt-cAMP (500 μM) in the absence or presence of staurosporine (100 pM) applied externally and/or through the pipette as indicated. Values are mean ± SEM of indicated number of determinations.

were superfused with ACTH or 8-pcpt-cAMP in the presence of an A-kinase inhibitor, a typically small (<20%) transient increase in I_{AC} amplitude preceded the prolonged inhibition (Figs. 5 *A* and 4 *B*).

The phosphorothioate derivative of cAMP, (Rp)-cAMPS, competitively antagonizes A-kinase activation by cAMP with an estimated inhibition constant of <10 μM (Van Haastert et al., 1984; Botelho et al., 1988). Like the other A-kinase inhibitors, (Rp)-cAMPS failed to suppress inhibition of I_{AC} by ACTH. When 500 μM (Rp)-cAMPS was applied through the patch electrode, ACTH inhibited I_{AC} by 88 ± 3.2% (n = 3) (data not shown).

A-Kinase Activation and Inhibition

In whole-cell patch clamp experiments, the protein kinase inhibitors H-89, PKI(5-24), and staurosporine were applied intracellularly to the cytoplasm through the patch pipette for 10–20 min before superfusing 8-pcpt-cAMP. To establish that the three kinase antagonists ef-

fectively inhibited A-kinase under conditions similar to those of our experiments, we measured 8-pcpt-cAMP–stimulated A-kinase activity in cytoplasmic extracts from AZF cells in untreated extracts and in extracts which were pretreated for 15 min with the kinase inhibitors at the identical concentrations used in patch clamp experiments. In the experiment illustrated in Fig. 6 *A*, 8-pcpt-cAMP (200 μM) increased A-kinase activity 62-fold over control. H-89, and staurosporine inhibited this increase by 97–100%.

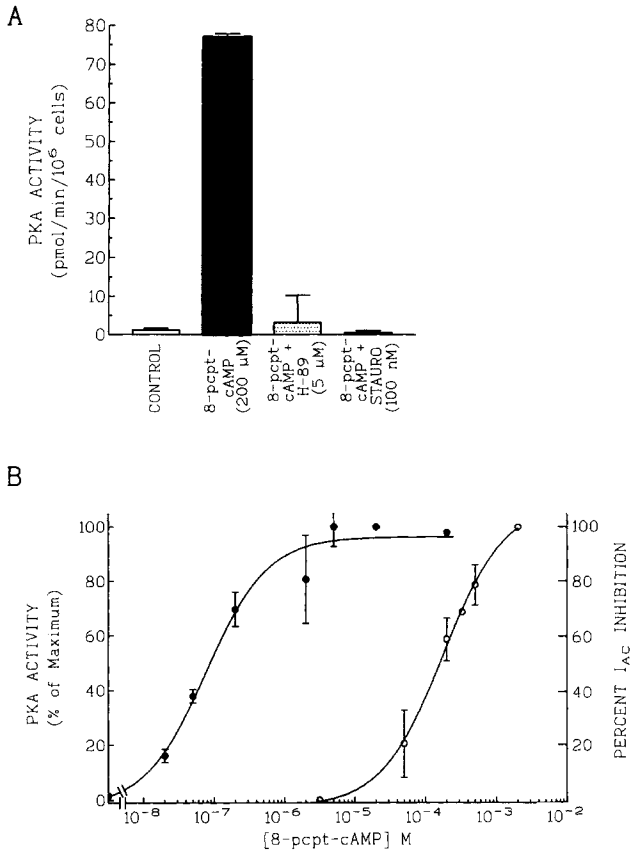

FIGURE 6. Effect of 8-pcpt-cAMP and kinase inhibitors on A-kinase activity. (*A*) AZF cells were cultured on duplicate fibronectin-coated 60-mm plates at a density of 3–5 × 10^6 cells/plate in serum-supplemented DMEM/F12. After 24 h, media was replaced with serum-free DMEM/F12 (*CONTROL*) or this same media containing 8-pcpt-cAMP. After 15 min, cells were harvested and extracts prepared as described in MATERIALS AND METHODS for measurement of A-kinase activity. Aliquots of extracts from cells were treated with 5 μM H-89 or 100 nM staurosporine for 15 min before measuring A-kinase activity. A-kinase (*PKA*) activity is expressed as pmol of ^{32}P incorporated into substrate per min per million AZF cells. (*B*) Concentration-dependent stimulation of A-kinase activity by 8-pcpt-cAMP. AZF cells were cultured as described above in *A*. After 24 h, media was replaced with serum-free DMEM/F12 containing no further additions or 8-pcpt-cAMP at various concentrations ranging from 20 nM to 200 μM. After 15 min, cells were harvested for measurement of A-kinase activity. Protein kinase activity, expressed as percentage of maximum activity, is plotted against 8-pcpt-cAMP concentration (*closed circles*). For comparison, the concentration-dependent inhibition of I_{AC} by 8-pcpt-cAMP is plotted on this same graph (*open circles*). Data is the same as that shown in Fig. 2 *C*. Both sets of data were fit with an equation of the form: $y = 1/[1 + K_d/B]$, where y is the percent maximal response, B is the 8-pcpt-cAMP concentration, and K_d is the equilibrium dissociation constant.

If inhibition of I_{AC} by 8-pcpt-cAMP required activation of A-kinase then 8-pcpt-cAMP–mediated activation of A-kinase in intact AZF cells and inhibition of I_{AC} K^+ current should occur over a similar range of concentrations. 8-pcpt-cAMP activated A-kinase in AZF cells with an EC_{50} of 77 nM, a concentration >2,000-fold lower than that needed to inhibit I_{AC} half maximally (Fig. 6 *B*). Consequently, 8-pcpt-cAMP maximally activated A-kinase at concentrations which did not measurably inhibit I_{AC}. Further, in these experiments application of 8-pcpt-cAMP to intact AZF cells at maximally effective concentrations activated virtually all (95.1 ± 4.8%, n = 5) of the A-kinase in these cells. Application of 10 μM cAMP (100× the EC_{50}) directly to cellular extracts from cells treated with 8-pcpt-cAMP did not further increase A-kinase activity.

Measurements of A-kinase activity in AZF cells clearly indicated that A-kinase activation was neither necessary nor sufficient for cAMP-mediated inhibition of I_{AC}. In another series of experiments, we added the catalytic subunit of cAMP-dependent protein kinase (5 μg/ml) to the patch pipette solution and measured I_{AC} at 30-s intervals. As illustrated in Fig. 7, the active catalytic subunit did not prevent the time-dependent expression of I_{AC}, nor did it alter inhibition of this current by ACTH. Similar results were obtained in each of three cells.

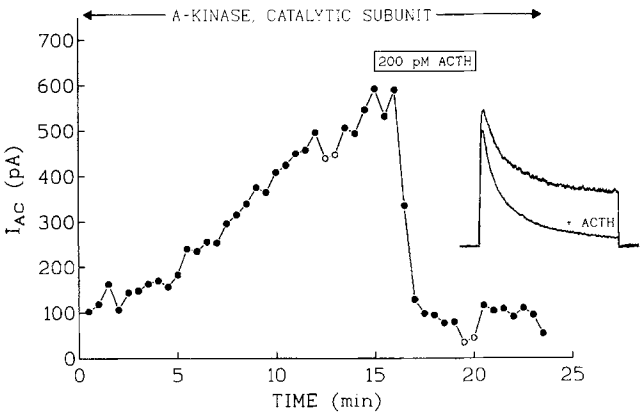

FIGURE 7. A-kinase catalytic subunit and I_{AC} inhibition. K^+ currents were recorded with a patch electrode containing 5 μg/ml of A-kinase catalytic subunit (bovine heart) with phosphorylating activity of 46 picomolar U/μg of protein. AZF cell was superfused with 200 pM of ACTH at the indicated time. Voltage clamp steps were applied from −80 mV to a test potential of +20 mV with (*open circles*) or without (*closed circles*) inactivating prepulses as described in the legend of Fig. 1. I_{AC} amplitude is plotted against time. (*Inset*) I_{AC} currents recorded immediately before and after maximum inhibition by ACTH.

I_{AC} Inhibition by ACTH and 8-pcpt-cAMP Requires ATP Hydrolysis

Experiments with protein kinase inhibitors indicated that I_{AC} inhibition by both ACTH and cAMP did not require phosphorylation by A kinase or other common serine/threonine kinases. However, I_{AC} inhibition by both ACTH and cAMP was dependent on the hydrolysis of ATP. When the nonhydrolyzable ATP analogue AMP-PNP (1 mM) replace ATP in the pipette solution, ACTH and 8-pcpt-cAMP were ineffective at inhibiting I_{AC}. In the presence of AMP-PNP, ACTH (100 nM) inhibited I_{AC} by only 10.9 ± 6.2% (n = 7) compared to 95.3 ± 1.3% (n = 12) observed in the presence of ATP (Fig. 8 *B*).

Similarly, with AMP-PNP in the recording pipette, 200 μM 8-pcpt-cAMP inhibited I_{AC} by only 4.0 ± 4.2% (n = 5) compared with 58.8 ± 7.8% (n = 8) in the control pipette solution (Fig. 8, *A* and *B*). As shown in Fig. 8 *A*, substituting AMP-PNP for ATP in the recording pipette did not alter the time-dependent expression of I_{AC} typically observed in whole-cell recordings, but eliminated inhibition by 200 μM 8-pcpt-cAMP. Inhibition of I_{AC} by ACTH, 8-pcpt-cAMP, and forskolin was also prevented in a total of six cells when recordings were made using pipette solutions that contained neither ATP nor AMP-PNP (data not shown).

Ca^{2+} Dependence

ACTH-stimulated activation of adenylate cyclases in AZF cells requires the presence of extracellular Ca^{2+} (Lefkowitz et al., 1970; Lefkowitz et al., 1971). To determine if inhibition of I_{AC} by ACTH shared this Ca^{2+} dependence, we exposed cells to the peptide in Ca^{2+}-free saline containing 0.1 mM EGTA. The time-dependent growth of I_{AC} was not suppressed in the "0" Ca^{2+} saline, but the inhibition of this current by ACTH was prevented completely (Fig. 9). In each of three cells, 100 pM ACTH produced no inhibition of I_{AC} in the absence of external Ca^{2+}. This effect was specific to ACTH. Inhibition of I_{AC} by forskolin was not affected. Fig. 9 *A* shows the results of an experiment performed in Ca^{2+}-free external solution, in which the cell was sequentially superfused with ACTH and forskolin. ACTH was ineffective, while forskolin inhibited I_{AC} almost completely. In three experiments performed in "0" Ca^{2+}, forskolin inhibited I_{AC} by 95.1 ± 5% (Fig. 9 *C*). A second peptide hormone, AII, retained its ability to inhibit I_{AC} in the absence of extracellular Ca^{2+} (Fig. 9, *B* and *C*). Thus, the removal of extracellular Ca^{2+} did not result in a nonspecific inhibition of adenylate-cyclase activation or peptide hormone binding. It is unlikely that the ineffectiveness of ACTH in zero external Ca^{2+} resulted from a secondary reduction in intracellular Ca^{2+}. When the internal pipette solution was reduced to nominally zero by raising

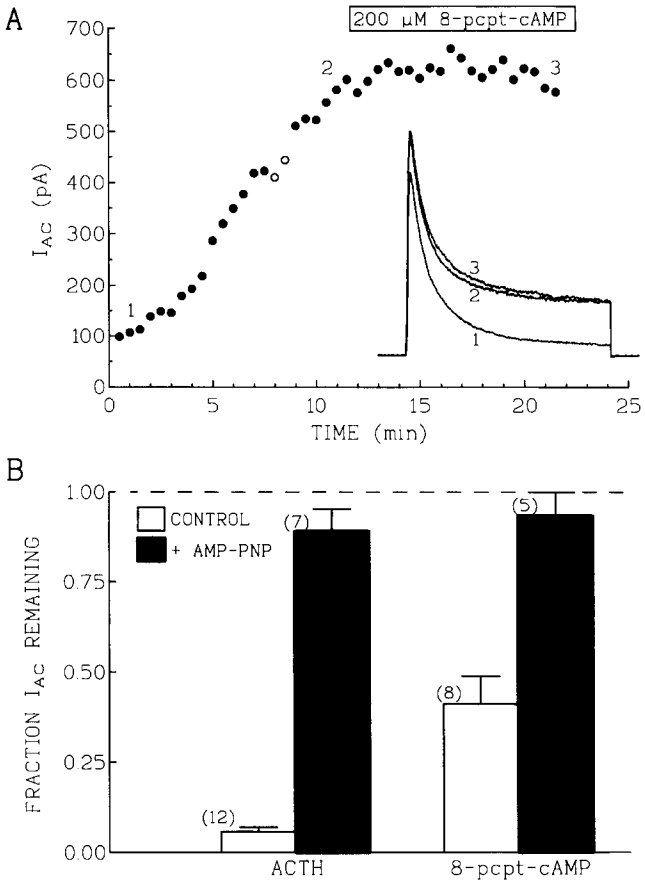

FIGURE 8. Effect of AMP-PNP on I_{AC} inhibition. Inhibition of I_{AC} by ACTH and 8-pcpt-cAMP was measured in whole-cell recordings with standard pipette solution and using pipette solution containing 1 mM AMP-PNP and no ATP. (*A*) AMP-PNP prevents inhibition of I_{AC} by 8-pcpt-cAMP. K^+ currents were activated at 30-s intervals by voltage steps to +20 mV from a holding potential of −80 mV with or without inactivating prepulse (*open circles*). Pipette contained 1 mM AMP-PNP. After I_{AC} reached a maximum value, cell was superfused with 200 μM 8-pcpt-cAMP for the indicated time. Traces are currents recorded at indicated times. (*B*) Summary of results from experiments similar to those in *A*. Bars indicate fraction of I_{AC} remaining after steady-state block by 100 pM ACTH or 200 μM 8-pcpt-cAMP with standard pipette solution (control) or pipette solution containing 1 mM AMP-PNP. Values are mean ± SEM of indicated number of determinations.

BAPTA from 11 to 20 mM with no Ca^{2+} added to the pipette, ACTH inhibited I_{AC} by 91% in two cells.

T-type Ca^{2+} Channels and 8-pcpt-cAMP–stimulated Cortisol Secretion

It is well established that cAMP-induced steroidogenesis is obligatorily dependent on the cAMP-dependent protein kinase signalling cascade. In addition, the results of the present study suggest that cAMP may inhibit I_{AC} K^+ current through a separate A-kinase–independent pathway. Previously, we proposed a model for ACTH-stimulated cortisol secretion whereby I_{AC} inhibition leads to membrane depolarization and Ca^{2+} entry

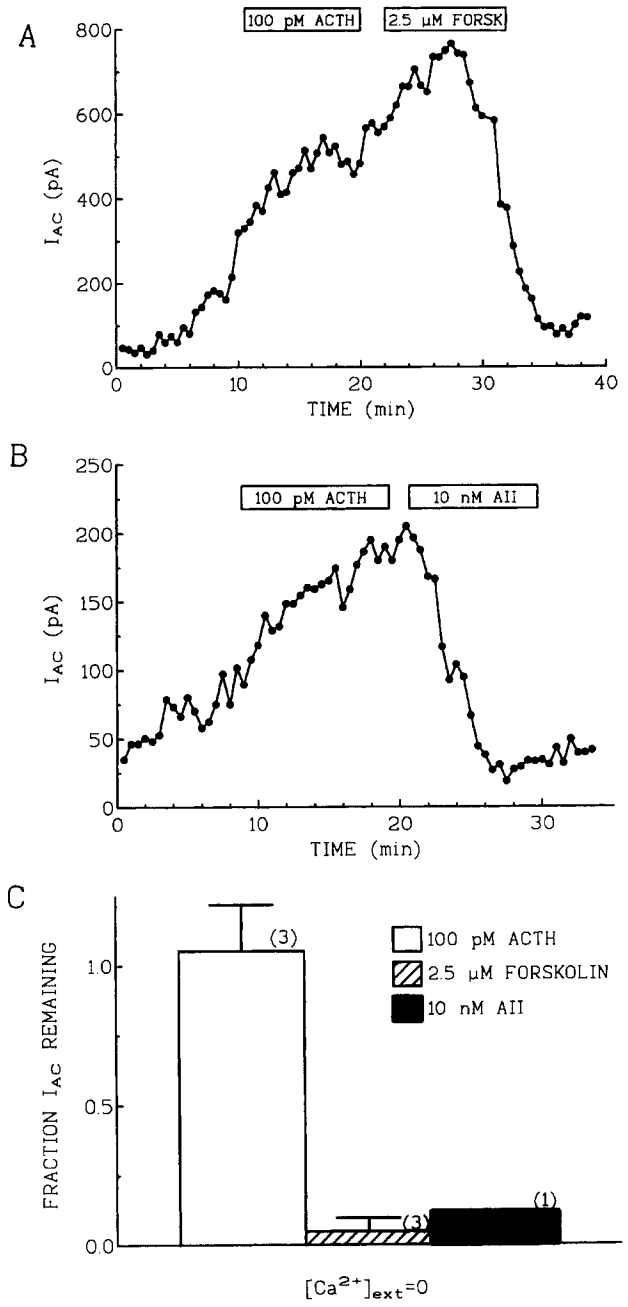

FIGURE 9. Ca^{2+} dependence of I_{AC} inhibition. K^+ current was activated by voltage steps to +20 mV from a holding potential of −80 mV in saline containing no added Ca^{2+} and 0.1 mM EGTA or normal saline containing no Ca^{2+}. (*A*) Effect of ACTH and forskolin in "0" Ca^{2+}. Cell was superfused sequentially with 100 pM ACTH and 2.5 μM forskolin at the indicated times. I_{AC} amplitude is plotted against time. (*B*) Effect of ACTH and AII in "0" Ca^{2+}. Cell was superfused sequentially with 100 pM ACTH and 10 nM AII at the indicated times. I_{AC} amplitude is plotted against time. (*C*) Data obtained from experiments in which cells were superfused with ACTH (100 pM), forskolin (2.5 μM), or AII (10 nM) in saline containing 0 Ca^{2+}. Bars illustrate fraction of I_{AC} remaining after steady-state block by ACTH, forskolin or AII as indicated. Values are mean ± SEM.

through T-type Ca^{2+} channels (Enyeart et al., 1993; Mlinar et al., 1993*a*). Accordingly, the diphenylbutylpiperidine penfluridol inhibits T-type Ca^{2+} channels and ACTH-stimulated cortisol secretion in bovine AZF cells with similar potency and IC_{50}s of 0.3 and 0.16 μM, respectively (Enyeart et al., 1993). We used penfluridol to determine whether 8-pcpt-cAMP–stimulated cortisol secretion might also require Ca^{2+} entry through T-type Ca^{2+} channels. 8-pcpt-cAMP stimulated large (>50-fold) increases in cortisol-secreted by AZF cells. Penfluridol inhibited 8-pcpt-cAMP–stimulated cortisol secretion measured after 24 h with an IC_{50} of 0.33 μM (Fig. 10). Cortisol secreted during a 4 h exposure to 8-pcpt-cAMP was also inhibited with similar potency.

DISCUSSION

In this study, I_{AC} was identified as an outwardly rectifying K^+-selective current that is inhibited by both ACTH and cAMP through a novel A-kinase-independent mechanism requiring ATP hydrolysis. The results suggest a model for ACTH-stimulated cortisol secretion wherein cAMP binds to two separate effectors to activate parallel steroidogenic signalling pathways in AZF

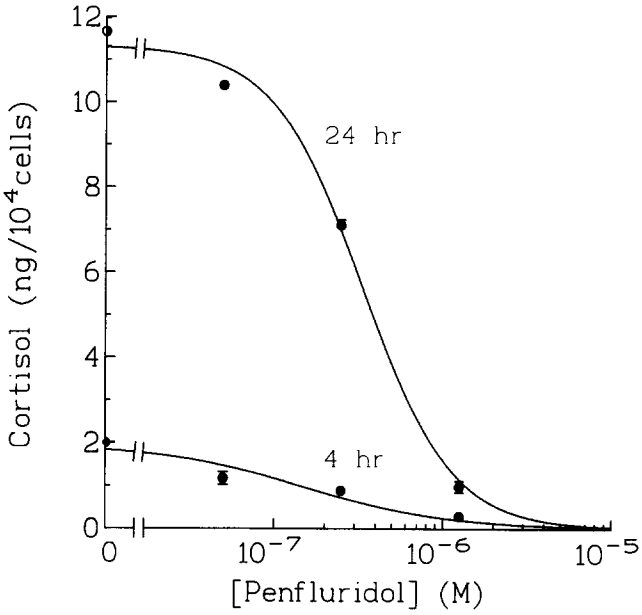

FIGURE 10. Inhibition of cortisol secretion by penfluridol. Cultured AZF cells were incubated for 24 h in serum-containing media as described in the legend of Fig. 6 before switching to test media containing various concentrations of penfluridol. After a 1-h preincubation with penfluridol, media was replaced with one containing penfluridol and 8-pcpt-cAMP (1 mM). Media samples were collected and assayed for cortisol after 4 and 24 h incubation. Results are mean ± SEM of triplicate determinations. Curves were fit with an equation of the form: $y = 1/[1 + B/K_d]$, where y is the quantity of cortisol secreted, B is the penfluridol concentration, and K_d is the equilibrium dissociation constant.

cells. These include the traditional A-kinase-dependent signalling cascade and a novel pathway wherein cAMP binding to I_{AC} K^+ channels leads to depolarization-dependent Ca^{2+} entry.

Properties of I_{AC} and AZF Membrane Potential

I_{AC} displayed properties that are unusual among most K^+ currents described thus far. It is one of several outwardly rectifying K^+ currents that have been characterized (McCloskey and Cahalan, 1990; Ketchum et al., 1995). Unlike other voltage-gated K^+ currents, I_{AC} activation kinetics were not marked by a sigmoidal onset but instead could be fit by a single exponential function. By comparison, activation kinetics for the A-type K^+ current in AZF cells were classically sigmoidal and fit by an exponential function raised to a power of 4 (Mlinar and Enyeart, 1993).

The instantaneous component of I_{AC} observed in whole-cell recordings indicated that a substantial fraction of I_{AC} channels are active at holding potentials negative to −35 mV. Although these results demonstrate that I_{AC} would oppose membrane depolarization and contribute to negative membrane potentials, they do not prove that I_{AC} is primarily responsible for setting the membrane potential of I_{AC} cells. Several lines of evidence suggest that it is. The resting potential of I_{AC} varies with K_o/K_i as predicted by the Nernst equation. Of the two types of K^+ channels that can be identified in whole-cell recordings from AZF cells, only I_{AC} is inhibited by ACTH and cAMP. ACTH inhibits I_{AC} with a temporal pattern and concentration dependence that are nearly identical to ACTH-stimulated membrane depolarization (Mlinar et al, 1993*a*).

Inhibition of I_{AC} by cAMP

The results of experiments with GDP-β-S and Ptx were consistent with the hypothesis that ACTH-mediated inhibition of I_{AC} required a G_S-dependent activation of adenylate cyclase. Accordingly, 8-pcpt-cAMP produced a concentration-dependent inhibition of I_{AC} when applied by bath perfusion. The suppression of the time-dependent expression of I_{AC} in whole-cell recordings made with pipettes containing cAMP and the inhibition of I_{AC} by forskolin indicate that cAMP acts at an intracellular site rather than through a receptor located on the cell surface. This point is significant since cAMP has been reported to modulate a Na^+ channel on cardiac myocytes through G-protein–coupled cell surface receptor (Sorbera and Morad, 1991). G-protein–coupled cAMP receptors are present on the surface of slime molds where they control development (Klein et al., 1988).

Forskolin has been reported to inhibit K^+ currents by a cAMP-independent mechanism. However, forskolin concentrations much greater than 2.5 μM were required for significant inhibition to be observed (Zunkler et al., 1988). Further, forskolin, like cAMP, selectively inhibited I_{AC} in AZF cells, without suppressing the transient A-type K^+ current. Thus, it is likely that suppression of I_{AC} by forskolin is specific and mediated by cAMP.

In nearly all reported cases, the modulation of voltage and Ca^{2+}-activated K^+ channels by cAMP occurs through phosphorylation by A kinase (Hille, 1992; Pedarzani and Storm, 1993; Drain et al., 1994; Levitan, 1994). I_{AC} K^+ channels appear to be distinctive in this respect. An overwhelming body of evidence indicated that A-kinase activation was neither necessary nor sufficient for 8-pcpt-cAMP- or ACTH-mediated inhibition of I_{AC}. The three A-kinase antagonists, H-89, PKI (5-24), (Rp)-cAMPS, and the nonspecific kinase inhibitor staurosporine applied intracellularly, externally, or by both routes at concentrations 10–1,000 times their reported IC_{50}s failed to prevent inhibition by either 8-pcpt-cAMP or ACTH. Since diffusional equilibrium between pipette and cell is reached within seconds for small molecules (Pusch and Neher, 1988), it is likely that A-kinase antagonists were present in large excess at the intended target. When applied directly to AZF cell extracts for comparable times at identical concentrations, these kinase antagonists eliminated 8-pcpt-cAMP–stimulated A-kinase activation.

Two separate lines of evidence demonstrated that A-kinase activation did not mediate I_{AC} inhibition by 8-pcpt-cAMP. Most importantly, 8-pcpt-cAMP activated A-kinase in AZF cells with an EC_{50} >2,000-fold lower than that necessary to inhibit I_{AC} half maximally. Therefore, 8-pcpt-cAMP fully activated A-kinase at concentrations that had virtually no effect on I_{AC}. Accordingly, the active catalytic subunit of bovine A-kinase applied intracellularly through the patch pipette failed to suppress expression of I_{AC}. Although our results show that A-kinase activation is not required for I_{AC} inhibition by cAMP, they do not exclude a role for this enzyme in I_{AC} modulation in an undialyzed cell.

Our evidence suggests that cAMP may act directly on I_{AC} K^+ channels to inhibit their function. I_{AC} K^+ current resembles other cyclic nucleotide-gated channels with respect to its sensitivity to cAMP and weak voltage-dependent activation. A large and growing family of ion channels directly activated by cAMP has been identified, including those present in retinal and olfactory neurons. These channels are nonselective cation pores with only a weak voltage dependence (Dhallen et al., 1990; Kaupp, 1991; Hille, 1992; Yau, 1994). However, the overall sequence similarity between cyclic nucleotide-gated ion channels and voltage-activated K^+ channels (Heginbotham et al., 1992) suggests origination from a common ancestor. In this regard, several cloned K^+ channel genes from *Drosophila* and the plant *Arabi-*

dopsis thaliana are more closely related in sequence to cyclic nucleotide-gated channels than to other K^+ channels. Importantly, both the *Arabidopsis* and the *Drosophila* K^+ channels contain a consensus cAMP binding domain (Schachtman et al., 1992; Bruggemann et al., 1993). Perhaps, interposed between the voltage-gated K^+ channels and the related cyclic nucleotide-gated cation channels, there exists a continuum of intermediate forms that include K^+-selective channels whose gating is directly controlled or modulated by cAMP. A cAMP-activated K^+ selective channel in *Drosophila* larvae has been characterized with patch clamp (Delgado et al., 1991). I_{AC} may be the first ion channel directly inhibited by cAMP.

Mechanism of ACTH Inhibition of I_{AC}

The results of patch clamp experiments were consistent with the interpretation that I_{AC} inhibition by ACTH is mediated by cAMP. Experiments with GDP-β-S and Ptx demonstrated that activation of a Ptx-insensitive G protein such as G_S was required for I_{AC} inhibition by ACTH. In AZF cells, ACTH receptors are coupled to adenylate cyclase through G_s (Mountjoy et al., 1992).

ACTH stimulates A-kinase and by inference cAMP synthesis at concentrations identical to those which inhibit I_{AC} (unpublished observations). The inability of ACTH to inhibit I_{AC} in the absence of extracellular Ca^{2+} supports the hypothesis that inhibition is mediated by cAMP. It had previously been demonstrated that while binding of ACTH to its receptor was not affected by removal of external Ca^{2+}, activation of adenylate cyclase was completely prevented in the absence of this divalent cation (Lefkowitz et al., 1970; Lefkowitz et al., 1971). Thus, ACTH-mediated activation of adenylate cyclase and I_{AC} inhibition share a common dependence on external Ca^{2+}. cAMP appears to be the messenger that links ACTH receptors to I_{AC} channels.

Patch clamp studies with the A-kinase antagonists failed to provide evidence that ACTH and 8-pcpt-cAMP inhibited I_{AC} by separate mechanisms. Each of the three protein kinase antagonists were ineffective at preventing I_{AC} inhibition by either agent. The complete elimination of ACTH- and 8-pcpt-cAMP–mediated inhibition of I_{AC} by AMP-PNP also suggests a common mechanism is involved.

Although all of the results in this study are consistent with the hypothesis that cAMP directly mediates inhibition of I_{AC} by ACTH, they do not exclude the possibility of a separate signalling pathway. In this regard, inhibition of I_{AC} by ACTH was more difficult to reverse than 8-pcpt-cAMP–mediated reduction of this current. However, this difference could reflect the slower dissociation of ACTH from its receptor rather than distinct inhibitory mechanisms. If ACTH at any concentration suppresses I_{AC} through a cAMP-independent mechanism, it is unlikely that activation of serine-threonine kinase is involved. Staurosporine, at concentrations of 100–200 nM, failed to significantly alter inhibition of I_{AC} by ACTH. Staurosporine inhibits some tyrosine kinases only at higher micromolar concentrations. The possibility that ACTH suppresses I_{AC} by an unknown tyrosine kinase cannot be excluded. Interestingly, we found that staurosporine does suppress AII-mediated inhibition of I_{AC} (Mlinar et al., 1995). Apparently, AII- and ACTH-mediated inhibition of this current occur through distinct signalling pathways.

I_{AC} Inhibition and ATP Hydrolysis

The complete suppression of 8-pcpt-cAMP–mediated inhibition of I_{AC} observed upon substituting the nonhydrolyzable ATP analogue AMP-PNP for ATP in the recording pipette suggests that cAMP-induced channel closing is linked to an ATP hydrolysis cycle. Several channels have been discovered whose functional expression and gating are regulated by A-kinase and ATP-hydrolysis (Anderson et al., 1991; Baukrowitz et al., 1994; Fakler et al., 1994). Gating of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels is coupled to an ATP hydrolysis cycle. For CFTR Cl^- channels to open they must be phosphorylated by A-kinase and then exposed to a hydrolyzable nucleoside triphosphate such as ATP (Baukrowitz et al., 1994). The functional activity of K_{ir} 2.1 inward rectified K^+ channels also requires phosphorylation by PKA and ATP hydrolysis (Fakler et al., 1994).

cAMP-mediated inhibition of I_{AC} K^+ channels may involve a novel mechanism that requires ATP hydrolysis but not phosphorylation by A-kinase. The results are consistent with a mechanism where the binding of cAMP to an I_{AC} K^+ channel is coupled to the conformational change that closes the channel by a process fueled by ATP hydrolysis.

The elimination of ACTH-mediated inhibition of I_{AC} observed upon substituting AMP-PNP for ATP in the recording pipette is readily explained within the framework of a mechanism that requires both cAMP synthesis and ATP hydrolysis for channel inhibition. In addition to eliminating ATP-hydrolysis, the nonhydrolyzable adenine nucleoside does not serve as a substrate for adenylate cyclase, preventing the synthesis of cAMP. Our results do not exclude the possibility that ACTH inhibition of I_{AC} occurs through a cAMP-independent mechanism such as a staurosporine-insensitive protein kinase, which would become inactive in the absence of a nonhydrolyzable substrate.

Model for Cortisol Secretion Stimulated by ACTH and cAMP

The T-type Ca^{2+} channel antagonist penfluridol inhibited cAMP-stimulated cortisol secretion at concentrations similar to those that we previously found sup-

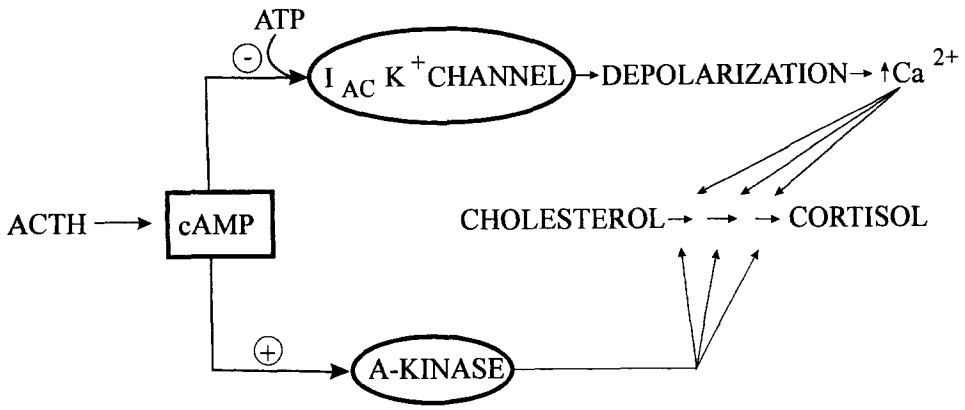

FIGURE 11. Model for cAMP-mediated stimulation of corticosteroid synthesis through activation of parallel signalling pathways.

pressed ACTH-stimulated cortisol secretion and blocked T-type Ca^{2+} channels in bovine AZF cells (Enyeart et al., 1993). The results of combined patch clamp and secretion studies suggest a model for ACTH-stimulated cortisol secretion wherein cAMP functions in a dual capacity as a second messenger to activate parallel signalling pathways (Fig. 11). Along one path, cAMP directly combines with I_{AC} K^+ channels leading to their inhibition. This inhibition triggers the sequence of membrane depolarization T-type Ca^{2+} channel opening, and Ca^{2+}-dependent activation, and ultimately, induction of steroidogenic enzymes. cAMP activates the second pathway through the conventional interaction with A-kinase followed by activation and induction of the same steroidogenic enzymes. Although it is clear that cAMP can mediate electrical and biochemical events in cortisol secretion, the possibility remains that addition signalling pathways could be involved in ACTH-stimulated cortisol secretion.

Aside from its apparent importance in regulating cortisol secretion, I_{AC} is an extremely interesting K^+ current whose gating is regulated by an unusual combination of voltage and metabolic factors. The cAMP-dependent inhibition of this K^+ current through an A-kinase–independent pathway requiring ATP hydrolysis is a unique mechanism for modulating the function of any ion channel. The control of I_{AC} K^+ channel gating by metabolic factors including cAMP and ATP suggests a mechanism where the membrane potential of these secretory cells could be tightly coupled to the metabolic state of the cell and modulated by biochemical signals originating at the cell membrane.

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