

Adenosine Triphosphate Activates a Noninactivating K⁺ Current in Adrenal Cortical Cells through Nonhydrolytic Binding

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ABSTRACT Bovine adrenal zona fasciculata (AZF) cells express a noninactivating K⁺ current (I_{AC}) that is inhibited by adrenocorticotrophic hormone and angiotensin II at subnanomolar concentrations. Since I_{AC} appears to set the membrane potential of AZF cells, these channels may function critically in coupling peptide receptors to membrane depolarization, Ca²⁺ entry, and cortisol secretion. I_{AC} channel activity may be tightly linked to the metabolic state of the cell. In whole cell patch clamp recordings, MgATP applied intracellularly through the patch electrode at concentrations above 1 mM dramatically enhanced the expression of I_{AC} K⁺ current. The maximum I_{AC} current density varied from a low of 8.45 ± 2.74 pA/pF (*n* = 17) to a high of 109.2 ± 26.3 pA/pF (*n* = 6) at pipette MgATP concentrations of 0.1 and 10 mM, respectively. In the presence of 5 mM MgATP, I_{AC} K⁺ channels were tonically active over a wide range of membrane potentials, and voltage-dependent open probability increased by only ~30% between -40 and +40 mV. ATP (5 mM) in the absence of Mg²⁺ and the nonhydrolyzable ATP analog AMP-PNP (5 mM) were also effective at enhancing the expression of I_{AC}, from a control value of 3.7 ± 0.1 pA/pF (*n* = 3) to maximum values of 48.5 ± 9.8 pA/pF (*n* = 11) and 67.3 ± 23.2 pA/pF (*n* = 6), respectively. At the single channel level, the unitary I_{AC} current amplitude did not vary with the ATP concentration or substitution with AMP-PNP. In addition to ATP and AMP-PNP, a number of other nucleotides including GTP, UTP, GDP, and UDP all increased the outwardly rectifying I_{AC} current with an apparent order of effectiveness: MgATP > ATP = AMP-PNP > GTP = UTP > ADP >> GDP > AMP and ATP-γ-S. Although ATP, GTP, and UTP all enhanced I_{AC} amplitude with similar effectiveness, inhibition of I_{AC} by ACTH (200 pM) occurred only in the presence of ATP. As little as 50 μM MgATP restored complete inhibition of I_{AC}, which had been activated by 5 mM UTP. Although the opening of I_{AC} channels may require only ATP binding, its inhibition by ACTH appears to involve a mechanism other than hydrolysis of this nucleotide. These findings describe a novel form of K⁺ channel modulation by which I_{AC} channels are activated through the nonhydrolytic binding of ATP. Because they are activated rather than inhibited by ATP binding, I_{AC} K⁺ channels may represent a distinctive new variety of K⁺ channel. The combined features of I_{AC} channels that allow it to sense and respond to changing ATP levels and to set the resting potential of AZF cells, suggest a mechanism where membrane potential, Ca²⁺ entry, and cortisol secretion could be tightly coupled to the metabolic state of the cell through the activity of I_{AC} K⁺ channels.

KEY WORDS: adenosine triphosphate • potassium channel • adrenocorticotrophic hormone • nucleotide

INTRODUCTION

I_{AC} is a novel noninactivating K⁺ current that may set the resting potential of bovine adrenal zona fasciculata (AZF)¹ cells. Angiotensin II (AII) and adrenocorticotrophic hormone (ACTH) inhibit I_{AC} and depolarize AZF cells at concentrations identical to those that stimulate cortisol production (Mlinar et al., 1993a). This K⁺ channel appears to act pivotally in coupling these

peptide hormone receptors to depolarization-dependent Ca²⁺ entry and corticosteroid production (Enyeart et al., 1993).

I_{AC} K⁺ channel activity may be regulated by the complex interaction of biochemical factors and membrane voltage. In whole-cell patch clamp recordings from AZF cells, we found that I_{AC} K⁺ current measured during depolarizing voltage steps increases dramatically (10–100-fold) over a period of many minutes (Mlinar et al., 1993a). Inhibitory factors present in the cytoplasm may be diluted during dialysis of the cell by pipette solution, allowing the functional expression of the I_{AC} current. In this regard, the time-dependent growth of I_{AC} is suppressed by including the nonhydrolyzable GTP analog GTP-γ-S in the recording pipette, indicating the presence of an inhibitory mechanism requiring a GTP-binding protein (Mlinar et al., 1993a). Accordingly, inhibition of I_{AC} by both ACTH and AII require G-pro-

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¹Abbreviations used in this paper: ACTH, adrenocorticotrophic hormone; AII, angiotensin II; AZF, bovine adrenal fasciculata; CFTR, cystic fibrosis transmembrane conductance regulator; IV, current-voltage relationship.

tein intermediates (Mlinar et al., 1995; Enyeart et al., 1996b).

ACTH receptors are coupled to adenylate cyclase through G_s . Although most cAMP-dependent actions of ACTH are mediated through cAMP-dependent protein kinase, inhibition of I_{AC} by both ACTH and cAMP occur through an A-kinase-independent mechanism requiring ATP hydrolysis (Enyeart et al., 1996b). This result suggests that opening and closing of I_{AC} K^+ channels could be coupled to an ATP hydrolysis cycle, similar to that which controls the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (Hwang et al., 1994; Baukowitz et al., 1994; Quinton and Reddy, 1992). If so, then I_{AC} K^+ channels could be activated by the hydrolytic or nonhydrolytic binding of ATP to these channels or associated proteins.

A large number of ATP-sensitive K^+ channels are expressed by a variety of cells ranging from cardiac and skeletal muscle cells to neurons and insulin-secreting pancreatic β cells (Ashcroft, 1988a; Hilgemann, 1997; Terzic et al., 1994; Takano and Noma, 1993). However, these inwardly rectifying channels are uniformly inhibited by ATP through nonhydrolytic binding to the channel or an associated protein. In contrast, whole-cell and single channel patch clamp recordings from bovine AZF cells showed that ATP, over a physiological range of concentrations, dramatically enhanced I_{AC} K^+ current.

MATERIALS AND METHODS

Tissue culture media, antibiotics, fibronectin, and fetal bovine sera were obtained from Gibco Laboratories (Grand Island, NY). Culture dishes were purchased from Corning Glass Works (Corning, NY). Coverslips were from Bellco Glass, Inc. (Vineland, NJ). Enzymes, ACTH (1-24), NaATP, MgATP, KATP, KADP, AMP, 5'-adenylyl-imidodiphosphate (AMP-PNP, lithium salt), adenosine 5'-O-3-thio-triphosphate (ATP- γ -S, tetra-lithium salt), EDTA, NaGTP, NaGDP, NaUTP, and NaUDP were obtained from Sigma Chemical Co. (St. Louis, MO). Pinacidil was obtained from Research Biochemicals International (Natick, MA).

Isolation and Culture of AZF Cells

Bovine adrenal glands were obtained from steers (age 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 with some modifications (Gospodarowicz et al., 1977). 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 $\sim 1.0 \times 1.0 \times 0.5$ cm. A Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ) 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. 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 (neutral protease activity not exceeding 100 U/mg of solid), 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 two layers of sterile cheesecloth, and then centrifuged to pellet cells at $100 g$ for 5 min. Undigested tissue remaining in the cheesecloth was collagenase treated for an additional hour. Pelleted cells were washed twice with DMEM/0.2% BSA, centrifuging as before. Cells were filtered through $200\text{-}\mu\text{m}$ 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, and 0.1 mg/ml streptomycin, and plated for immediate use, or resuspended in FBS/5% DMSO, divided into 1-ml aliquots, each containing $\sim 2 \times 10^6$ 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 ($10 \mu\text{g/ml}$) at 37°C for 30 min, and then rinsed with warm, sterile PBS immediately before adding cells. Dishes were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2 .

To minimize variability in I_{AC} currents, all of the cells used in experiments measuring current density (~ 200 cells) were obtained in a single isolation from six bovine adrenal glands and stored in liquid N_2 , as described above, in ~ 70 vials. For each experiment, cells were plated on coverslips from a single vial. Experimental results reported in this paper were obtained in recordings made over an 8-mo period. Cells stored in liquid N_2 , as described above, retained electrophysiological and biochemical properties for at least 1 yr from freezing date. Specifically, AZF cells stored in this way expressed I_{AC} K^+ current as well as I_A K^+ current with no obvious deterioration during the course of this study. Further, as previously reported, ACTH and AII both inhibited I_{AC} K^+ current in these cells with a potency not distinguishable from that observed in freshly isolated cells (Enyeart et al., 1996b; Mlinar et al., 1993a, 1995). After months in liquid N_2 , cultured AZF cells responded to ACTH and AII with large increases in cortisol secretion, and expression of orphan receptor mRNAs (Enyeart et al., 1993, 1996a, 1996b; Mlinar et al., 1995).

Patch Clamp Experiments

Patch clamp recordings of K^+ channel currents were made in the whole-cell and outside-out patch configurations. For both recording configurations, the standard pipette solution was 120 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM HEPES, 11 mM BAPTA (1,2-bis-(2-aminophenoxy)ethane- N,N,N',N'' -tetraacetic acid), and 200 μM GTP, with pH buffered to 7.2 using KOH. Addition of various nucleotides and other deviations from the standard solution are described in the text. Pipette $[\text{Ca}^{2+}]$ was determined using the "Bound and Determined" program (Brooks and Storey, 1992). The external solution consisted of (mM): 140 NaCl, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, and 5 glucose, pH 7.4 using NaOH. All solutions were filtered through $0.22\text{-}\mu\text{m}$ cellulose acetate filters.

AZF cells were used for patch clamp experiments 2–12 h after plating. Typically, cells with diameters of $<15 \mu\text{m}$ and capacitances of 8–12 picofarads were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (1.5 ml vol) that was continuously perfused by gravity at a rate of 3–5 ml/min. For whole cell recordings, patch electrodes with resistances of 1.0–2.0 megohms were fabricated from Corning glass (7052 or 0010; Garner Glass Co., Claremont, CA). These routinely yielded access resistances of 1.5–4 megohms and voltage clamp time constants of $<100 \mu\text{s}$. For single channel recordings, patch electrodes with higher resistances of 3–5 megohms were used. K^+ currents were recorded at room temperature (22 – 25°C)

following the procedure of Hamill et al. (1981) using an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany).

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 Inc., Haverhill, MA). Linear leak and capacity currents were subtracted from current records using scaled hyperpolarizing steps of 1/3–1/4 amplitude. Data were analyzed and plotted using PCLAMP 5.5 and 6.02 (CLAMPAN, CLAMPFIT, FETCHAN, and PSTAT) and GraphPAD InPLOT. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve. Series resistance compensation was not used in experiments where the I_{AC} currents were <1 nA. A current of this size in combination with a 4 M Ω access resistance produces a voltage error of only 4 mV that was not corrected.

RESULTS

Effects of ATP and Analogs on I_{AC} K^+ Current

Bovine AZF cells express two types of K^+ currents that are easily distinguished in whole cell recordings. These include a rapidly inactivating A type K^+ current and a noninactivating K^+ current that grows continually over a period of many minutes in whole cell recordings (Mlinar et al., 1993a; Enyeart et al., 1996b). The absence of time-dependent inactivation of the I_{AC} K^+ current allowed it to be easily isolated for measurement in whole cell recordings using either of two voltage clamp protocols. When voltage steps of 300-ms duration were ap-

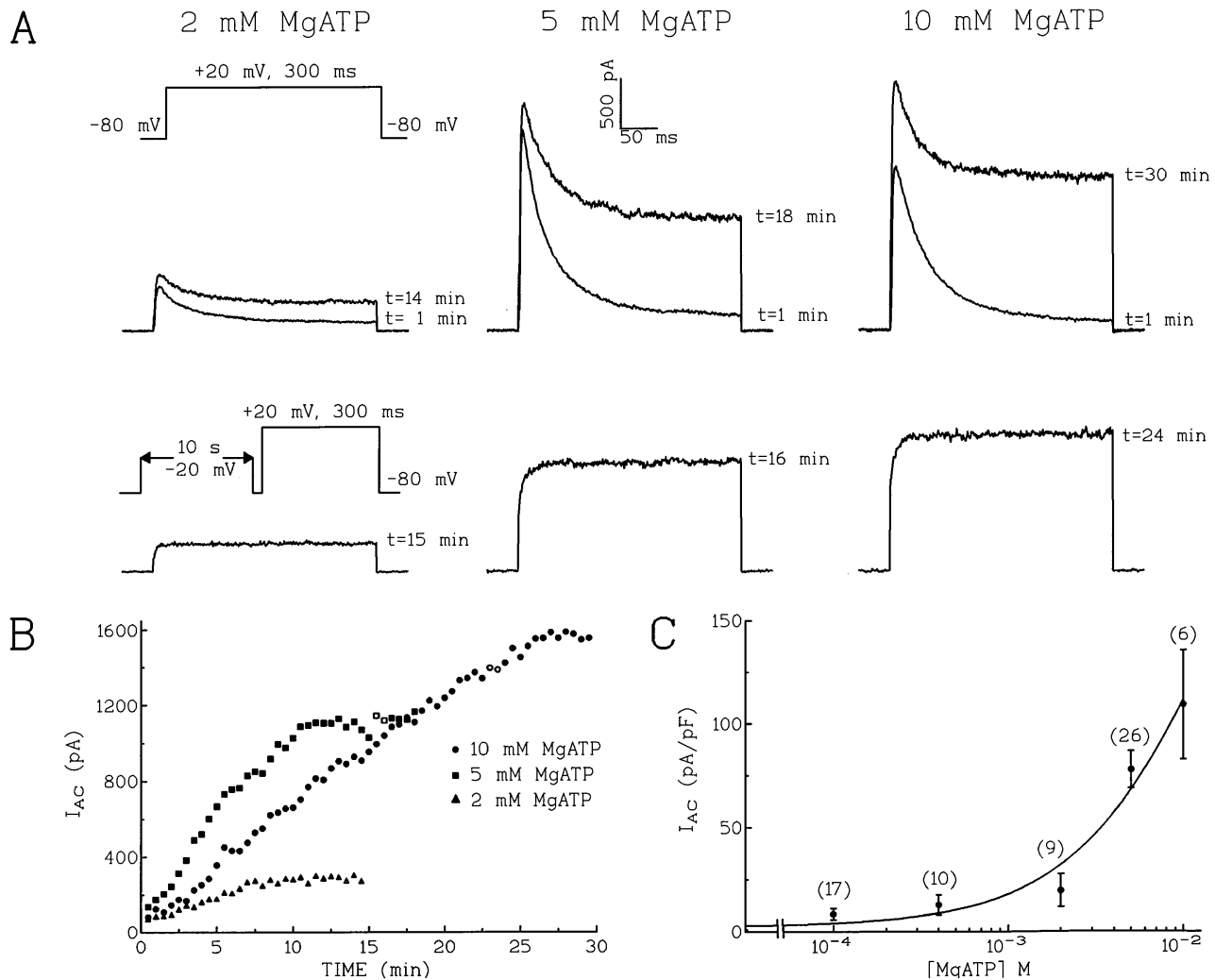


FIGURE 1. MgATP enhances time-dependent expression of I_{AC} K^+ current. (A) Current records showing K^+ currents at indicated times after initiating whole-cell recording with patch pipettes containing standard pipette solution (see METHODS) supplemented with 2, 5, or 10 mM MgATP as indicated. (top) K^+ currents were activated by voltage steps to +20 mV from a holding potential of -80 mV applied at 30-s intervals. (bottom) Voltage clamp steps to +20 mV were preceded by 10-s prepulses to -20 mV, inactivating the A-type K^+ current. (B) I_{AC} currents activated at 30-s intervals by either of the above voltage protocols were measured and plotted against time for three representative cells in which the pipette contained 2 (\blacktriangle), 5 (\blacksquare), or 10 (\bullet) mM MgATP. Open symbols show current amplitudes after inactivating prepulse. (C) Max I_{AC} current density (expressed as pA/pF) was obtained by dividing the maximum I_{AC} current amplitude obtained in experiments such as those illustrated in A and B by the cell capacitance determined from transient cancellation controls of patch clamp amplifier. I_{AC} current density is plotted against pipette MgATP concentration. Results are mean \pm SEM for the indicated number of cells.

plied from a holding potential of -80 mV to a test potential of $+20$ mV, I_{AC} could be selectively measured near the end of a step, at a point where the A type K^+ current had inactivated entirely (Fig. 1 A, top traces). Using the second protocol, I_{AC} was selectively activated with an identical voltage step, after a 10-s prepulse to -20 mV had fully inactivated the A type current (Fig. 1 A, bottom traces).

The time-dependent increase in I_{AC} amplitude observed in whole-cell recordings depended on the concentration of ATP in the pipette solution. At concentrations >1 mM, MgATP dramatically increased the maximum I_{AC} amplitude attained (Fig. 1). At lower concentrations, MgATP was much less effective. With MgATP present in the pipette at 0.1 and 0.4 mM, I_{AC} reached maximum current densities of 8.4 ± 2.7 ($n = 7$) and 12.2 ± 4.9 pA/pF ($n = 10$), respectively. In contrast, at concentrations of 5 and 10 mM MgATP, I_{AC} reached, after 10–25 min, maximums of 78.0 ± 8.9 ($n = 26$) and 109.2 ± 26.3 pA/pF ($n = 6$), respectively (Fig. 1 C).

The dramatic enhancement of I_{AC} current by MgATP observed in whole-cell recordings could have several explanations. Modulation of ion channel function by protein kinases is widespread, while ion channel modulation through an ATP hydrolysis cycle involving ATPases has more recently been reported (Baukowitz et al., 1994; Fakler et al., 1994; Levitan, 1994). Alternatively, ATP could modulate I_{AC} activity through nonhydrolytic binding to the channel or an associated protein, as occurs with ATP-inhibited inward rectifier K^+ channels found in many cells (Ashcroft, 1988a; Takano and Noma, 1993). To distinguish between these possibilities, we eliminated all Mg^{2+} from the pipette solution by substituting KCl and KATP for $MgCl_2$ and MgATP, respectively. Both protein kinases and ATPases require ATP complexed with Mg^{2+} as a substrate (Eckstein, 1985; Levitan, 1994; Hilgemann, 1997). In the absence of Mg^{2+} , 5 mM ATP promoted large increases in I_{AC} , although not as large as those observed with MgATP (Fig. 2, A and C). The nonhydrolyzable ATP analog AMP-PNP (5 mM) also effectively stimulated I_{AC} expression. At a concentration of 0.1 mM, AMP-PNP and MgATP were equally ineffective at enhancing I_{AC} activity (Fig. 2 C).

ATP- γ -S is a poorly hydrolyzable ATP analog that is, like AMP-PNP, a poor substrate for cellular ATPases. In contrast to AMP-PNP, ATP- γ -S is a good substrate for many protein kinases, while the transferred phosphorothioate group is resistant to hydrolysis by phosphatases (Eckstein, 1985). ATP- γ -S produced effects on K^+ currents in AZF cells that were dramatically different from those observed with AMP-PNP. Specifically, with 2 mM ATP- γ -S in the pipette, I_{AC} did not grow in whole-cell recordings. Instead, any noninactivating I_{AC} current that was present upon initiating whole-cell recording was inhibited over a period of several minutes

(Fig. 3 A). At the same time, the decay kinetics of the rapidly inactivating A type K^+ current often slowed dramatically in the presence of ATP- γ -S. In the experiment illustrated in Fig. 3 B, 4 min after commencing whole-cell recording, I_{AC} inactivated with a time constant (τ_i) of 26.1 ms. By 13 min, τ_i had slowed to 254 ms. In spite of the slowed inactivation kinetics, a 10-s prepulse to -20 mV was effective at inactivating nearly all of the I_A current (Fig. 3 C). Overall, with 2 mM ATP- γ -S in the recording pipette, I_{AC} was not clearly detectable in any of 20 cells at any time between 5 and 30 min. In nine of these cells, I_A inactivation kinetics slowed dramatically during the recordings.

ATP and Unitary I_{AC} Currents

Single channel recordings made from AZF cells in the outside-out configuration showed that, in contrast to whole-cell currents, unitary I_{AC} current amplitudes were not increased by raising the “intracellular” MgATP concentration from 2 to 5 mM. In these experiments, I_{AC} currents were first recorded in the whole-cell configuration to allow I_{AC} to reach a stable amplitude. After obtaining an outside-out patch, the holding potential was set to -40 mV, a potential where nearly all I_A channels are inactivated (Mlinar and Enyeart, 1993b). Under these conditions, a single type of K^+ channel was typically present in the patch membrane. Fig. 4 A shows unitary currents activated by voltage steps to $+30$ mV from a holding potential of -40 mV in the presence of either 2 or 5 mM MgATP. Histogram analysis of unitary current amplitudes showed a single major peak for the lower and higher MgATP concentrations with respective means of 3.81 ± 0.62 pA and 3.92 ± 0.81 pA (Fig. 4 A). A second minor peak, with a mean of approximately twice the unitary amplitude, was also present (data not shown).

In whole-cell recordings, MgATP and AMP-PNP both induced expression of noninactivating outward currents presumed to be I_{AC} . Single channel current-voltage (IV) relationships recorded in the presence of the hydrolyzable and nonhydrolyzable nucleotides showed that the corresponding unitary K^+ currents were identical. Single channel IVs for I_{AC} K^+ channels were obtained by applying voltage steps in 10-mV increments to outside-out patches from a holding potential of -40 mV. The single channel IV relationship obtained with 5 mM MgATP or 2 mM AMP-PNP in the pipette were nearly identical (Fig. 4 B). In each case, with otherwise standard pipette and external solutions, I_{AC} channel currents displayed a unitary conductance of ~ 70 pS, estimated between potentials of 0 and $+40$ mV. Thus, the unitary currents activated by low and high concentrations of MgATP or a nonhydrolyzable ATP analog appear to flow through the same I_{AC} channel.

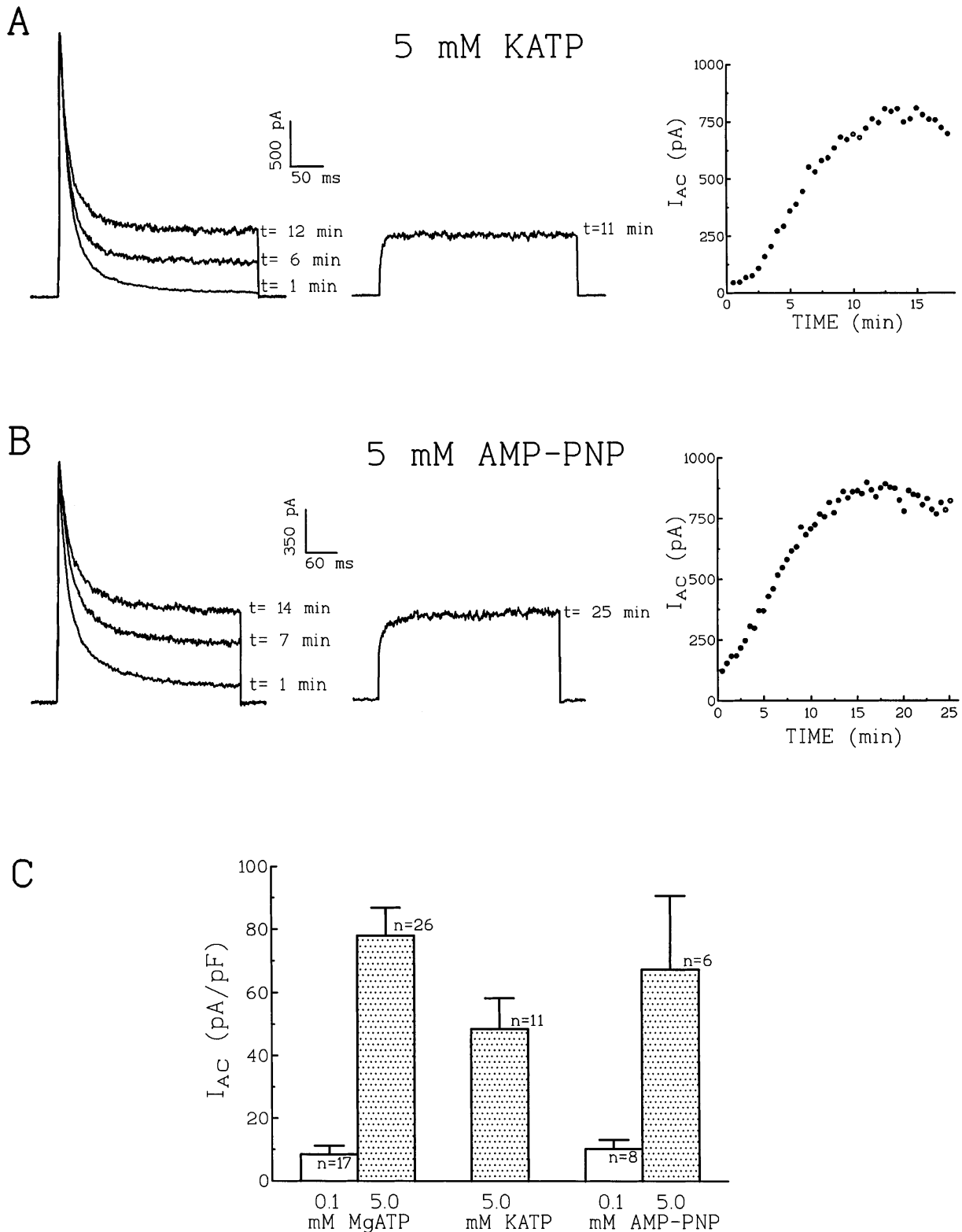


FIGURE 2. Effect of K⁺ ATP and AMP-PNP on I_{AC} expression. The time-dependent increase of I_{AC} K⁺ current was monitored using patch pipettes containing standard solution supplemented with AMP-PNP, or a modified pipette solution containing no MgCl₂ with ATP added as a K⁺ salt. (A and B) K⁺ currents were activated at 30-s intervals from a holding potential of -80 mV using patch electrodes containing 5 mM KATP (A) or 5 mM AMP-PNP (B). K⁺ current records at the indicated times after obtaining the whole-cell configuration with (middle) and without (left) inactivating prepulses as described in Fig. 1. I_{AC} current amplitude is plotted against time at right. (C) Effect of KATP and AMP-PNP on maximum I_{AC} current density. Maximum I_{AC} current density in the presence of 5 mM KATP and 5 mM AMP-PNP was 48.5 ± 9.8 pA/pF (n = 11) and 67.3 ± 23.2 pA/pF (n = 6), respectively, compared with 78.0 ± 8.9 pA/pF (n = 26) with 5 mM MgATP in the pipette solution. Maximum I_{AC} current density was calculated from experiments as in A and B above. MgATP, KATP, and AMP-PNP were applied intracellularly through the pipette solution at either 0.1 or 5.0 mM. Results are mean ± SEM for the indicated number of individual determinations.

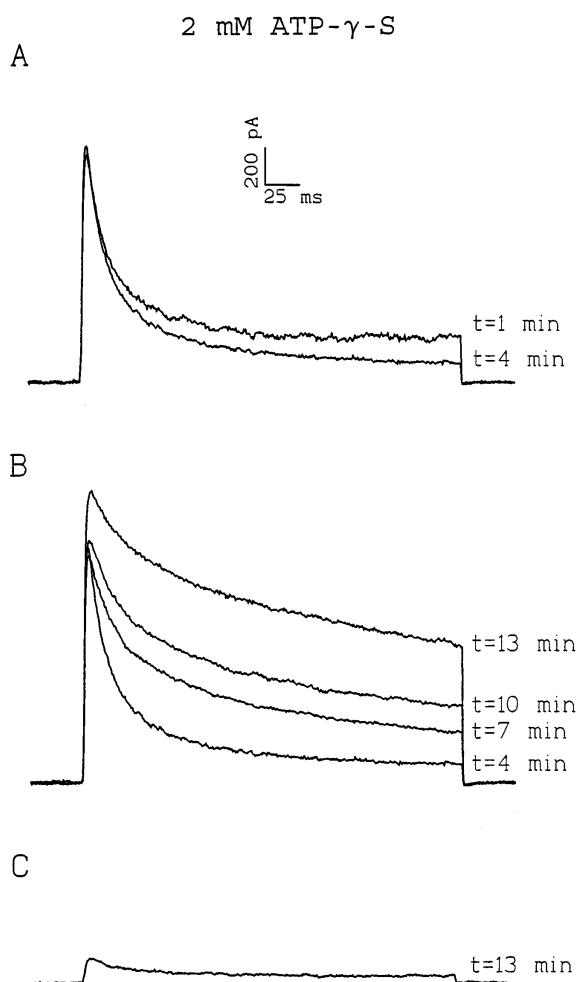


FIGURE 3. Effect of ATP- γ -S on K^+ currents in AZF cells. Cells were voltage clamped in the whole-cell configuration with patch pipette containing 2 mM ATP- γ -S. K^+ currents were activated at 30-s intervals by voltage steps to +20 mV from a holding potential of -80 mV with (C) or without (A and B) a 10-s depolarizing prepulse to -20 mV. Current records were recorded at times after initiating whole-cell recording.

I_{AC} Increase by Other Nucleotides

Experiments with KATP and AMP-PNP indicated that nonhydrolytic binding of ATP was sufficient to convert I_{AC} channels to an active or activatable form. To determine whether other adenine nucleotides would increase I_{AC} amplitude in whole cell recordings, we compared MgATP with ADP and AMP. A time-dependent increase in I_{AC} amplitude occurred in the presence of ADP but not AMP (Fig. 5, A and C). When the pipette contained 5 mM AMP, I_{AC} failed to grow above the control amplitude measured immediately after initiating whole-cell recording (Fig. 5, B and C). Further, in each of five cells, the rapidly inactivating A type K^+ current was observed to decrease with time in the presence of AMP (Fig. 5 B). Overall, when the pipette contained 5 mM ADP, I_{AC} grew to a maximum current density of

36.1 ± 10.8 pA/pF ($n = 12$), compared with 78.0 ± 8.9 pA/pF ($n = 26$) observed with 5 mM MgATP in the patch electrode (Fig. 5 D). When the patch electrode contained 0.1 mM ADP, I_{AC} reached a maximum of only 10.7 ± 2.4 pA/pF ($n = 4$), a value not significantly different from that observed with 0.1 mM MgATP.

In addition to ATP, we found that other nucleotide triphosphates, including GTP and UTP, were also effective in promoting I_{AC} activity in whole cell recordings. In these experiments, sodium salts of ATP, UTP, or GTP were added to the pipette solution at a concentration of 5 mM. With each of these agents, a time-dependent increase in I_{AC} amplitude, which usually reached a maximum value in 10–25 min, was observed (Fig. 6). Overall, these three nucleotides produced similar effects on I_{AC} current densities with maximum values of 72.0 ± 27.5 ($n = 5$), 57.7 ± 23.8 ($n = 7$), and 53.7 ± 18.0 pA/pF ($n = 8$), for ATP, GTP, and UTP respectively (Fig. 6 B). The nucleotide diphosphates GDP and UDP were much less effective than the nucleotide triphosphates at enhancing I_{AC} , but both did significantly increase I_{AC} over the control value observed in the absence of nucleotides (Fig. 6 B).

Current-Voltage Characteristics of Nucleotide-activated K^+ Current

The IV relationships for the noninactivating K^+ currents induced by ATP, GTP, and UTP were similar and indicated that, regardless of the nucleotide, a large fraction of I_{AC} channels are open at membrane potentials at least as negative as -40 mV. In these experiments, I_{AC} was selectively activated by applying voltage steps of varying size from a holding potential of -40 mV. Fig. 7, A and B illustrates typical IV relationships obtained with 5 mM ATP and 5 mM UTP. Sustained outward currents were present at the holding potential. The K^+ current observed in response to depolarizing steps consisted of an apparently instantaneous component and a time-dependent fraction that became more prominent with stronger depolarizations (Fig. 7 A). Similar outwardly rectifying currents were observed with all three nucleotide triphosphates.

The characteristics of I_{AC} currents observed in current-voltage relationships suggested that I_{AC} K^+ channels are at most weakly voltage dependent over potentials ranging from -40 to +40 mV. In an effort to determine to what extent the outwardly rectifying properties of I_{AC} were due to the conductance properties of open channels as opposed to voltage-dependent activation, we compared the steady state IV for the I_{AC} current to the instantaneous current-voltage relationship. The open channel IV (IIV) provides a measure of the open channel conductance properties.

The IIV for I_{AC} was obtained by selectively activating this current from a holding potential of -40 mV with

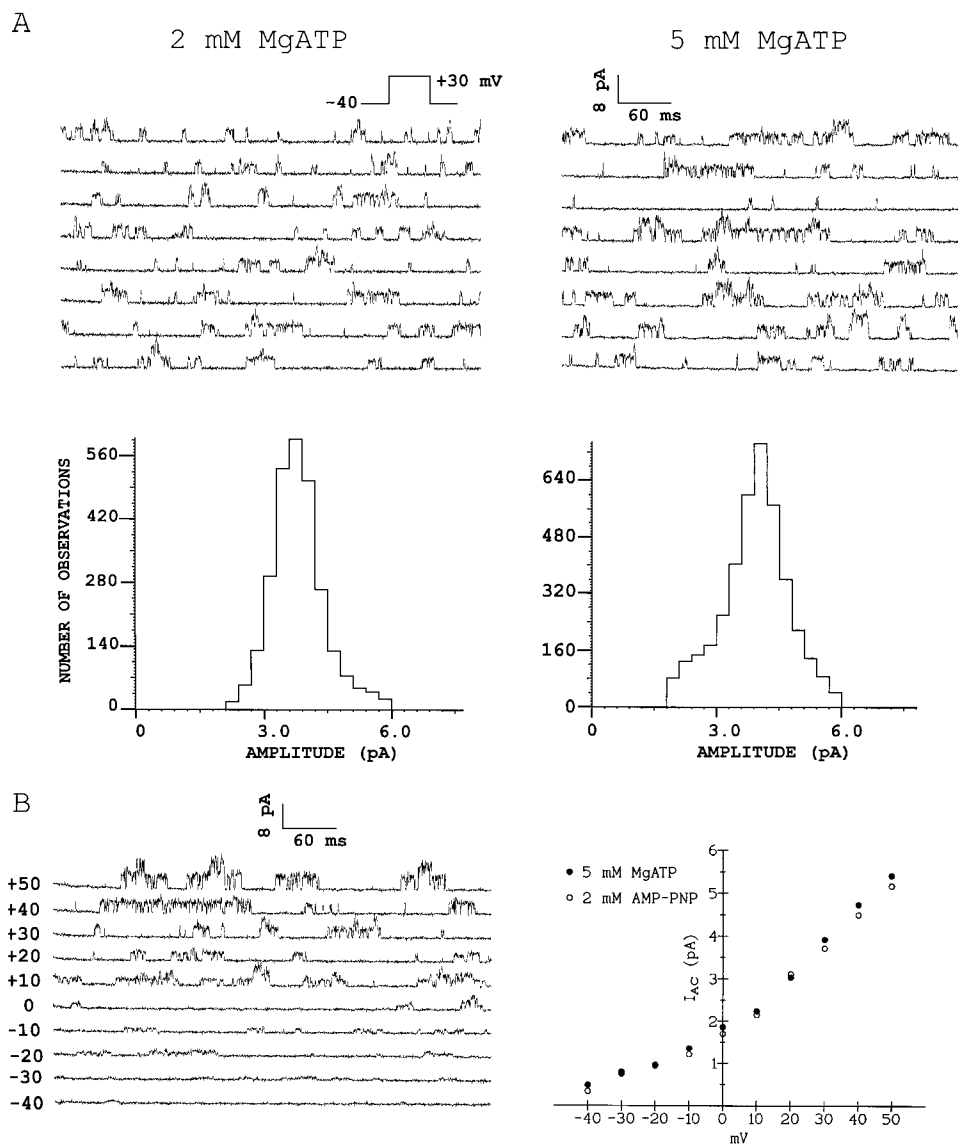


FIGURE 4. MgATP and AMP-PNP on unitary I_{AC} currents. (A) Unitary I_{AC} currents were recorded in the outside-out configuration using pipettes containing standard solution supplemented with either 2 or 5 mM MgATP as indicated. Voltage steps to +20 mV were applied at 10-s intervals from a holding potential of -40 mV. Each amplitude histogram was constructed from unitary currents recorded in response to 90 voltage steps of 300-ms duration. Unitary current amplitudes were distributed into bins of 0.3 pA width. Mean unitary amplitudes of 3.90 ± 0.62 (2,706 events) and 3.92 ± 0.81 pA (3,955 events) were obtained for 2 and 5 mM MgATP, respectively. A second smaller peak of approximately twice the mean amplitude of the major peak is not shown. (B) Single channel IVs with MgATP and AMP-PNP. Current-voltage relationship for unitary I_{AC} currents were obtained in outside-out patches by applying voltage steps in 10-mV increments from a holding potential of -40 mV to potentials up to +50 mV. (left) Traces show unitary currents recorded with standard pipette solution supplemented with 5 mM MgATP. (right) Mean unitary current amplitudes are plotted against voltage for currents recorded in the presence of 5 mM MgATP or 2 mM AMP-PNP. Currents were sampled at 5 KHz and filtered at a cutoff frequency of 1.5 KHz.

150-s depolarizing steps to +50 mV, after which the membrane potential was stepped to new levels between +40 and -120 mV. The I_{AC} "tail current" was measured after 1 ms, before a significant change in the number of open channels occurred (Fig. 7 C).

An estimate of the voltage dependence of I_{AC} activation over the range of potentials from -40 to +40 mV was obtained by dividing current amplitudes taken from the steady state IV relationship by corresponding amplitudes from the IIVs. Dividing the steady state IV values obtained with ATP in Fig. 7 B by the IIV values from Fig. 7 C indicated that the open probability of I_{AC} channels increased by only 30% over this 80-mV range of potentials.

The weak voltage dependence of I_{AC} open probability indicated that the current-voltage characteristics of I_{AC} are due to the conductance properties of open chan-

nels. Accordingly, in a previous study using elevated external K^+ , we have shown I_{AC} to be an outwardly rectifying current (Enyeart et al., 1996b).

Effect of Other Agents on I_{AC}

Each of the nucleotides that activated I_{AC} is a polyvalent anion that can bind polyvalent metal cations. Nucleotide binding of polyvalent trace metals may activate a calcium-activated chloride current in *Xenopus* oocytes (Hilgemann, 1997). It is unlikely that nucleotide chelation of metals activates I_{AC} in AZF cells since EDTA, a nonspecific metal ion chelator, failed to activate this K^+ current when included in the pipette solution. When whole cell recordings were made with standard pipette solution supplemented with 0.1 mM MgATP, I_{AC} reached a maximum density of 8.45 ± 2.74 pA/pF

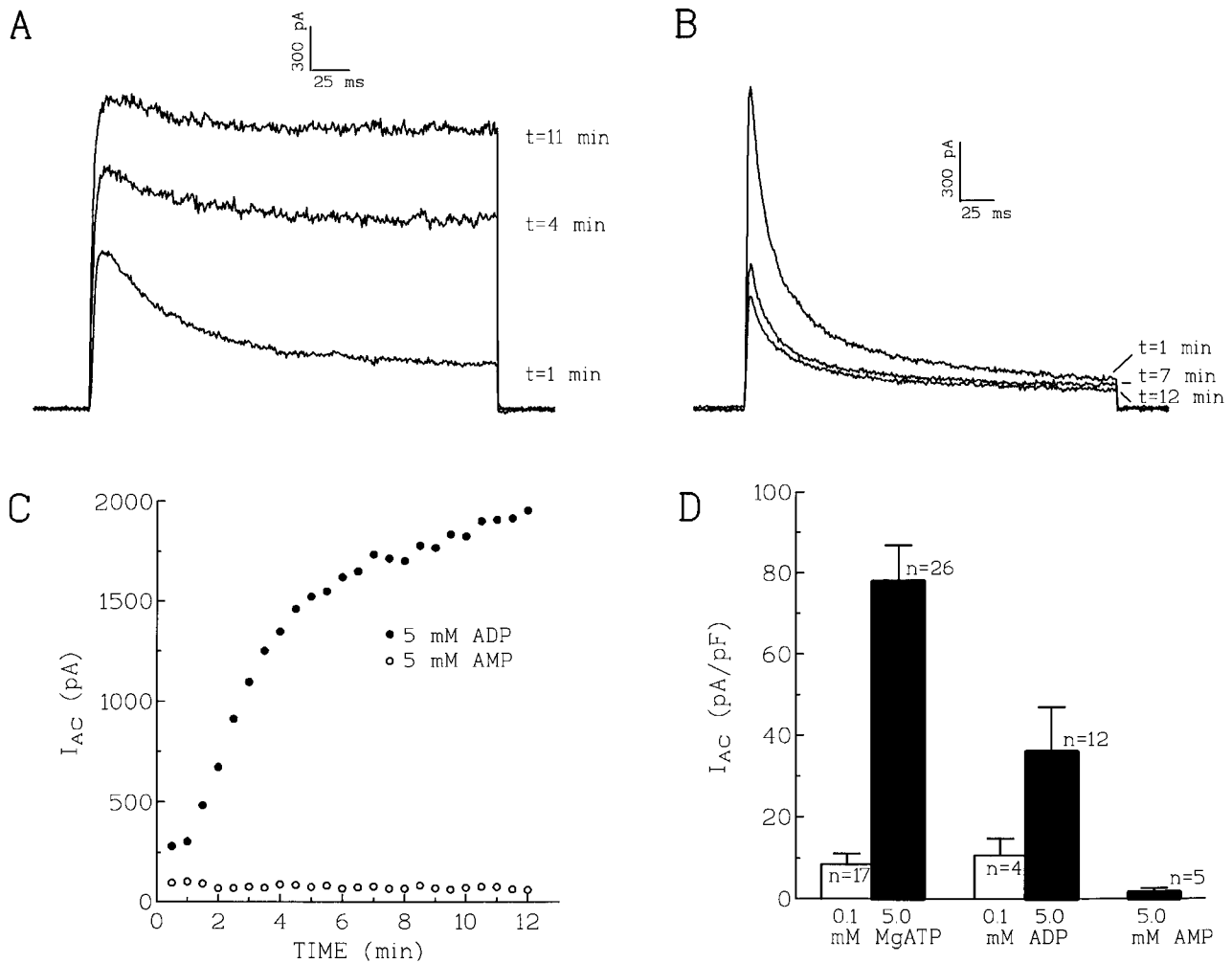


FIGURE 5. Effect of ADP and AMP on AZF cell K^+ currents. K^+ currents were activated by voltage steps to +20 mV applied at 30-s intervals from a holding potential of -80 mV using patch pipettes containing standard pipette solution supplemented with either ADP or AMP. (A and B) K^+ current records at indicated times after initiating whole-cell recording with pipettes containing 5 mM ADP (A) or 5 mM AMP (B). (C) I_{AC} amplitude is plotted against time for cells shown in A and B. (D) Effect of ADP and AMP on maximum I_{AC} current density. Maximum I_{AC} current density was calculated from recordings in which MgATP, ADP, and AMP were applied intracellularly through the pipette solution at either 0.1 or 5.0 mM. Total Mg^{2+} was maintained constant for solutions containing 5 mM nucleotide by addition of $MgCl_2$. Results are mean \pm SEM for the indicated number of individual determinations.

($n = 17$). The addition of EDTA (1 mM) to this pipette solution did not increase I_{AC} . In the presence of 1 mM EDTA, I_{AC} reached a maximum density of 6.24 ± 4.56 pA/pF ($n = 6$).

Although I_{AC} channels are activated rather than inhibited by ATP, modulation by this nucleotide could indicate that I_{AC} channels are structurally similar to ATP-sensitive K^+ channels. Inwardly rectifying ATP-sensitive K^+ channels such as those found in pancreatic β cells display a distinctive pharmacology. K^+ channel activators such as pinacidil dramatically enhance the activity of these channels (Takano and Noma, 1993). However, pinacidil at concentrations of 30 ($n = 4$) and 100 ($n = 2$) μ M failed to measurably increase I_{AC} amplitude in whole-cell recordings.

ACTH and ATP Hydrolysis

The activity of I_{AC} K^+ channels is promoted by a number of nucleotide triphosphates including the nonhydrolyzable ATP analog AMP-PNP. Apparently, activation of I_{AC} channels by these nucleotides requires only binding to the channel or an associated protein. Relatedly, ACTH and its primary intracellular messenger, cAMP, can both inhibit I_{AC} by an A-kinase-independent mechanism requiring ATP hydrolysis (Enyeart et al., 1996b). These results are consistent with a model in which I_{AC} opening and closing are controlled through an ATP hydrolysis cycle involving ATP binding and metabolism via an ATPase.

To test this model and clarify the mechanism of

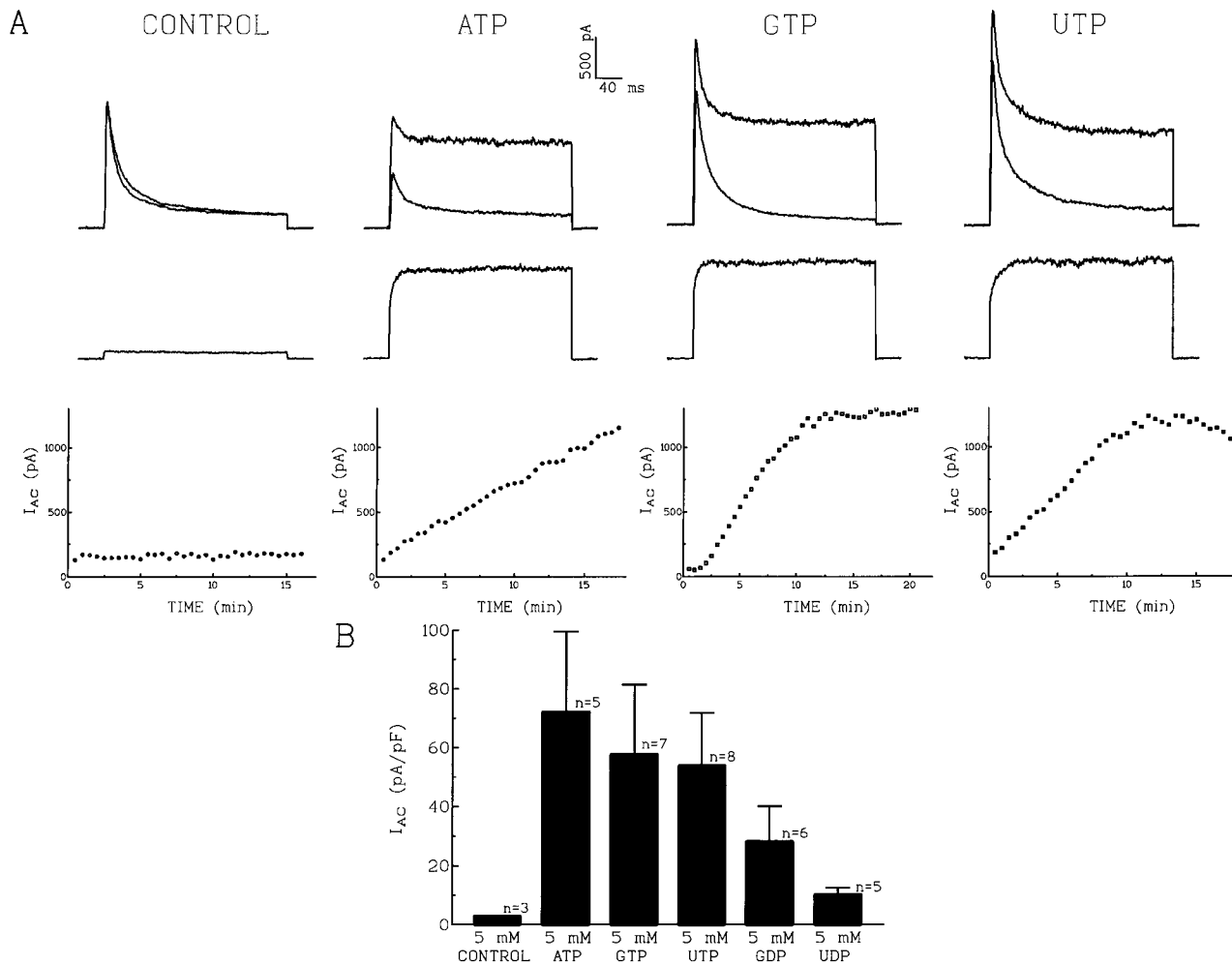


FIGURE 6. Effect of ATP, GTP, UTP, GDP, and UDP on I_{AC} expression. (A) AZF cells were clamped in the whole-cell configuration and voltage steps were applied at 30-s intervals from a holding potential of -80 mV with (*bottom*) or without (*top*) 10-s prepulses to -20 mV. Patch pipettes contained standard solution (*CONTROL*) supplemented with either NaATP, NaGTP, or NaUTP at a concentration of 5 mM. Current traces and corresponding plots of I_{AC} amplitude against time for four representative cells are shown. (B) Maximum I_{AC} current density (expressed as pA/pF) was obtained by dividing the maximum I_{AC} current by cell capacitance in experiments such as those illustrated in A. Results are mean \pm SEM for the indicated number of cells for each nucleotide.

ACTH, we took advantage of our finding that UTP (and GTP) could activate I_{AC} almost as effectively as ATP. However, unlike ATP, UTP is not a substrate for enzymes including adenylate cyclase, protein kinases, and most ATPases. As previously reported (Mlinar et al., 1993a; Enyeart et al., 1996b), when the pipette contains standard solution supplemented with 5 mM ATP and 200 μ M GTP, ACTH inhibits I_{AC} almost completely within 3–5 min ($n > 50$) (Fig. 8 A). When UTP replaced ATP in the recording pipette, ACTH was totally ineffective ($n = 3$) (Fig. 8 B). ACTH was also ineffective when GTP replaced ATP ($n = 3$) (data not shown). The addition of only 50 μ M ATP to a pipette solution containing 5 mM UTP restored the characteristic near complete inhibition of I_{AC} by ACTH (Fig. 8 C). In each of three cells, I_{AC} was inhibited by $>90\%$ under these

conditions.

These results indicate that ACTH does not close I_{AC} channels through ATPase-catalyzed hydrolysis of ATP at the nucleotide binding site since the activating site presumably remained occupied by UTP. The findings also convincingly demonstrate that the outwardly rectifying, noninactivating K^+ current activated by ATP and UTP in AZF cells are the identical ACTH-inhibited I_{AC} current.

DISCUSSION

The central finding of this study is that over a physiological range of concentrations, ATP, in hydrolyzable or nonhydrolyzable forms, dramatically increases I_{AC} K^+ current in bovine AZF cells through a mechanism

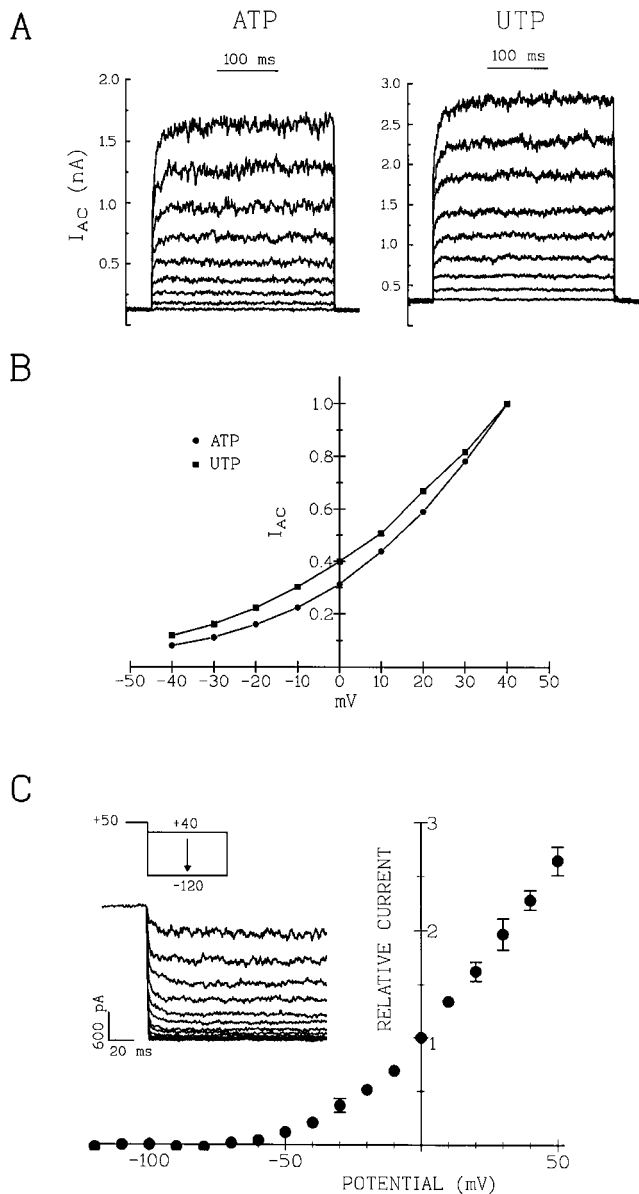


FIGURE 7. I_{AC} current-voltage relationships for ATP and UTP. (A) Whole-cell recordings were made from AZF cells using patch pipettes containing standard solution supplemented with NaATP or NaUTP, each at a concentration of 5 mM. After I_{AC} K^+ current had reached a stable amplitude, the current-voltage relationship was obtained from a holding potential of -40 mV by applying voltage steps at 15-s intervals in 10-mV increments to test potentials up to $+40$ mV. (B) I_{AC} current amplitudes were normalized to the maximum current and plotted against test voltage. (C) Instantaneous current-voltage relationship (IV). After activation of I_{AC} by voltage steps of 150-ms duration to $+50$ mV from a holding potential of -40 mV, the membrane potential was stepped to values between $+40$ and -120 mV, where tail currents were recorded. Maximum tail currents at each potential are expressed as a fraction of the tail current measured at 0 mV and plotted against membrane potential. Values are mean \pm SEM of measurements from three separate cells. (inset) Tail-current records from a single cell.

that presumably requires only the binding of this nucleotide to the channel or a related protein. A number of other nucleotides, including ADP, UTP, and GTP were also effective at enhancing I_{AC} activity. With any of these nucleotides in the pipette, a significant fraction of I_{AC} channels remained tonically active at membrane potentials at least as negative as -40 mV and the open probability increased little between -40 and $+40$ mV. Although the opening of I_{AC} K^+ channels under physiological conditions may require only the binding of ATP to the I_{AC} channel, its inhibition by ACTH appears to proceed through a mechanism other than the hydrolysis of this nucleotide. Due to their capacity to sense cellular ATP levels and set the membrane potential of AZF cells, I_{AC} K^+ channels may act as transducers that couple metabolic signals to membrane depolarization and cortisol secretion.

Modulation of I_{AC} K^+ Current by Adenine Nucleotides

The activation of I_{AC} K^+ channels by both hydrolyzable and nonhydrolyzable forms of ATP clearly distinguishes these channels from the ATP-sensitive K^+ channels described in many other cells. These inwardly rectifying channels are uniformly inhibited by the nonhydrolytic binding of ATP and analogs (Ashcroft, 1988a). However, in a number of ATP-inhibited K^+ channels, low concentrations of MgATP (<100 μ M) are actually required to maintain K^+ channel activity. Phosphorylation of the channel by a protein kinase is the mechanism involved (Lederer and Nichols, 1989; Terzic et al., 1994; Levitan, 1994). Regardless, when ATP concentrations are raised to the millimolar range, inwardly rectifying ATP-sensitive K^+ channels are uniformly inhibited. In contrast, outwardly rectifying I_{AC} channels are activated.

There are very few reports of ion channels that are directly activated by ATP binding. In human sweat glands, the CFTR Cl^- channel has been reported to be activated by both hydrolyzable and nonhydrolyzable forms of ATP at millimolar concentrations (Quinton and Reddy, 1992). However, this channel appears to differ from the I_{AC} channel in that the CFTR channel requires A-kinase-dependent phosphorylation, as well as ATP binding, for activity. Enhanced activity of voltage-gated L-type Ca^{2+} channels in heart cells by nonhydrolyzable ATP analogs has also been reported (O'Rourke et al., 1992). I_{AC} may be the first example of a K^+ -selective channel that is directly activated by nonhydrolytic binding of ATP.

In a variety of cells that express ATP-sensitive K^+ channels, including myocytes, pancreatic β cells, and neurons, the inhibitory actions of ATP are antagonized by ADP, thereby tightly coupling channel activity to the energetic state of the cell (Ashcroft, 1988a; Takano and Noma, 1993; Terzic et al., 1994). In AZF cells, ADP and ATP each enhance the I_{AC} K^+ current, although ADP is

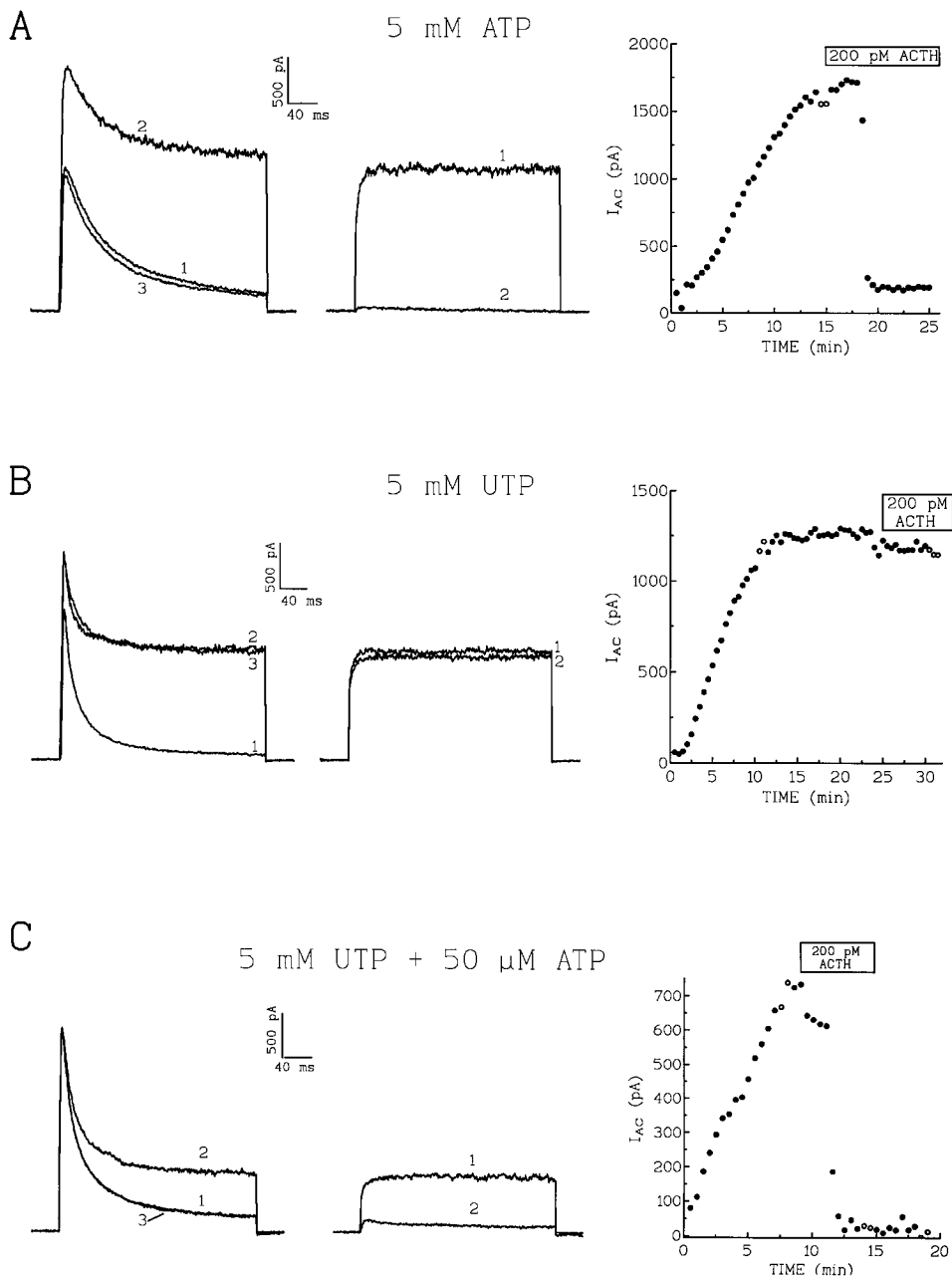


FIGURE 8. Effect of nucleotide triphosphates on I_{AC} inhibition by ACTH. I_{AC} was recorded with pipettes containing standard solution supplemented with 200 μ M GTP and either 5 mM NaATP (A), 5 mM NaUTP (B), or 5 mM NaUTP and 50 μ M NaATP (C). I_{AC} was recorded at 30-s intervals from a holding potential of -80 mV in response to voltage steps to $+20$ mV with (middle) or without (left) 10-s prepulses to -20 mV. After I_{AC} reached a maximum amplitude, cells were superfused with 200 pM ACTH. Trace numbering: (1) initial K^+ current; (2) maximum current before ACTH; (3) current after steady state block with ACTH. I_{AC} amplitudes are plotted against time for each of the three cells. Open symbols show I_{AC} amplitudes measured using 10-s prepulse to -20 mV.

less effective. Thus, in contrast to many cells where ATP and ADP exert opposing actions on ATP-sensitive K^+ channels, in bovine AZF cells both nucleotides are agonists. It is possible that at some $[ATP]/[ADP]$ ratios, the less effective ADP might antagonize the stimulatory action of ATP on I_{AC} channels.

In contrast to ATP and AMP-PNP, the poorly hydrolyzable ATP analog ATP- γ -S failed to enhance I_{AC} current and was actually inhibitory. While ATP- γ -S is a poor substrate for ATPases and phosphatases, it is a surprisingly good substrate for most kinases (Eckstein, 1985). Further, the phosphorothioate that is transferred from ATP- γ -S is poorly hydrolyzed. Proteins normally regulated by a phosphorylation/dephosphoryla-

tion cycle end up highly thiophosphorylated. This would suggest that phosphorylation of I_{AC} channels by an unidentified protein kinase inhibits I_{AC} and overrides enhancement of channel activity by ATP. The reduction, rather than increase, in I_{AC} K^+ current amplitude observed in the first few minutes of whole-cell recording with pipettes containing ATP- γ -S is consistent with this model. Presumably, ATP- γ -S binds to the nucleotide binding site associated with enhanced I_{AC} activity. However, this action is negated by a slowly reversible phosphorylation. This model would also account for the dramatically different effects of ATP- γ -S and AMP-PNP on K^+ current in AZF cells.

The slowing of I_A inactivation kinetics induced by

ATP- γ -S may also be due to the slowly hydrolyzable thiophosphorylation of the normally rapidly inactivating I_A K^+ channels. The inactivation kinetics of several A type K^+ channels are regulated by protein kinase-mediated phosphorylation that can either accelerate or slow inactivation (Covarrubias et al., 1994; Drain et al., 1994).

Activation of I_{AC} by Other Nucleotides

Other nucleotide triphosphates including GTP and UTP increased I_{AC} K^+ current in a manner similar to ATP. Accordingly, ATP-sensitive K^+ channels in skeletal muscle and ventricular myocytes are inhibited by GTP and UTP, although less effectively than by ATP itself (Spruce et al., 1987; Lederer and Nichols, 1989). In contrast to the inhibitory effects of nucleotide triphosphates on many ATP-sensitive channels, various nucleotide diphosphates including UDP and GDP activate these same channels (Takano and Noma, 1993; Terzic et al., 1994). Thus, while nucleotide di- and triphosphates exert opposing effects on the activity of classic ATP-sensitive channels, these nucleotides have qualitatively similar effects on I_{AC} channel activity.

Our results indicate that a diverse group of nucleotides, including purine and pyrimidine triphosphates and diphosphates, alone or complexed with Mg^{2+} , can activate I_{AC} K^+ channels through nonhydrolytic binding. Two nucleotide binding domains are present on ATP-sensitive K^+ channel-associated sulfonylurea receptors that form the β subunit of these channels in various cells (Aguilar-Bryan et al., 1995). Although the nucleotide binding site(s) on I_{AC} channels seem to be similar with regard to lack of nucleotide specificity, the number and location of these sites on I_{AC} channels remain to be identified.

The sulfonylurea receptor-coupled ATP-sensitive K^+ channels are inwardly rectifying and, unlike most K^+ channels, include two rather than six membrane-spanning domains (Ho et al., 1993; Aguilar-Bryan et al., 1995). The structure of the novel outwardly rectifying I_{AC} channel has not been determined. However, the ineffectiveness of pinacidil in enhancing I_{AC} current does not suggest a similarity between I_{AC} and ATP-inhibited channels. Further, in other experiments, we have found that the sulfonylurea glibenclamide, which potently inhibits inwardly rectifying ATP-sensitive K^+ channels, is much less effective at inhibiting I_{AC} K^+ channels (our unpublished observations).

ATP and I_{AC} Channel Gating

Gating of I_{AC} channels appears to be controlled by the complex interaction of metabolic factors in conjunction with a weak voltage dependence. In spite of the dramatic enhancement of I_{AC} K^+ current by ATP, the mechanism involved is not yet clear. Perhaps the bind-

ing of ATP alone is sufficient to activate the channel, regardless of membrane potential. Whole-cell and single-channel recordings did indicate that, with ATP and other nucleotides in the patch electrode, a large fraction of I_{AC} channels are open at -40 mV. Channel open probability increased by only ~ 0.3 between -40 and $+40$ mV. In this regard, when ATP is excluded from the pipette in whole-cell recordings, I_{AC} current cannot be activated at test potentials as positive as $+70$ mV. Thus, if the binding of ATP shifts the voltage dependence of the channel such that I_{AC} channels open at more negative potentials, then this shift must be very large (i.e., >100 mV). A quantitative study of I_{AC} activation at more negative potentials and the possible effects of ATP on the process is hampered by the copresence of the I_A K^+ current as well as the outwardly rectifying nature of I_{AC} itself.

It may be possible to study the modulation of single I_{AC} channels by ATP in excised inside-out patches. However, in excised patches, the activity of I_{AC} channels is quite variable and unstable. ACTH and membrane-permeable forms of cAMP do not reliably inhibit single I_{AC} channels in outside-out patches as they do in whole-cell recordings (our unpublished observations). Likewise, in an extensive study of ATP-sensitive K^+ channels in rat heart, a large variability in ATP sensitivity and Hill coefficients was observed. Apparent $K_{0.5}$ s for ATP ranged from 9–580 μ M. ATP sensitivity of the cardiac channel also decreased during the course of an experiment (Findlay and Faivre, 1991). Overall, in excised patches from a variety of cells, ATP inhibits these inward rectifier K^+ channels at concentrations far below physiological concentrations of this nucleotide (Ashcroft, 1988a; Terzic et al., 1994; Takano and Noma, 1993). In contrast, it is clear from our studies using whole-cell recording in AZF cells that only physiological concentrations of ATP enhance the activity of I_{AC} channels over a wide range of potentials.

ACTH and ATP Hydrolysis

In the present study, we have demonstrated that the binding of ATP dramatically enhances the activity of I_{AC} channels measured in response to membrane depolarization. Previously, we had demonstrated that ACTH and cAMP can each inhibit I_{AC} K^+ current by a mechanism that is independent of A kinase activation while requiring ATP hydrolysis. Taken together, these results suggested that the gating of I_{AC} could be controlled through an ATP hydrolysis cycle, as has been observed for CFTR Cl^- channels of the heart (Baukowitz et al., 1994). If ACTH-mediated hydrolysis of ATP at its binding site on the I_{AC} channel resulted in channel closing, then it is unlikely that channels activated by UTP would be effected since UTP is not a substrate for most ATPases (Azhar and Menon, 1975; Krebs and Beavo, 1979;

Edelman et al., 1987). Therefore, the effective inhibition of UTP-activated I_{AC} channels by ACTH in the presence of only 50 μ M ATP argues strongly against a model for channel closing requiring hydrolysis of this nucleotide by an ATPase. However, an unusual class of ATPases that hydrolyze a variety of nucleotides including UTP has been described (Beukers et al., 1993).

In contrast to most ATPases that have K_m s of one to several millimolar, protein kinases are typically activated by ATP at 100–1,000-fold lower concentrations (Krebs and Beavo, 1979). ACTH may inhibit I_{AC} channels through activation of an unidentified protein kinase. The suppression of I_{AC} by ATP- γ -S is consistent with this model.

ATP Sensing, Membrane Potential, and Cortisol Secretion

The resting potential of bovine AZF cells approaches the Nernst equilibrium potential for K^+ (Mlinar et al., 1993a). Of the two detectable K^+ currents expressed by these cells, I_{AC} channels display properties consistent with one that would contribute strongly to the resting potential. The activation of I_{AC} K^+ channels by physiological levels of ATP suggests that these membrane proteins could act as sensors coupling the metabolic state of the cell to membrane potential and ultimately, cortisol secretion (Enyeart et al., 1993).

In insulin-secreting cells of the pancreas, ATP-sensitive K^+ channels play a key role in excitation–secretion coupling. High blood glucose levels are associated with elevated ATP, K^+ channel inhibition, and membrane depolarization leading to Ca^{2+} entry and insulin secretion (Ashcroft, 1988a, 1988b). In bovine AZF cells where I_{AC} channels are activated rather than inhibited by ATP, elevated glucose would be associated with I_{AC}

activation and membrane hyperpolarization, thereby suppressing Ca^{2+} entry and cortisol secretion (Enyeart et al., 1993). Thus, because ATP-sensitive K^+ channel activity in AZF cells and pancreatic β cells is modulated in opposite directions by ATP, metabolic conditions inducing insulin secretion might be expected to suppress cortisol production. In this regard, cortisol is secreted under conditions of metabolic stress such as starvation, whereas insulin secretion is suppressed (Bondy, 1985).

Metabolically, the glucose-conserving hormone cortisol has effects opposing those of insulin. Cortisol stimulates gluconeogenesis and inhibits glucose uptake and use in many tissues (Bondy, 1985). Because of the antagonistic actions of these two hormones on glucose metabolism, it may then be appropriate that the cells that secrete them express ATP-sensitive K^+ channels whose activity is regulated by ATP in opposite directions.

Although the above scheme linking cellular ATP levels to K^+ channels and secretion of two opposing hormones is attractive, cortisol secretion occurs primarily under the control of ACTH released by the pituitary. However, in bovine AZF cells, ACTH triggers depolarization-dependent Ca^{2+} entry and cortisol secretion through inhibition of I_{AC} (Enyeart et al., 1993). It would be interesting to determine whether ACTH-mediated I_{AC} inhibition could be modulated by altering external glucose concentration. Irrespective of its function in cortisol secretion, I_{AC} is a distinctive new type of K^+ channel that is activated rather than inhibited by ATP. Perhaps other cells that secrete hormones with antiinsulin effects (e.g., glucagon, catecholamines) also express ATP-activated K^+ channels.

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