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# Purine and Pyrimidine Nucleotides Inhibit a Noninactivating K+ Current and Depolarize Adrenal Cortical Cells through a G Protein-Coupled Receptor

LIN XU and JOHN J. ENYEART

*Department of Pharmacology (L.X., J.J.E.) and Neuroscience Program (J.J.E.), The Ohio State University College of Medicine, Columbus, Ohio*

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## ABSTRACT

Bovine adrenal zona fasciculata (AZF) cells express a noninactivating $K^+$ current ($I_{AC}$) that sets the resting membrane potential and may mediate depolarization-dependent cortisol secretion. External ATP stimulates cortisol secretion through activation of a nucleotide receptor. In whole-cell patch clamp recordings from bovine AZF cells, we found that ATP selectively inhibited $I_{AC}$ $K^+$ current by a maximum of 75.7 ± 3% ($n$ = 13) with a 50% inhibitory concentration of 1.3 $\mu$M. A rapidly inactivating A-type $K^+$ current was not inhibited by ATP. Other nucleotides, including ADP and the pyrimidines UTP and UDP, also inhibited $I_{AC}$, whereas 2-methylthio-ATP (2-MeSATP) and CTP were completely ineffective. The rank order of potency for six nucleotides was UTP = ADP > ATP > UDP ≫ 2-MeSATP = CTP. At maximally effective concentrations, UTP, ADP, and UDP inhibited $I_{AC}$ current by 81.4 ± 5.2% ($n$ = 7), 70.7 ± 7.2% ($n$ = 4), and 65.2 ± 7.9% ($n$ = 5), respectively. Inhibition of $I_{AC}$ by external ATP was reduced from 71.3 ± 3.2% to 22.8 ± 4.5% ($n$ = 18) by substituting guanosine 5′-*O*-2-(thio) diphosphate for GTP in the patch pipette. Inhibition of $I_{AC}$ by external ATP (10 $\mu$M) was markedly suppressed (to 17.3 ± 5.5%, $n$ = 9) by the nonspecific protein kinase antagonist staurosporine (1 $\mu$M) and eliminated by substituting the nonhydrolyzable ATP analog 5-adenylyl-imidodiphosphate or UTP for ATP in the pipette. ATP-mediated inhibition of $I_{AC}$ was not altered by the kinase C antagonist calphostin C, the calmodulin inhibitory peptide, or by buffering the intracellular (pipette) $Ca^{++}$ with 20 mM 1,2-bis-(2-aminophenoxy)ethane-*N, N,N′,N′*-tetraacetic acid. In current clamp recordings, ATP and UTP (but not CTP) depolarized AZF cells at concentrations that inhibited $I_{AC}$ $K^+$ current. These results demonstrate that bovine AZF cells express a nucleotide receptor with a $P2Y_3$ agonist profile that is coupled to the inhibition of $I_{AC}$ $K^+$ channels through a GTP-binding protein. The inhibition of $I_{AC}$ $K^+$ current and associated membrane depolarization are the first cellular responses demonstrated to be mediated through this receptor. Nucleotide inhibition of $I_{AC}$ proceeds through a pathway that is independent of phospholipase C, but that requires ATP hydrolysis. The identification of a new signaling pathway in AZF cells, whereby activation of a nucleotide receptor is coupled to membrane depolarization through inhibition of a specific $K^+$ channel, suggests a mechanism for ATP-stimulated corticosteroid secretion that depends on depolarization-dependent $Ca^{++}$ entry. This may be a means of synchronizing the stress-induced secretion of corticosteroids and catecholamines from the adrenal gland.





Extracellular ATP acts as a neurotransmitter or local hormone to elicit responses in a variety of cells. By activation of specific receptors, ATP mediates or modulates a range of physiological processes that include neurotransmission, contraction of smooth and cardiac muscle cells, inflammatory and immune responses, and secretion of hormones including insulin, glucagon, catecholamines, and corticosteroid hormones (Dalziel and Westfall, 1994; Fredholm et al., 1994; Hoey et al., 1994; Williams and Burnstock, 1997).

Membrane receptors for ATP and other nucleotides can be grouped into two major classes. The P2X receptors form ligand-gated, nonselective cation channels, whereas P2Y receptors are G protein-coupled, membrane proteins (Dubyak and El-Moatassim, 1993; Dalziel and Westfall, 1994; Williams and Burnstock, 1997). Within each of these two major groups, multiple subtypes exist that can be distinguished based on their sensitivity to ATP and other nucleotides. In particular, G protein-coupled $P2Y_1$ receptors include those that are preferentially activated by purines such as ATP and ADP, and the pyrimidines UTP and UDP (Dubyak and El-Moatassim, 1993; Dalziel and Westfall, 1994; Williams and Burnstock, 1997). The discovery that P2Y receptors included

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**ABBREVIATIONS:** $I_{AC}$, noninactivating potassium current in bovine adrenal fasciculata cells; 2-MeSATP, 2-methylthio-ATP; AZF, bovine adrenal fasciculata; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N, N,N′,N′*-tetraacetic acid; $IC_{50}$, 50% inhibitory concentration; AMP-PNP, 5′-adenylyl-imidodiphosphate; GDP-$\beta$-S, guanosine 5′-*O*-2-(thio) diphosphate; DMEM, Dulbecco's modified Eagle's medium; PLC, phospholipase C; ACTH, adrencorticotropic hormone; PBS, phosphate-buffered saline.

a subtype that was preferentially activated by both purines and pyrimidines led to the subclassification of this family into $P2Y_1$ and $P2Y_2$ receptors (Lustig et al., 1993; Parr et al., 1994; Williams and Burnstock, 1997). The cloning of additional P2Y receptors with distinct structures and pharmacological profiles has resulted in the addition of at least six new subtypes to this family ($P2Y_3$–$P2Y_8$) (reviewed in Filtz et al., 1997 and Williams and Burnstock, 1997).

In bovine adrenal zona fasciculata (AZF) cells, ATP, UTP, and ADP stimulate cortisol secretion at concentrations between $10^{-6}$ and $10^{-4}$ M through a $Ca^{++}$-dependent process (Niitsu, 1992; Hoey et al., 1994). The cellular mechanisms by which ATP and other nucleotides stimulate cortisol secretion are unknown. AZF cells express a novel $K^+$ channel ($I_{AC}$) that appears to set the resting membrane potential (Mlinar et al., 1993a; Enyeart et al., 1996). Corticotropin [adrencorticotropic hormone (ACTH)] and AII, two peptide hormones that physiologically regulate cortisol secretion, inhibit $I_{AC}$ and depolarize AZF cells at concentrations identical with those that stimulate steroidogenesis (Mlinar et al., 1993a). $I_{AC}$ channels couple peptide receptor activation to membrane depolarization, $Ca^{++}$ entry, and cortisol secretion (Enyeart et al., 1993; Mlinar et al., 1993a). To determine whether nucleotide receptors on AZF cells might also be linked to $I_{AC}$ inhibition, we have studied the effect of ATP and other nucleotides on $I_{AC}$ current in whole-cell patch clamp recordings from bovine AZF cells.

## Materials and Methods

Tissue culture media, antibiotics, fibronectin, and fetal bovine sera were obtained from Gibco (Grand Island, NY). Coverslips were purchased from Bellco Glass, Inc. (Vineland, NJ). Enzymes, ACTH(1–24), MgATP, NaATP, NaUTP, NaUDP, KADP, 5-adenylylimidodiphosphate (AMP-PNP, lithium salt), NaCTP, NaGTP, guanosine 5′-*O*-2-(thio)-diphosphate (GDP-β-S), 2-MeSATP, 1,2-bis-(2-aminophenoxy)ethane-*N, N,N′,N′*-tetraacetic acid (BAPTA), and staurosporine were obtained from Sigma Chemical Company (St. Louis, MO). Calmodulin inhibitory peptide (residues 290–309 of CaM kinase II) was obtained from Biomol (Plymouth Meeting, PA).

**Isolation and Culture of Adrenocortical Cells.** Bovine adrenal glands were obtained from steers (age range, 1–3 years) within 60 min of slaughter at a local slaughterhouse. Fatty tissue was removed immediately and the glands were transported to the laboratory in ice-cold phosphate-buffered saline (PBS) containing 0.2% dextrose. Isolated AZF cells were prepared as described previously (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 was trimmed away from the cortex and discarded. The capsule with attached glomerulosa, and thicker fasciculata layer were then dissected into pieces approximately 1.0 × 1.0 × 0.5 cm. A Stadie-Riggs tissue slicer (Thomas Scientific) was used to separate fasciculata tissue from the glomerulosa layers by slicing 0.3- to 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 (about 0.5 mm) and capsule with attached glomerulosa were discarded. Fasciculata tissue slices were then diced into 0.5-$mm^3$ pieces and dissociated with 2 mg/ml (about 200–300 U/ml) of Type I collagenase (neutral protease activity not exceeding 100 units/mg of solid), 0.2 mg/ml deoxyribonuclease in Dulbecco's modified Eagle's medium (DMEM)/F12 for approximately 1 h at 37°C, triturating after 30 and 45 min with a sterile, plastic transfer pipette. The tissue/cell 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 with DMEM/0.2% BSA, centrifuging as before. After resuspension in DMEM, cells were filtered through 200-μm stainless steel mesh to remove clumps. Dispersed cells were again centrifuged and either resuspended in DMEM/F12 (1:1) with 10% fetal bovine serum (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 about $4 \times 10^6$ 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 μg/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 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, and 200 μM GTP, and 5 mM MgATP with pH buffered to 7.2 using KOH. Deviations from the standard solution are described in the text. Pipette [$Ca^{++}$] 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. All solutions were filtered through 0.22-μm cellulose acetate filters. The $Na^+$ salts of ATP, UTP, and CTP were applied externally by bath perfusion controlled manually by a six-way rotary valve.

AZF cells were used for patch clamp experiments 2 to 12 h after plating. Typically, cells with diameters of <15 μm and capacitances of 8 to 15 pF were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml), which was perfused continuously by gravity at a rate of 3 to 5 ml/min. Patch electrodes with resistances of 1.0 to 2.0 MΩ were fabricated from Corning 0010 glass (Garner Glass Co., Claremont, CA). These routinely yielded access resistances of 1.5 to 2 MΩ and voltage clamp time constants of less than 100 μs. $K^+$ currents were recorded at room temperature (22–25°C) following the procedure of Hamill et al. (1981) using an Axopatch 1D patch clamp amplifier (Axon Instruments, Burlingame, CA).

Pulse generation and data acquisition were done using a personal computer and PCLAMP software with a TL-1 interface (Axon Instruments). Currents were digitized at 1 to 20 kHz after filtering with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using scaled hyperpolarizing steps of one-third to one-fourth amplitude. Data were analyzed and plotted using PCLAMP 5.5 and 6.02 (CLAMPAN and CLAMPFIT) and SigmaPlot (version 4.0). Series-resistance compensation was not used in most experiments. The mean amplitude of $I_{AC}$ current in AZF cells was less than 750 pA. A current of this size in combination with a 4 MΩ access resistance produces a voltage error of only 3 mV, which was not corrected.

## Results

Bovine AZF cells express two types of $K^+$ current, a rapidly inactivating, voltage-gated A-type $K^+$ current, and a noninactivating weakly voltage-dependent current ($I_{AC}$). $I_{AC}$ is present initially, but grows dramatically over a period of minutes in whole-cell recordings, provided that ATP or other nucleotides are present at millimolar concentrations in the recording pipette (Mlinar and Enyeart, 1993; Mlinar et al., 1993a; Enyeart et al., 1996; Enyeart et al., 1997). The absence of time and voltage-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 are applied from a holding potential of −80 mV to a test potential of +20

mV, $I_{AC}$ could be measured selectively near the end of a voltage step where the rapidly inactivating A-type current had completely inactivated (Fig. 1A, left traces). Alternatively, $I_{AC}$ was selectively activated with an identical voltage step, after a 10-s prepulse to −20 mV had fully inactivated the A-type $K^+$ current (Fig. 1A, right traces). Results reported in this study were obtained in recordings from more than 200 AZF cells.

**Selective Inhibition of $I_{AC}$ by External Nucleotides.** ATP applied to AZF cells externally by bath perfusionproduced a selective, concentration-dependent inhibition of $I_{AC}$ (Fig. 1). In these experiments, $I_{AC}$ was recorded at 30-s intervals. When this current reached a stable maximum amplitude, ATP was superfused at concentrations ranging from 0.1 to 100 $\mu$M. Inhibition of $I_{AC}$ by ATP began after a delay of 1.5 to 3 min and required several additional minutes to reach a steady-state value (Fig. 1B). ATP inhibited $I_{AC}$ half-maximally with an estimated 50% inhibitory concentration ($IC_{50}$) of 1.28 $\mu$M. Even at maximally effective concentrations, ATP did not completely inhibit $I_{AC}$, reducing this current by 71.3 ± 3.2% ($n$ = 26) and 73.9 ± 3.5% ($n$ = 13) at concentrations of 10 and 100 $\mu$M, respectively (Fig. 1C). Inhibition of $I_{AC}$ by ATP was, in general, poorly reversible even with prolonged washing (Fig. 1B). In contrast to $I_{AC}$ current, the rapidly inactivating voltage-gated A-type current was not reduced by ATP (Fig. 1A).

Inhibition of $I_{AC}$ by ATP showed evidence of desensitization. When ATP (10 $\mu$M) was superfused without previous exposure to this nucleotide, $I_{AC}$ was inhibited by a maximum of 83.1 ± 3.3% ($n$ = 11). By comparison, when cells were first exposed to 1 $\mu$M ATP before superfusing 10 $\mu$M ATP, $I_{AC}$ was inhibited by a maximum of 62.7 ± 3.8% ($n$ = 15) relative to the current amplitude in control saline.

The selective inhibition of $I_{AC}$ by ATP was independent of test voltage. In the experiment illustrated in Fig. 2, $I_{AC}$ was allowed to grow to a stable value before recording $K^+$ currents at test potentials ranging from −60 to +60 mV in control saline and after inhibition by 10 $\mu$M ATP. $I_{AC}$ was inhibited by 84 to 90% at all test potentials where $I_{AC}$ current was large enough for accurate measurement (−10 to 100 mV) (Fig. 2B). In contrast, the inactivating A-type $K^+$ current was insensitive to ATP at each test voltage (Fig. 2A, right traces).

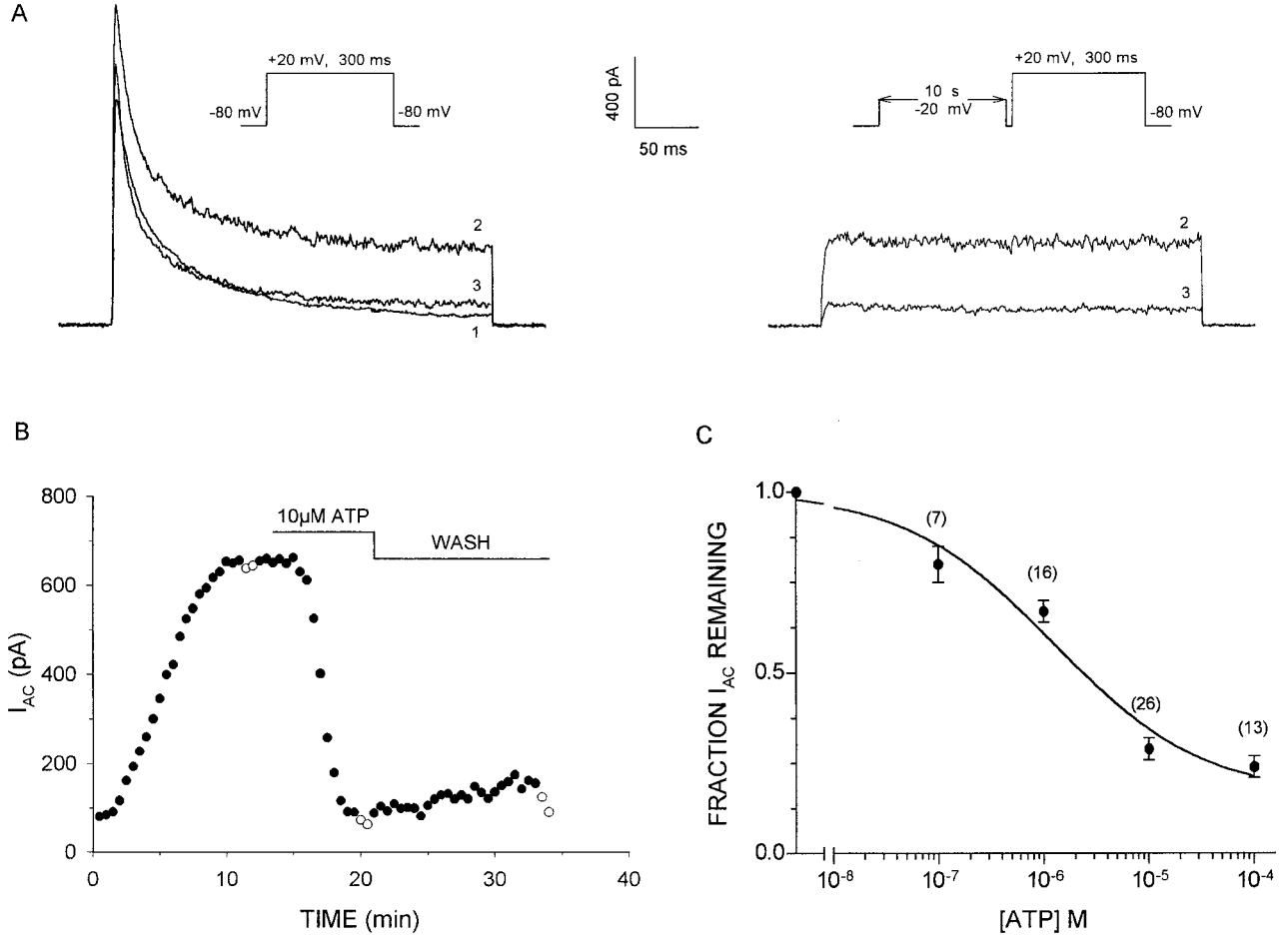


**Fig. 1.** Time- and concentration-dependent inhibition of $I_{AC}$ by external ATP. Whole-cell $K^+$ currents were recorded from bovine AZF cells at 30-s intervals in response to voltage steps to +20 mV applied from a holding potential of −80 mV with or without 10-s prepulses to −20 mV, which inactivated A-type $K^+$ current. After $I_{AC}$ reached a stable amplitude, cells were superfused with NaATP at various concentrations. A, $K^+$ current records made with patch pipettes containing standard pipette solution containing 5 mM MgATP and 200 $\mu$M GTP, with (right) or without (left) 10-s prepulses to −20 mV. Numbers correspond to currents immediately after initiating whole-cell recording (1), after $I_{AC}$ had reached a maximum amplitude (2), and after inhibition by ATP (10 $\mu$M) (3). B, $I_{AC}$ amplitudes recorded with (open circles) or without (solid circles) depolarizing prepulses are plotted against time. NaATP (10 $\mu$M) was perfused as indicated. C, inhibition curve. Fraction of unblocked $I_{AC}$ is plotted against ATP concentration. Data were fit with an equation of the form: $I/I_{max} = 1/[1+(B/K_d)^x]$, where B is NaATP concentration, $K_d$ is the equilibrium dissociation constant, and x is the Hill coefficient.

In addition to ATP, several other nucleotides, including ADP, UTP, and UDP, were found to inhibit $I_{AC}$ with $IC_{50}$ values of 0.24, 0.34, and 3.94 $\mu$M, respectively (Fig. 3,A and C). As observed for ATP, inhibition by these nucleotides was incomplete, even at maximally effective concentrations. At a concentration of 10 $\mu$M, ATP inhibited $I_{AC}$ by 71.3 ± 3.2% ($n$ = 26) whereas ADP, UTP, and UDP reduced $I_{AC}$ by 69.1 ± 7.2% ($n$ = 4), 81.4 ± 5.2% ($n$ = 7), and 65.1 ± 7.9% ($n$ = 4), respectively (Fig. 3B). Both 2-MeSATP and CTP at concentrations as high as 100 $\mu$M failed to significantly inhibit $I_{AC}$ (Fig. 3, B and C). The overall order of effectiveness for $I_{AC}$ inhibition was UTP ≥ ATP ≥ ADP ≥ UDP [tmt] 2-MeSATP = CTP. Nucleotide receptors were present on each AZF cell, because ATP, UTP, ADP, and UDP significantly inhibited $I_{AC}$ in each of more than 100 cells tested.

**Inhibition of $I_{AC}$ by ATP Involves a G Protein-Coupled Receptor.** When AZF cells were voltage clamped at the standardholding potential of −80 mV, superfusion of ATP never elicited an inward current, as would have occurred if an ATP-gated nonselective cation channel were activated. This finding, coupled with the observed order of effectiveness of nucleotides as inhibitors of $I_{AC}$ current, suggested that these activated a G protein-coupled nucleotide receptor of the P2Y family (Filtz et al., 1997; Williams and Burnstock, 1997). To determine whether ATP-mediated inhibition of $I_{AC}$ required activation of a G protein, GTP in the pipette solution was replaced with the inactive guanine nucleotide GDP-$\beta$-S. With 1 mM GDP-$\beta$-S in the patch pipette, ATP (10 $\mu$M) inhibited $I_{AC}$ by only 22.8 ± 4.5% ($n$ = 18), compared with 71.3 ± 3.2% ($n$ = 26) under control conditions (Fig. 4).

**$I_{AC}$ Inhibition by ATP Is Independent of Phospholipase C (PLC)-Generated Second Messengers.** Experiments with GDP-$\beta$-S indicated that ATP-inhibited $I_{AC}$ through a G protein-coupled receptor. In many cells, the binding of ATP to G protein-dependent nucleotide receptors is coupled to activation of PLC (Dubyak and El-Moatassim, 1993). PLC-catalyzed cleavage of phosphatidyl inositol 4,5-bisphosphate generates second messengers, including diacylglycerol and $IP_3$, which, respectively, activate protein kinase C and release intracellular $Ca^{++}$. We have explored the possibility that ATP-mediated inhibition of $I_{AC}$ occurs through a PLC-activated pathway.

Calphostin C is a potent, specific antagonist of protein kinase C ($IC_{50}$ ≈ 50 nM) (Tamaoki, 1991). When applied

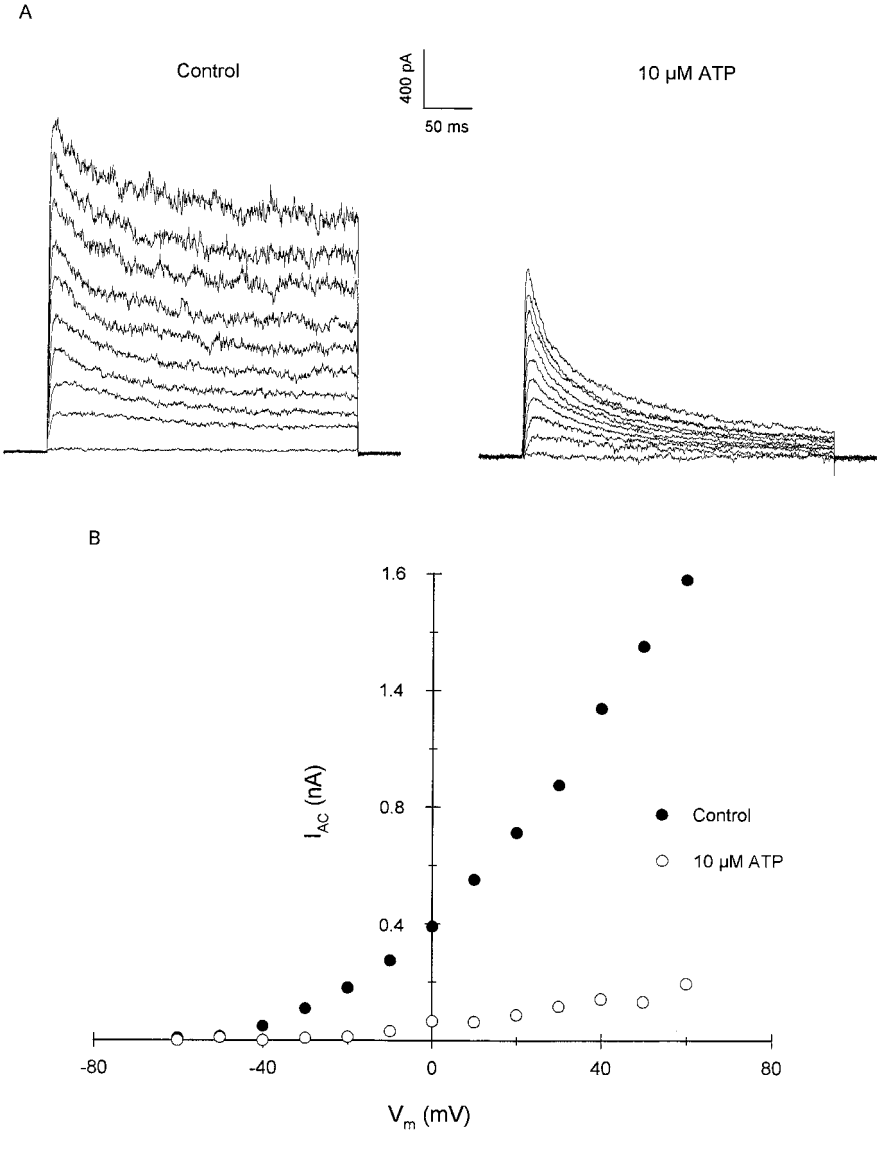


**Fig. 2.** Voltage-independent inhibition of $I_{AC}$ by ATP. $K^+$ currents were activated at 30-s intervals by voltage steps of varying size from a holding potential of −80 mV before and after superfusing the cell with 10 $\mu$M NaATP. A, current records at test potentials between −60 and +60 mV (in 10-mV increments) before and after superfusing ATP, as indicated. B, current-voltage relationships. $I_{AC}$ current amplitudes from experiment shown in A are plotted against test voltage.

directly to the cytoplasm of the AZF cell by addition to the pipette solution at a concentration of 500 nM, calphostin C failed to significantly alter inhibition of $I_{AC}$ by 10 $\mu$M ATP (Fig. 5,A and C). In the presence of this antagonist, external ATP inhibited $I_{AC}$ by 66.4 ± 6.8% ($n$ = 11), compared with the control value of 71.3 ± 3.2% ($n$ = 26) (Fig. 5C).

ATP-stimulated activation of PLC leads to $IP_3$-induced release of intracellular $Ca^{++}$. $Ca^{++}$ can modulate the function of ion channels through several different mechanisms. These include direct interaction with the channel, modulation by a $Ca^{++}$-calmodulin complex, or through a $Ca^{++}$-calmodulin-activated enzyme such as $Ca^{++}$-calmodulin kinase II (CaM Kinase II) (Levitan, 1994; Liu et al., 1994; Selyanko and Brown, 1996; Cui et al., 1997). To determine whether ATP-mediated inhibition of $I_{AC}$ occurred through a $Ca^{++}$ or calmodulin-dependent process, we measured the inhibition of $I_{AC}$ by ATP using a high-capacity $Ca^{++}$-buffering pipette solution in which $Ca^{++}$ was buffered with 20 rather than 11 mM BAPTA. This pipette solution was supplemented further with a calmodulin inhibitory peptide (residues 290–309 of CaM Kinase II) that potently inhibits CaM Kinase II and other calmodulin-dependent processes with an $IC_{50}$ of approximately 50 nM (Payne et al., 1988).

The high-BAPTA pipette solution supplemented with 2.5 $\mu$M calmodulin inhibitory peptide failed to suppress ATP-mediated inhibition of $I_{AC}$. As illustrated in Fig. 5B, the modified pipette solution had no effect on the $I_{AC}$ growth or its selective inhibition by 10 $\mu$M ATP. Overall, in five similar experiments, ATP (10 $\mu$M) inhibited $I_{AC}$ by 75.3 ± 8.0%, compared with the control value of 71.3 ± 3.2% ($n$ = 26) (Fig. 5C). These results indicate that ATP-mediated inhibition of $I_{AC}$ can occur independently of increases in intracellular $Ca^{++}$ or activation of calmodulin.

Overall, the results of studies with kinase C and the $Ca^{++}$/calmodulin antagonists indicate that $I_{AC}$ inhibition by ATP is independent of PLC-generated second messengers. U73122 is a PLC antagonist that inhibits agonist-induced activation of PLC, with an $IC_{50}$ of 1 to 2 $\mu$M (Smith et al., 1990). When U73122 (10 $\mu$M) was included in the pipette solution, it suppressed almost completely the time-dependent increase

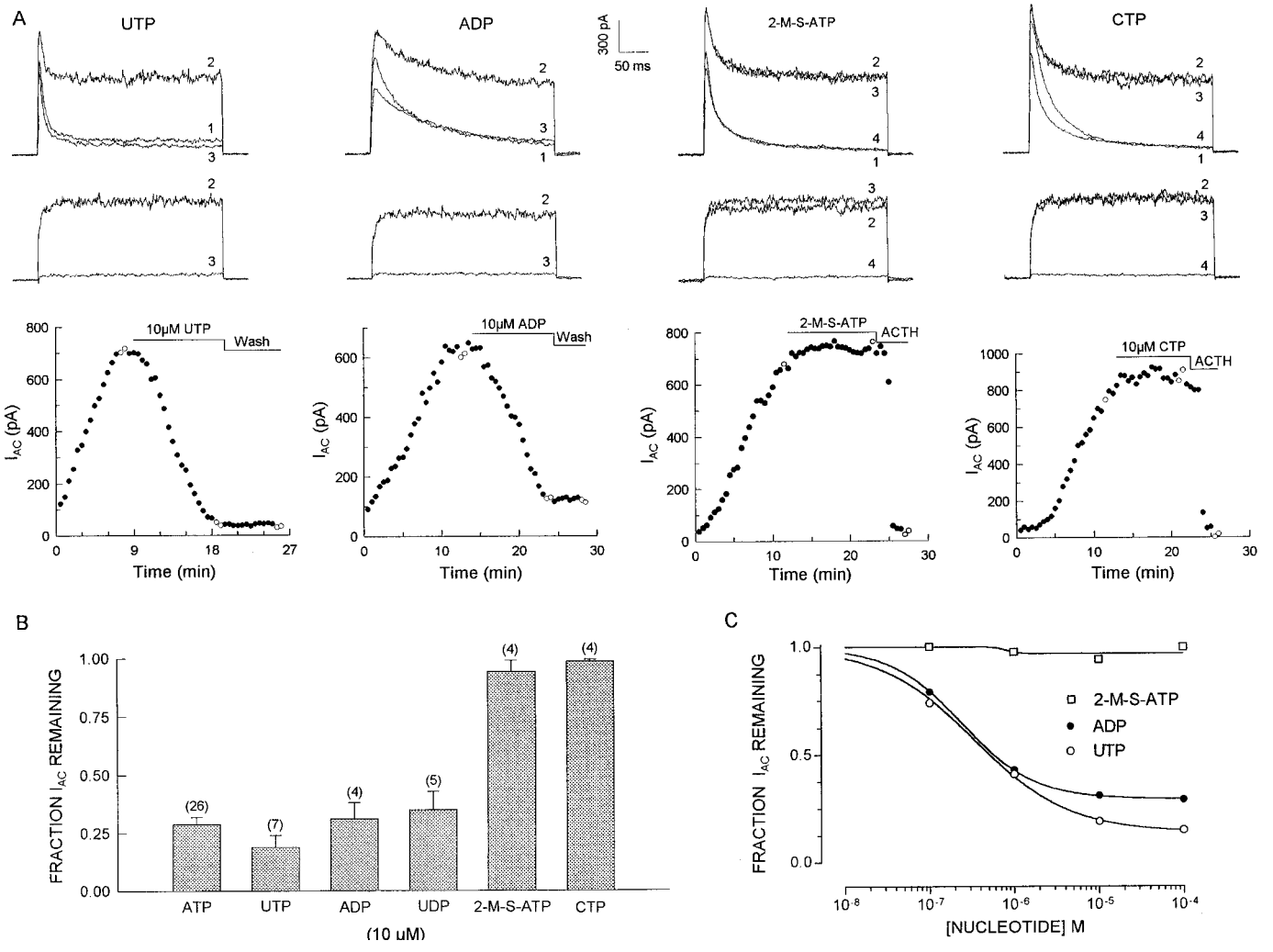


**Fig. 3.** Inhibition of $I_{AC}$ $K^+$ current by purines and pyrimidines. A, AZF cells were clamped in whole-cell configuration and voltage steps were applied at 30-s intervals from a holding potential of −80 mV with (middle) or without (top) 10-s prepulses to −20 mV. After $I_{AC}$ reached a maximum amplitude, cells were superfused with UTP, ADP, UDP, 2-MeSATP, or CTP each at a concentration of 10 $\mu$M or 200 pM ACTH, as indicated. Current traces and corresponding plots of $I_{AC}$ amplitude against time before and after superfusion of nucleotides for four representative cells. B, summary of results from experiments such as in A. Bars indicate fraction of $I_{AC}$ remaining after steady-state block by each nucleotide as indicated. Values are mean ± S.E.M. for the indicated number of cells. C, inhibition curves for 2-MeSATP, ADP, and UTP. Fraction of $I_{AC}$ remaining is plotted against nucleotide concentration. Data were fit with an equation of the same form as in Fig. 1. $N$ values range from 3 to 7 for each concentration.

in $I_{AC}$ that is typically observed with pipette solutions containing ATP at millimolar concentrations ($n = 26$). This result is inconsistent with a mechanism in which PLC activation leads to inhibition of $I_{AC}$ $K^+$ current.

**Effect of Staurosporine and AMP-PNP on $I_{AC}$ Inhibition by ATP.** Staurosporine is a potent nonselective protein kinase antagonist. This microbial alkaloid inhibits most serine/threonine protein kinases, including A kinase, C kinase, and $Ca^{++}$/CaM kinase II with $IC_{50}$ values of <20 nM (Tamaoki, 1991). As illustrated in Fig. 6A, staurosporine (1 $\mu$M) applied intracellularly through the pipette solution markedly reduced the inhibition of $I_{AC}$ $K^+$ current by 10 $\mu$M ATP. In this experiment, ATP inhibited $I_{AC}$ by less than 5%. In contrast ACTH (100 pM), which functions through a staurosporine-insensitive mechanism (Enyeart et al., 1996), inhibits $I_{AC}$ almost completely. In a total of nine similar experiments, ATP (10 $\mu$M) inhibited $I_{AC}$ by only 17.3 ± 5.5% when the pipette solution contained 1 $\mu$M staurosporine (Fig. 6C), compared with the control value of 71.3 ± 3.2% ($n = 26$).

Experiments with staurosporine indicated that ATP-mediated inhibition of $I_{AC}$ required the activity of an unidentified protein kinase. Accordingly, when the nonhydrolyzable ATP analog AMP-PNP (1 mM) replaced MgATP in the recording pipette, external NaATP was totally ineffective at inhibiting $I_{AC}$ (Fig. 6B). Overall, in a total of seven cells, ATP inhibited $I_{AC}$ by only 2.1 ± 1.6% when AMP-PNP replaced ATP in the pipette solution (Fig. 6C).

**Effect of Intracellular UTP on $I_{AC}$ Inhibition by External ATP.** In addition to intracellular ATP, other nucleotides including UTP can, when added to the pipette at millimolar concentrations, activate $I_{AC}$ $K^+$ channels (Enyeart et al., 1997). Although UTP can bind to an intracellular site to activate $I_{AC}$ $K^+$ channels, it does not replace ATP as a substrate for most kinases or ATPases (Glynn and Hoffman, 1971; Lemaire et al., 1974). When 5 mM UTP was substituted for ATP in the pipette solution, externally applied ATP (10 $\mu$M) failed to inhibit $I_{AC}$ channels. In five experiments in which the pipette solution contained only 5 mM UTP and no ATP, external ATP (10 $\mu$M) inhibited $I_{AC}$ by only 2.0 ± 1.7% (Fig. 7). As previously reported, ACTH (100 pM) also failed to inhibit $I_{AC}$ under these conditions (Enyeart et al., 1997).

When the pipette solution was supplemented with 50 $\mu$M

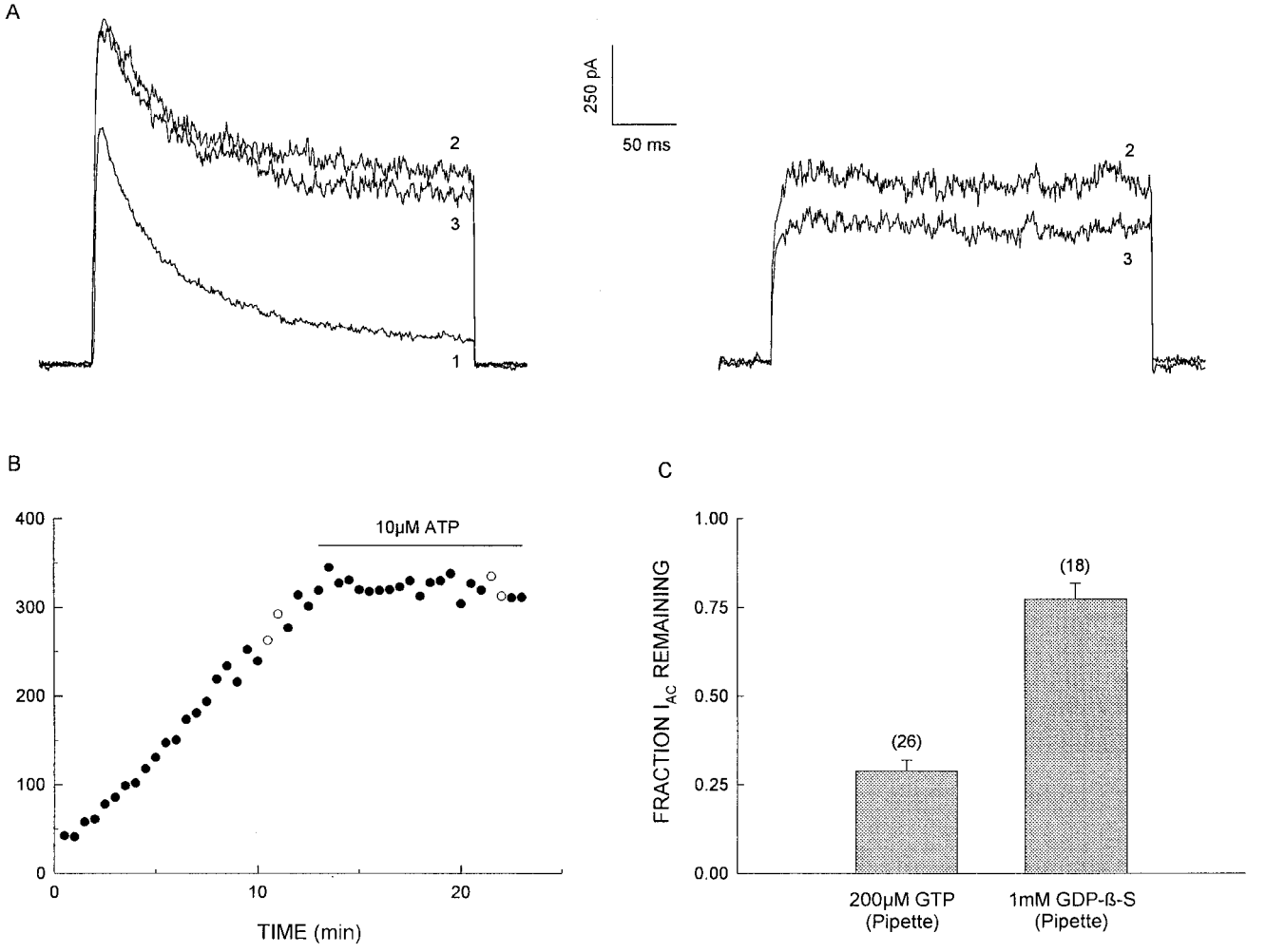


**Fig. 4.** Effect of guanine nucleotides on ATP-mediated $I_{AC}$ inhibition. $I_{AC}$ was measured at 30-s intervals as described in the legend of Fig. 1 with standard pipette solution (200 $\mu$M GTP) or the same solution containing 1 mM GDP-$\beta$-S in place of GTP. After $I_{AC}$ reached a stable value, cells were superfused with 10 $\mu$M NaATP. A, $K^+$ currents were recorded in the presence of GDP-$\beta$-S with (right) and without (left) 10-s prepulses to −20 mV immediately after initiating whole-cell recording (1), after $I_{AC}$ had reached a maximum value (2), and after steady-state inhibition by 10 $\mu$M ATP (3). B, $I_{AC}$ amplitudes recorded using pipette solution containing GDP-$\beta$-S either with (open circles) or without (solid circles) depolarizing pulses are plotted against time. C, summary of results from experiments as in A or B. Bars indicate fraction of $I_{AC}$ remaining after steady-state block by 10 $\mu$M ATP with 200 $\mu$M GTP or 1 mM GDP-$\beta$-S in pipette as indicated. Results are mean ± S.E.M. of indicated number of cells.

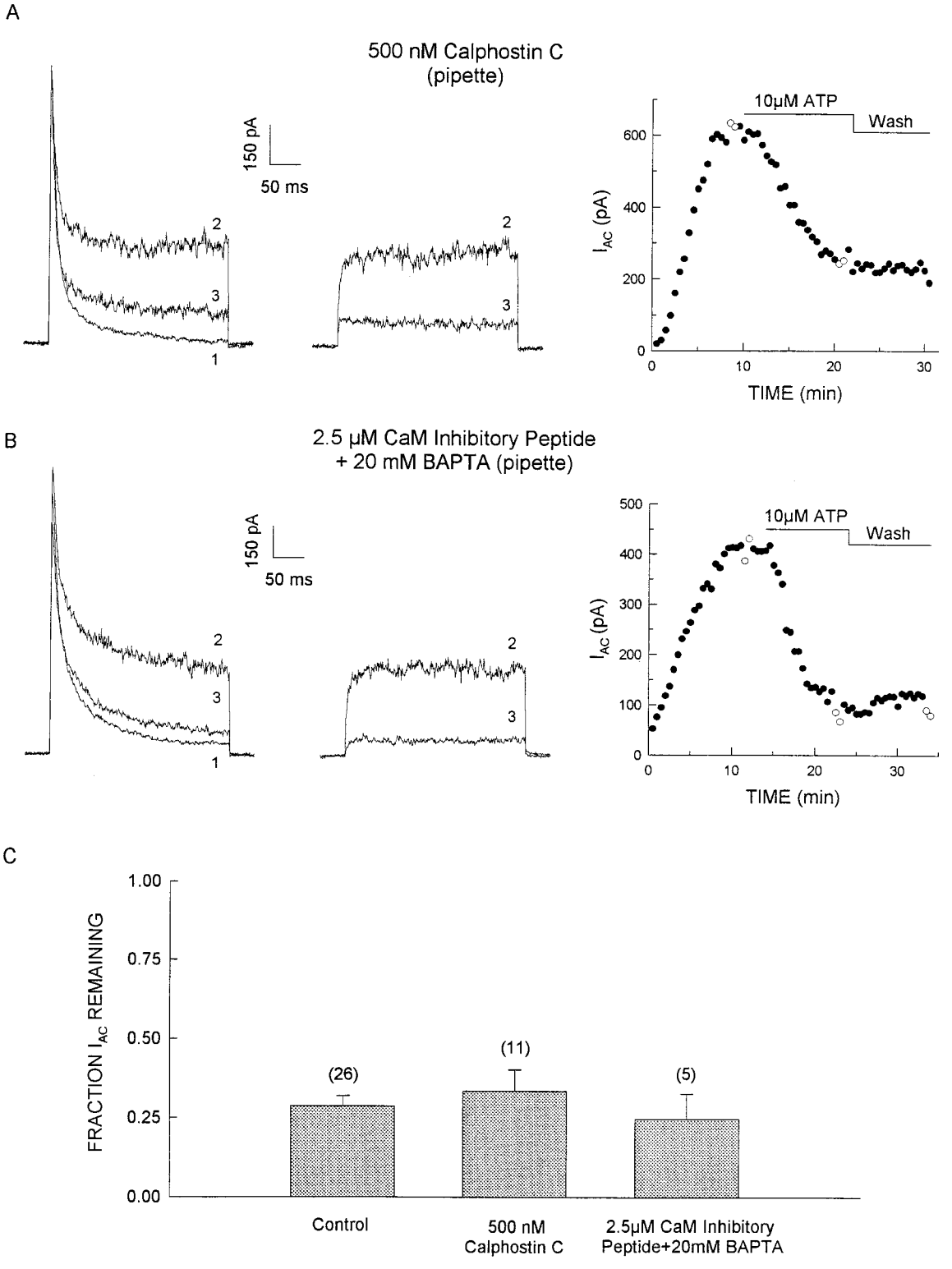


**Fig. 5.** $I_{AC}$ inhibition by ATP is independent of kinase C and $Ca^{++}$/calmodulin. $K^+$ currents were recorded from AZF cells using the two protocols described in the legend of Fig. 1. Pipettes contained standard internal solution each supplemented with 500 nM calphostin C (A) or 2.5 $\mu$M CaM Kinase inhibitory peptide and 20 mM BAPTA (B). After $I_{AC}$ reached a stable maximum value, cells were superfused with 10 $\mu$M NaATP. A and B, calphostin C and calmodulin inhibitory peptide. Current traces recorded with (middle) and without (left) 10-s prepulses to −20 mV immediately after initiating whole-cell recording (1), after $I_{AC}$ had reached a maximum value (2), and after block by 10 $\mu$M ATP. $I_{AC}$ amplitudes recorded either with (open circles) or without (solid circles) 10-s prepulses to −20 mV are plotted against time at right. C, summary of results from experiments as in A and B. Bars indicate fraction of $I_{AC}$ remaining after block by 10 $\mu$M ATP with pipette solutions containing calphostin or calmodulin inhibitory peptide and 20 mM BAPTA as indicated. Results are mean ± S.E.M. of indicated number of experiments.

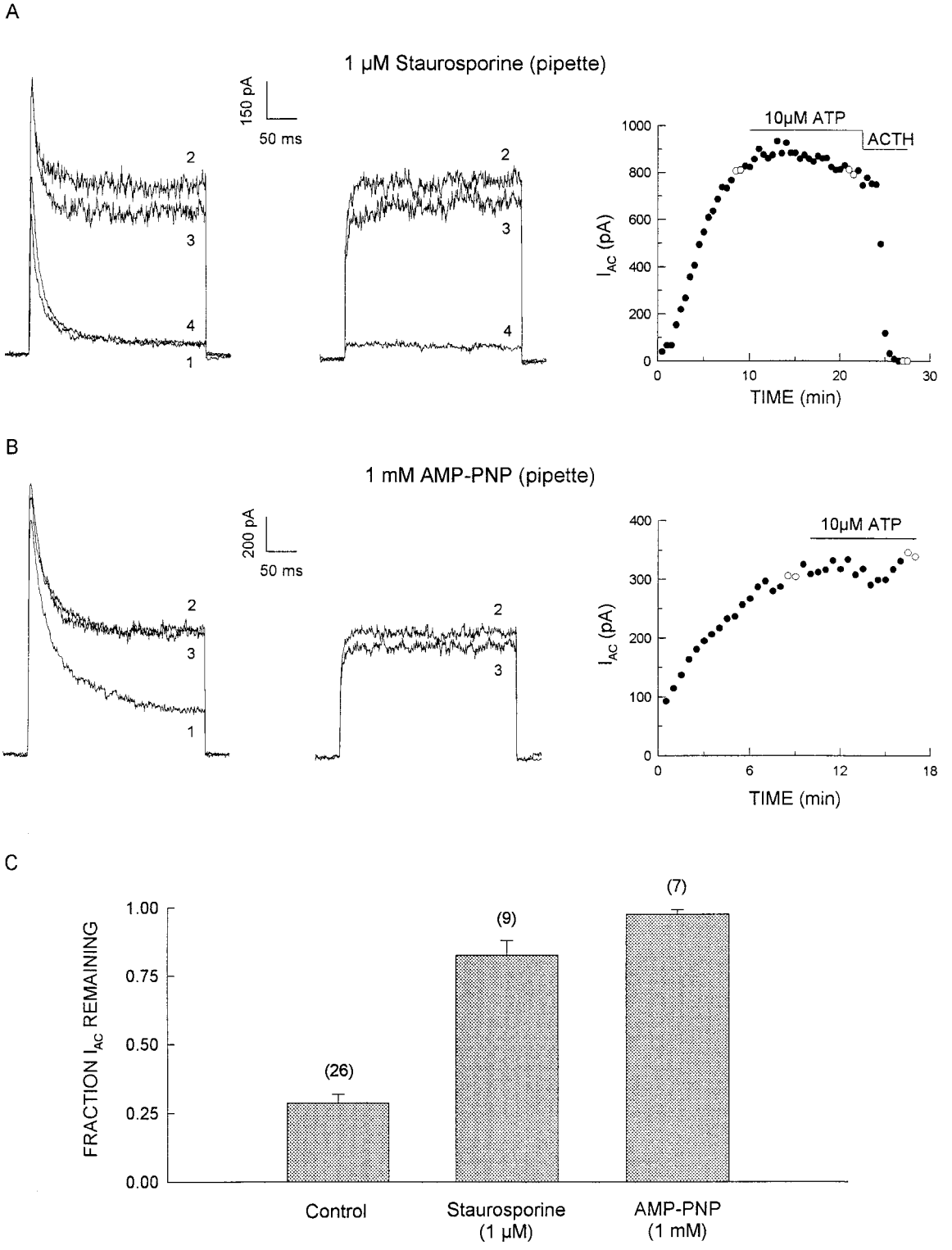


**Fig. 6.** Inhibition of $I_{AC}$ by ATP is suppressed by staurosporine and AMP-PNP. $K^+$ currents were recorded in AZF cells using the two protocols described in the legend of Fig. 1. Patch pipettes contained standard solution supplemented with 1 $\mu$M staurosporine (A) or the same solution in which AMP-PNP (1 mM) was substituted for MgATP (B). After $I_{AC}$ reached a stable amplitude, cells were superfused with 10 $\mu$M NaATP. A and B, current traces recorded with (middle) and without (left) 10-s prepulses to −20 mV immediately after initiating whole-cell recording (1), after $I_{AC}$ had reached a maximum value (2), after block by 10 $\mu$M ATP (3), and after block by 100 pM ACTH (4). $I_{AC}$ amplitudes recorded either with (open circles) or without (solid circles) 10-s prepulses to −20 mV are plotted against time at right. C, summary of results from experiments as in A and B. Bars indicate fraction of $I_{AC}$ remaining after block by 10 $\mu$M external ATP with standard pipette solution or the same solution supplemented with staurosporine or one in which AMP-PNP was substituted for MgATP.

or 200 $\mu$M MgATP, external ATP remained ineffective, inhibiting $I_{AC}$ by only 3.43 ± 2.22% ($n$ = 7) and 5.28 ± 3.61 ($n$ = 5), respectively. In contrast, the addition of only 50 $\mu$M MgATP to the pipette solution completely restored the near-complete inhibition of $I_{AC}$ by ACTH (100 pM) (Fig. 7). Raising pipette ATP to 2 or 5 mM in the continued presence of 5 mM UTP only partially restored $I_{AC}$ inhibition by external ATP. Specifically, with pipette solutions containing 5 mM ATP and UTP, external ATP (10 $\mu$M) inhibited $I_{AC}$ by 49.2 ± 2.7% ($n$ = 5) compared with 71.3 ± 3.2% ($n$ = 26) with pipettes containing only 5 mM ATP (Fig. 7B). These results indicate that ATP and ACTH inhibit $I_{AC}$ by different mechanisms and further suggest competition between UTP and ATP at an intracellular site involved in ATP-mediated inhibition of this current.

**Effect of Nucleotides on Membrane Potential.** In previous studies, we have shown that $I_{AC}$ $K^+$ channels display little voltage dependence and remain open at negative membrane potentials (Mlinar et al., 1993a; Enyeart et al., 1996), characteristics indicative of a channel that sets the resting potential. These results suggest that ATP and other nucleotides that inhibit $I_{AC}$, when applied externally, also should depolarize AZF cells.

In the experiment illustrated in Fig. 8, $I_{AC}$ was recorded at 30-s intervals until it reached a stable maximum (Fig. 8, trace 2, top and bottom). The membrane potential then was recorded after switching to current clamp (Fig. 8, bottom), and the cell was superfused with either ATP (10 $\mu$M) or UTP (10 $\mu$M), as indicated. After a delay of 1 to 2 min, both nucleotides produced a steady membrane depolarization from the resting potential of −65 to −70 mV. The depolarization reached a stable value at potentials between −15 and −5 mV. Upon switching back to voltage clamp, $I_{AC}$ had been inhibited by >90% in both cells. In cells where these nucleotides produced smaller inhibition of $I_{AC}$, membrane depolarization was proportionately less. Overall, ATP (10 $\mu$M) and UTP (10 $\mu$M) depolarized AZF cells by 44.0 ± 6.5 mV ($n$ = 6) and 47.3 ± 6.6 mV ($n$ = 6), respectively. In contrast, CTP (10 $\mu$M), which did not inhibit $I_{AC}$, depolarized AZF cells by only 1.33 ± 1.3 mV ($n$ = 3).

## Discussion

Bovine AZF cells express a nucleotide receptor that, when activated by ATP, ADP, UTP, and UDP, inhibits $I_{AC}$ $K^+$ current. The order of nucleotide potency and effectiveness in inhibiting $I_{AC}$ indicates that this receptor is distinct from purine-specific $P2Y_1$ and $P2Y_2$ receptors but similar to $P2Y_3$ receptors (Lustig et al., 1993; Parr et al., 1994; Webb et al., 1996; Filtz et al., 1997; Williams and Burnstock, 1997). Members of the P2Y family of receptors are coupled through a G protein-to-PLC activation. However, $I_{AC}$ inhibition by ATP appears to occur through a distinct mechanism involving a staurosporine-sensitive kinase and, possibly, an ATPase. The inhibition of $I_{AC}$ $K^+$ current by nucleotides was tightly correlated with their effectiveness in depolarizing AZF cells. ATP and UTP inhibit $I_{AC}$ current and depolarized AZF cells at nearly identical concentrations. 2-MeSATP and CTP were ineffective in both respects.

Overall, purine and pyrimidine nucleotides inhibit $I_{AC}$ and depolarize AZF cells at concentrations identical with those that stimulate cortisol secretion (Hoey et al., 1994). The common signaling mechanism that emerges from these studies suggests a model for cortisol secretion similar to that previously proposed for ACTH and AII, wherein $I_{AC}$ inhibition leads to membrane depolarization, $Ca^{++}$ entry through T-type channels, and cortisol secretion (Enyeart et al., 1993).

**Nucleotide Receptor Subtype.** Patch clamp experiments showed that the nucleotide receptor whose activation was linked to the inhibition of $I_{AC}$ $K^+$ current and membrane depolarization in AZF cells was a G protein-coupled receptor of the P2Y family. The results clearly demonstrate that ATP and other nucleotides do not function by activation of nonselective cation channels. In many cells, ATP-induced membrane depolarization is mediated through activation of these P2X receptors (Dalziel and Westfall, 1994; Williams and Burnstock, 1997). However, in the course of our studies, ATP and other adenine nucleotides were applied to more than 200 AZF cells at a holding potential of −80 mV. An inward current was never activated in any of these cells upon superfusing the nucleotides.

The rank order of potency and effectiveness of nucleotides as inhibitors of $I_{AC}$ demonstrate that a $P2Y_1$ receptor does not mediate this response. $P2Y_1$ receptors are potently activated by 2-MeSATP but are insensitive to UTP (Filtz et al., 1997; Williams and Burnstock, 1997), a profile quite different from that observed for $I_{AC}$ inhibition by nucleotides. By comparison, the potent inhibition of $I_{AC}$ by ATP and UTP combined with the ineffectiveness of $\alpha$-methyl-thio-ATP are characteristics typical of $P2Y_2$ receptors (Marrion et al., 1991; Parr et al., 1994; Filtz et al., 1997; Williams and Burnstock, 1997). However, $P2Y_2$ receptors are insensitive to ADP and UDP, although these nucleotides potently inhibited $I_{AC}$ (Lustig et al., 1993; Filtz et al., 1997). Thus, in spite of the similarities, the nucleotide sensitivity of this receptor, as measured by $I_{AC}$ block, is different from that of $P2Y_2$ receptors. In this regard, a novel G protein-coupled nucleotide receptor has been cloned from a chick brain cDNA library with an agonist profile similar to that observed for $I_{AC}$ inhibition (Webb et al., 1996). This $P2Y_3$ receptor is activated by ADP and UDP, in addition to ATP and UTP. The chick brain $P2Y_3$ receptor is coupled to unknown effectors.

The results of our study are consistent with the hypothesis that purine and pyrimidine nucleotides inhibit $I_{AC}$ through activation of a $P2Y_3$ receptor. However, they do not exclude the possibility that multiple receptors contributed to $I_{AC}$ inhibition in AZF cells.

**Modulation of Other $K^+$ Channels by Nucleotides.** Several reports have appeared demonstrating the modulation of specific $K^+$ channels by activation of G protein-coupled purinergic receptors. In cardiac myocytes, several different varieties of $K^+$ channels are modulated by purinergic receptor activation (Matsuura and Ehara, 1996; Matsuura et al., 1996). $Ca^{++}$-activated $K^+$ channels are activated by purinergic agonists in smooth muscle cells and in rat hepatocytes (Yamashita et al., 1996; Vogalis and Goyal, 1997). In none of these cells was the $K^+$ channel modulated by pyrimidine nucleotides. Thus, $I_{AC}$ $K^+$ channels are distinctive in their inhibition through $P2Y_3$ or closely related receptors.

**PLC-Independent Signaling Pathway.** Phospholipase C activation is the major signaling pathway that links various P2Y receptors to cellular responses (Dubyak and El-Moatassim, 1993; Filtz et al., 1997). Accordingly, extracellular ATP and UTP stimulate membrane phosphoinositol turnover and release of intracellular $Ca^{++}$ in AZF cells (Ni-

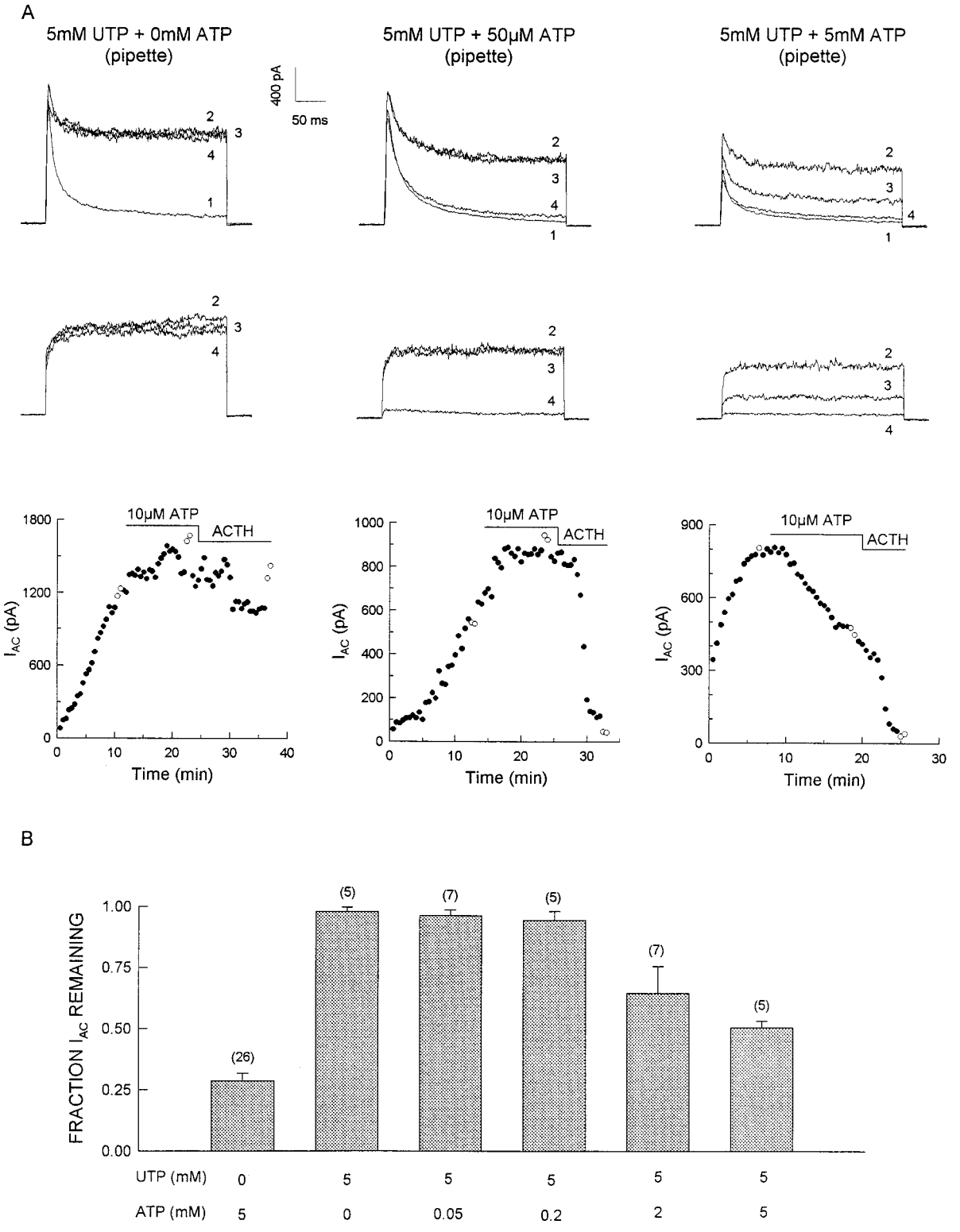


**Fig. 7.** Inhibition of $I_{AC}$ by external ATP requires intracellular ATP. $K^+$ currents were recorded with the two voltage protocols described in the legend of Fig. 1. Patch pipettes contained standard solution supplemented with 5 mM NaUTP, either alone or in combination with MgATP at concentrations of 50 $\mu$M, 200 $\mu$M, 2 mM, or 5 mM, as indicated. After $I_{AC}$ reached a maximum value, cells were superfused sequentially with ATP (10 $\mu$M) and ACTH (100 pM). A, traces show currents recorded with (bottom) and without (top) 10-s depolarizing prepulses to −20 mV immediately after initiating recording (1), after $I_{AC}$ had reached a maximum value (2), after superfusing 10 $\mu$M NaATP (3), and after superfusing 100 pM ACTH (4). Pipette ATP and UTP concentrations are as indicated. $I_{AC}$ amplitudes recorded either with (open circles) or without (solid circles) depolarizing prepulses are plotted beneath corresponding traces. B, summary of results of experiments as in A. Bars indicate fraction of $I_{AC}$ remaining after inhibition by 10 $\mu$M external NaATP with pipette solutions containing ATP and UTP at the indicated concentrations. Results are mean ± S.E.M. of the indicated number of determinations.

itsu, 1992; Hoey et al., 1994). However, ATP-mediated inhibition of $I_{AC}$ appears to occur through an alternative signaling pathway. PLC-mediated responses usually are mediated through PKC or $Ca^{++}$, acting either directly or through a calmodulin-dependent process (Berridge, 1993). The complete inability of the potent kinase C antagonist calphostin C to suppress ATP-mediated inhibition of $I_{AC}$ when applied directly to the cytoplasm through the patch electrode at 10 times the reported $IC_{50}$ is convincing evidence that activation of this enzyme is not necessary for this response. However, we cannot state with certainty that PKC was inhibited completely in these experiments.

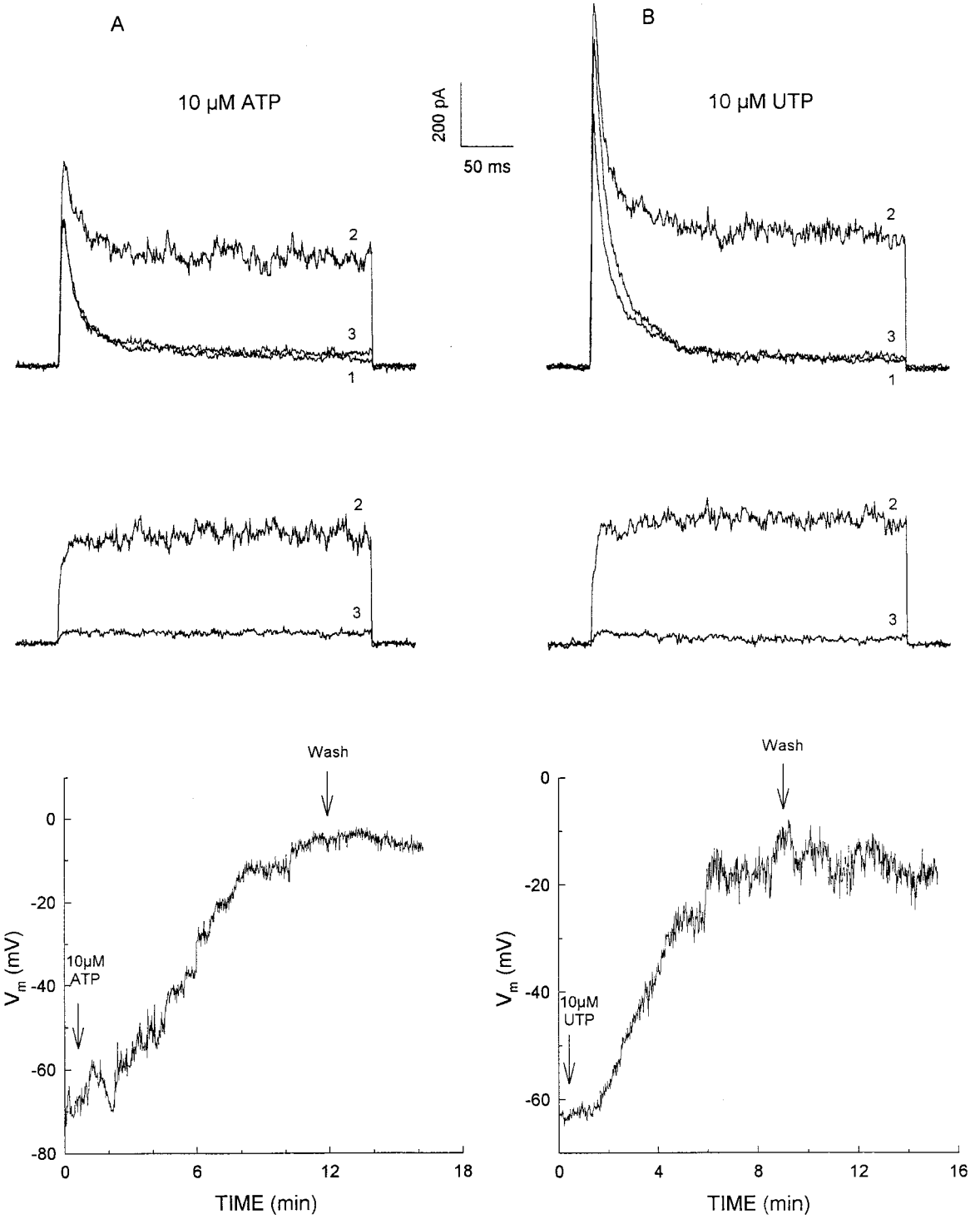


**Fig. 8.** Effect of ATP and UTP on $I_{AC}$ $K^+$ current and membrane potential. $K^+$ currents were recorded in the whole-cell mode using the two voltage clamp protocols described in the legend of Fig. 1. When $I_{AC}$ reached a stable maximum value, membrane potential was recorded upon switching to current clamp and cells were superfused with either 10 $\mu$M NaATP (A) or NaUTP (B). $K^+$ currents in A and B were recorded with (bottom traces) and without (top traces) 10-s depolarizing prepulses to −20 mV immediately after initiating whole-cell recording (1), after $I_{AC}$ had reached a maximum value (2), and after inhibition with ATP (left) or UTP (right) (3). Bottom panels show membrane potential recordings before, during, and after superfusing ATP or UTP as indicated.

In whole-cell patch clamp experiments, ion channel modulation mediated directly by $Ca^{++}$ or indirectly through calmodulin is usually suppressed or eliminated by strongly buffering $Ca^{++}$ in the pipette solution with 20 mM BAPTA or by adding a calmodulin antagonist to this solution (Marrion et al., 1991; Liu et al., 1994; Yu et al., 1994). Because both of these measures in combination failed to blunt ATP-mediated inhibition of $I_{AC}$, it is unlikely that $Ca^{++}$ released in response to PLC-mediated synthesis of $IP_3$ was involved in $I_{AC}$ inhibition.

Finally, if ATP-mediated inhibition of $I_{AC}$ occurred through activation of PLC, a PLC antagonist such as U73122 would be expected to enhance, rather than inhibit, $I_{AC}$ expression. However, it is possible that U73122 inhibited $I_{AC}$ by a mechanism independent of PLC. Overall, our results strongly indicate that ATP-mediated inhibition of $I_{AC}$ occurs through a PLC-independent pathway.

The specific pathway by which ATP and other nucleotides inhibit $I_{AC}$ remains to be identified. The ability of 1 $\mu$M staurosporine to prevent $I_{AC}$ inhibition by ATP indicates that a protein kinase is involved. However, at a concentration of 1 $\mu$M, staurosporine completely inhibits each of the serine/threonine kinases that have been described, as well as a number of tyrosine kinases (Tamaoki, 1991). The protein kinase whose activity is required for inhibition of $I_{AC}$ by external ATP remains to be identified.

**$I_{AC}$ Inhibition and ATP Hydrolysis.** Inhibition of $I_{AC}$ by external ATP also was prevented by substitution of the nonhydrolyzable ATP analog AMP-PNP or the pyrimidine UTP for ATP in the pipette. This result is not surprising, in view of the fact that neither of these nucleotides acts as a substrate for protein kinases (Azhar and Menon, 1975; Krebs and Beavo, 1979). However, the ineffectiveness of external ATP when the pipette contained 200 $\mu$M MgATP in addition to 5 mM UTP was unexpected. Protein kinases are fully activated by ATP at concentrations less than 50 $\mu$M (Glynn and Hoffman, 1971; Lemaire et al., 1974), whereas cellular ATPases have $K_m$ values for ATP of several millimolar (Hilgemann, 1997). Thus, inhibition of $I_{AC}$ by nucleotide receptor activation may require both active kinases as well as ATPases, as appears to be the case for the cystic fibrosis transmembrane conductance regulator $Cl^-$ channel, where channel gating is fueled by the energy of ATP hydrolysis but depends also on channel phosphorylation by a protein kinase (Baukrowitz et al., 1994). Alternatively, UTP might act as a competitive antagonist of ATP at its binding site on the protein kinase responsible for $I_{AC}$ inhibition. However, to our knowledge, UTP has not been shown to inhibit protein kinases.

**$I_{AC}$ Inhibition: Comparison of ATP, ACTH, and AII Signaling Pathways.** ATP-mediated inhibition of $I_{AC}$ occurs through a mechanism that is clearly different from that of ACTH. In contrast to ATP, inhibition of $I_{AC}$ by ACTH is unaffected by staurosporine. Furthermore, when $I_{AC}$ is activated by pipette solutions containing 5 mM UTP, 50 $\mu$M MgATP in the pipette is sufficient to restore complete inhibition of $I_{AC}$ by ACTH (100 pM). Under the same conditions, externally applied ATP (10 $\mu$M) produced no inhibition of $I_{AC}$.

Inhibition of $I_{AC}$ by externally applied ATP does resemble AII-mediated inhibition in several respects. AII inhibits $I_{AC}$ through a losartan-sensitive receptor that is known to be coupled to activation of PLC (Mlinar et al., 1995). However, AII-mediated inhibition of $I_{AC}$, similar to ATP-mediated inhibition, occurs through a PLC-independent pathway. Inhibition of $I_{AC}$ by AII is also suppressed by staurosporine (Mlinar et al., 1995). These results suggest that external ATP and AII are coupled to $I_{AC}$ inhibition by a novel, common pathway that is different from that used by ACTH.

**Nucleotide-Mediated Inhibition of $I_{AC}$ and Membrane Depolarization.** Bovine AZF cells express three types of ion channels, including rapidly inactivating $Ca^{++}$ and $K^+$ channels and noninactivating $K^+$ channels (Mlinar et al., 1993a,b; Mlinar and Enyeart, 1993). Of these, only $I_{AC}$ channels are open at negative membrane potentials and exhibit little voltage-dependent gating. It is likely that these channels are largely responsible for determining the resting potential of these cells (Mlinar et al., 1993a; Enyeart et al., 1996; Enyeart et al., 1997). Accordingly, ACTH, AII, ATP, and UTP all depolarize AZF cells at concentrations that maximally inhibit $I_{AC}$. Thus, nucleotides and peptide hormones that inhibit $I_{AC}$ through activation of three different receptors share the ability to depolarize AZF cells and stimulate large (>50-fold) increases in cortisol secretion (Enyeart et al., 1993; Mlinar et al., 1993a; Mlinar et al., 1995).

ACTH-stimulated cortisol secretion is inhibited by T-type $Ca^{++}$ channel antagonists at concentrations identical with those that inhibit T-type $Ca^{++}$ currents in these cells (Enyeart et al., 1993). These findings emphasize the importance of depolarization-dependent $Ca^{++}$ entry through voltage-gated $Ca^{++}$ channels in ACTH-stimulated cortisol secretion. Whether ATP-stimulated cortisol secretion also requires $Ca^{++}$ entry through T-type $Ca^{++}$ channels is not known, but a requirement for external $Ca^{++}$ in nucleotide-stimulated cortisol secretion has been established (Niitsu, 1992).

**Physiological Relevance.** The physiological significance of external ATP or other nucleotides in the regulation of cortisol secretion is not well established. ATP and other purines are stored and released, along with catecholamines, from secretory granules of adrenal medullary chromaffin cells (Glynn and Hoffman, 1971). In addition to nucleotide receptors, adrenal zona fasciculata cells also express $\beta$-adrenergic receptors that, when activated, stimulate cortisol secretion (Kawamura et al., 1984; Walker et al., 1988). Rays of adrenal medullary tissue have been reported to traverse the adrenal cortex, and clusters of chromaffin cells have been reported to exist in all three regions of the adrenal cortex (Nussdorfer, 1996). Overall, these results suggest that ATP and other nucleotides released from adrenal chromaffin cells, along with catecholamines, may act in a paracrine fashion to modulate $I_{AC}$ current and cortisol secretion in bovine AZF cells.

In this regard, it is important to point out that virtually all of the more than 200 normal AZF cells that we tested expressed the $P2Y_3$-type nucleotide receptor as measured by the inhibition of $I_{AC}$ current. These receptors were present in the freshly isolated AZF cells, because these cells were never cultured for more than several hours before use in patch clamp experiments.

A final possibility is that ATP released from AZF cells could act in an autocrine fashion to regulate $I_{AC}$ and cortisol secretion. A mechanism similar to this appears to function in airway epithelial cells, where the cystic fibrosis transmembrane conductance regulator triggers the transport of ATP

out of the cell, where it acts to activate chloride channels through activation of a P2U receptor (Schwiebert et al., 1995). It is interesting that the cystic fibrosis transmembrane conductance regulator-associated chloride channel, as well as the $I_{AC}$ $K^+$ channel, may both be activated by the nonhydrolytic binding of intracellular ATP (Quinton and Reddy, 1992), but inhibited by extracellular ATP through activation of a G protein-coupled nucleotide receptor.

In summary, we have discovered a new signaling pathway in which a nucleotide receptor with a $P2Y_3$ agonist is profile-coupled through a G protein to inhibition of $I_{AC}$ $K^+$ channels and depolarization of AZF cells. This suggests a specific mechanism for ATP-stimulated cortisol secretion that depends on depolarization-dependent $Ca^{++}$ entry. This may be an important physiological mechanism linking stress-induced chromaffin cell secretion to corticosteroid production.

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**Send reprint requests to:** Dr. John J. Enyeart, Department of Pharmacology, The Ohio State University College of Medicine, 5190 Graves Hall, 333 W. 10th Avenue, Columbus, OH 43210-1239. E-mail: enyeart.1@osu.edu