ATP Receptor Regulation of Adenylate Cyclase and Protein Kinase C Activity in Cultured Renal LLC-PK$_1$ Cells

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Abstract

In cultured intact LLC-PK$_1$ renal epithelial cells, a nonhydrolyzable ATP analogue, ATPγS, inhibits AVP-stimulated cAMP formation. In LLC-PK$_1$ membranes, several ATP analogues inhibit basal, GTP-, forskolin-, and AVP-stimulated adenylate cyclase activity in a dose-dependent manner. The rank order potency of inhibition by ATP analogues suggests that a P$_{2y}$ type of ATP receptor is involved in this inhibition. The compound ATPγS inhibits agonist-stimulated adenylate cyclase activity in solubilized and in isobutylmethylxanthine (IBMX) and quinacrine pretreated membranes, suggesting that ATPγS inhibition occurs independent of AVP and A$_1$ adenosine receptors and of phospholipase A$_2$ activity. The ATPγS inhibition of AVP-stimulated adenylate cyclase activity is not affected by pertussis toxin but is attenuated by GDPβS, suggesting a possible role for a pertussis toxin insensitive G protein in the inhibition. Exposure of intact LLC-PK cells to ATPγS results in a significant increase in protein kinase C activity. However, neither of two protein kinase C inhibitors (staurosporine and H-7) prevents ATPγS inhibition of AVP-stimulated adenylate cyclase activity, suggesting that this inhibition occurs by a protein kinase C independent mechanism. These findings suggest the presence of functional P$_{2y}$ purinoceptors coupled to two signal transduction pathways in cultured renal epithelial cells. The effect of P$_{2y}$ purinoceptors to inhibit AVP-stimulated adenylate cyclase activity may be mediated, at least in part, by a pertussis toxin insensitive G protein. (*J. Clin. Invest.* 1991. 87:1732–1738.) Key words: purinoceptors • vasopressin • adenosine triphosphate • cyclic AMP

Introduction

Extracellular purines such as adenosine and adenine nucleotides such as ATP interact with cell surface receptors to regulate several physiologic processes (1, 2). Two general classes of purinergic receptors exist (1–3). One class is activated by adenosine and blocked by methylxanthines. These receptors are coupled to adenylate cyclase via G proteins in an inhibitory (A$_1$ receptor) and a stimulatory (A$_2$ receptor) fashion. A second class of purinergic receptors is activated by ATP and is insensitive to methylxanthines. This class of receptor, referred to as P$_2$ purinergic receptors, can be further subdivided into P$_{2x}$ and P$_{2y}$ subtypes based on relative potencies of synthetic ATP analogues (3). There is limited information on the biochemical events involved in P$_2$ receptor action. In some cell types, P$_2$ receptors appear to be coupled via G proteins to activation of phosphoinositide- and phosphatidylcholine-specific phospholipase C activity (4–8).

Several studies have delineated an important role for A$_1$ and possibly A$_2$ adenosine receptors in regulation of renal blood flow, glomerular filtration rate, renin secretion, and renal tubular transport processes (9–15). There is, however, very limited information on the presence and functional significance of ATP responsive P$_2$ purinergic receptors on renal epithelial cells (16–21). These studies were undertaken to examine the effect of several ATP analogues on biochemical responses in cultured renal epithelial LLC-PK$_1$ cells. Our results suggest the presence of P$_{2y}$ purinergic receptors coupled to both inhibition of adenylate cyclase and stimulation of protein kinase C activity in these cells.

Methods

Materials. Arginine vasopressin, guanosine triphosphate (GTP), guanosine 5'-O-(2-thiodiphosphate, [GDPβS][1]), staurosporine, quinacrine dihydrochloride and 4β-phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co., St. Louis, MO. Adenosine 5'-O-3-thiotriphosphate, (ATPγS), β, γ-methyleneadenosine 5'-triphosphate, (β, γATP), and 2-methylthioadenosine 5'-triphosphosate, (2 MeATP) were purchased from Research Biochemicals, Natick, MA. Phosphatidylserine, diolein, and 1-oleoyl-2-acetyl-glycerol (OAG) were purchased from Avanti Polar Lipids, Inc., Birmingham, AL. Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and H-7 (1-15-isoquinolonylsulfonyl)-2-methylpiperazine) were purchased from Calbiochem-Behring Corp., San Diego, CA. Pertussis toxin was purchased from List and RO-201724 was obtained from Biomol Research Labs, Inc. [α-^{32}P]-ATP (30–40 Ci/mmol), [γ-^{32}P]ATP (25–35 Ci/mmol), [^{3}H]cAMP, and the cAMP radioimmunoassay kits were obtained from DuPont-New England Nuclear.

Cell culture and enzyme assays. LLC-PK$_1$ cells, crude membranes, and particulate and soluble cell fractions were prepared as previously described (22–24). Adenylate cyclase, cAMP phosphodiesterase, cAMP, protein kinase C, and total protein assays were performed exactly as described in recent publications from our laboratory (22–26).

Statistical analyses. For adenylate cyclase analyses, all assays were performed in triplicate and the mean of the three determinations considered an n of 1. For cAMP analyses, each tissue culture well was considered an n of 1. All calculations and analyses were carried out using an ATT PC-6300 desktop computer (Iverson, NJ) and ABSTAT software (Parker, CO). All data are expressed as the mean±SE. Statistical analyses were performed using paired or unpaired Student's t test and analysis of variance where appropriate. A P value < 0.05 is considered significant.

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1. *Abbreviations used in this paper:* GDPβS, guanosine thiodiphosphate staurosporine; IBMX, isobutyl methylxanthine; MeATP, methylthioadenosine triphosphate; OAG, oleoyl-acetyl-glycerol.

Results

Effect of ATPγS on AVP-stimulated cAMP formation in intact LLC-PK$_1$ cells. In initial studies, we measured the effect of ATPγS, a nonhydrolyzable ATP analogue with known P_{2y} receptor site activity, on hormone-stimulated cAMP formation in intact LLC-PK$_1$ cells. Cells were pretreated for 30 min with RO-201724 (10^{-3} M), a phosphodiesterase inhibitor that is not an adenosine receptor antagonist. As shown in Table I, ATPγS (10^{-4} M) significantly inhibits AVP-stimulated cAMP formation in intact LLC-PK$_1$ cells.

Effect of ATP analogues on basal and agonist-stimulated adenylate cyclase activity in LLC-PK$_1$ crude cell membranes. Our cAMP results suggest that ATPγS inhibits cAMP formation. To determine if ATPγS inhibits agonist-stimulated adenylate cyclase activity, the studies demonstrated in Fig. 1 were carried out in LLC-PK$_1$ crude membranes. At concentrations greater than 10^{-9} M, ATPγS significantly inhibits arginine vasopressin-stimulated adenylate cyclase activity. Between 10^{-4} and 10^{-8} M ATPγS, 25–40% inhibition occurs while 90% inhibition occurs at 10^{-3} M.

To better delineate the mechanism of ATP-induced inhibition of adenylate cyclase activity, we measured the effects of three ATP analogues (β, γATP, 2 MeATP, and ATPγS) on adenylate cyclase activity in crude LLC-PK$_1$ membranes in paired studies. These analogues were selected since previous studies suggest that the P_{2y} receptor subtype expresses a potency order of 2 MeATP ≫ ATP > β, γATP, whereas the P_{2x} receptor subtype expresses a rank order of potency of β, γATP > ATP > 2 MeATP (1–3). The results of synthetic ATP analogues on basal-, GTP-, forskolin-, and AVP-stimulated adenylate cyclase activity are in Figs. 2–5. All ATP analogues exert dose-dependent effects to significantly inhibit basal and agonist stimulated enzyme activity (Figs. 2 and 3). In general, 2 MeATP was the most potent inhibiting analogue. ATPγS at 10^{-4} inhibited the effects of maximal and submaximal concentrations of AVP and forskolin to stimulate adenylate cyclase activity (Figs. 4 *A* and 5 *A*).

Role of AVP and A$_1$ adenosine receptors and of phospholipase A$_2$ in ATPγS inhibition of adenylate cyclase activity in LLC-PK$_1$ cell membranes. To better delineate the mechanism of ATP inhibition of adenylate cyclase activity, LLC-PK$_1$ crude membranes were solubilized with 0.2% Lubrol PX (Table II).

Table I. Inhibition of AVP-stimulated cAMP Formation by ATPγS (10^{-4} M) in Intact LLC-PK$_1$ Cells

	Percentage increase from basal cAMP	
Concentration of AVP	(−) ATPγS	(+) ATPγS
−log M		
9	−6±1	−9±1
8	179±43	20±5*
7	314±23	39±10*

Cells were pretreated for 30 min with 10^{-3} M RO 201724 before exposure to ATPγS and AVP. The concentration of cAMP in RO 201724-pretreated cells before exposure to ATPγS and AVP ranged from 51.7 to 116.3 fmol/μg protein per min. The data represent the percentage change from six measurements performed under all conditions in two separate paired experiments. * $P < 0.05$ when compared with paired control.

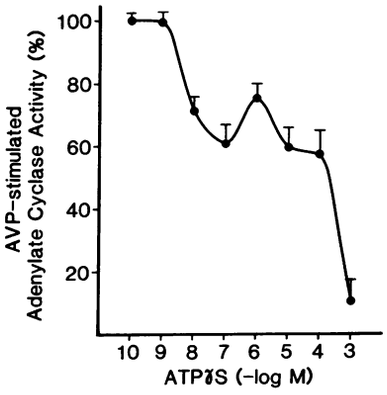

Figure 1. Dose-dependent effects of ATPγS on AVP- (10^{-7} M) stimulated adenylate cyclase activity in LLC-PK$_1$ membranes. The values represent the mean±SEM of four to six assays performed in triplicate.

Extensive Lubrol PX dose-ranging studies were done that found that lower concentrations did not inhibit AVP-stimulated adenylate cyclase activity and higher concentrations of Lubrol abolished GTP- and forskolin-stimulated adenylate cyclase activity. Solubilization of LLC-PK$_1$ membranes significantly reduces ($P < 0.05$) basal, GTP-, and forskolin-stimulated enzyme activity. In solubilized membranes, AVP no longer stimulates adenylate cyclase activity, while both GTP and forskolin significantly ($P < 0.05$) increase (4.5- and 5.8-fold, respectively) enzyme activity over basal values. ATPγS (10^{-4} M) significantly inhibits forskolin-stimulated adenylate cyclase activity by 56% in intact and 48% in solubilized membranes. These results suggest that ATPγS does not require an intact plasma membrane environment that contains a functional AVP receptor to inhibit adenylate cyclase activity.

ATPγS is a chemically stable ATP analogue that may be resistant to ecto-nucleotidase-mediated degradation to adenosine. However, to insure that ATPγS inhibition of adenylate cyclase is not mediated by an A$_1$ adenosine receptor, the effect of IBMX, an A$_1$ type adenosine receptor antagonist, was examined (Fig. 6, panel *B*). ATPγS inhibition of AVP-stimulated adenylate cyclase activity is the same in the presence and absence of IBMX suggesting an A$_1$ adenosine receptor-independent effect.

In some cell types, P_2 receptor agonists stimulate phospholipase A_2 activity (27). Activation of phospholipase A_2 could generate arachidonic acid metabolites which potentially inhibit

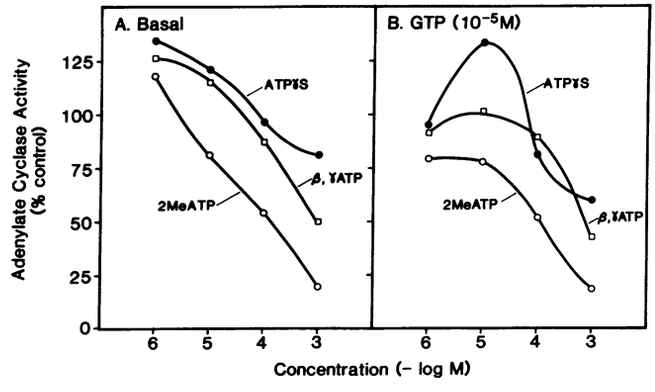

Figure 2. Dose-dependent effects of ATP analogues on basal (*A*) and GTP- (*B*) stimulated adenylate cyclase activity in LLC-PK$_1$ membranes. The values represent the mean of four separate assays performed in triplicate. The SEM values for individual data points ranged from 2 to 5%.

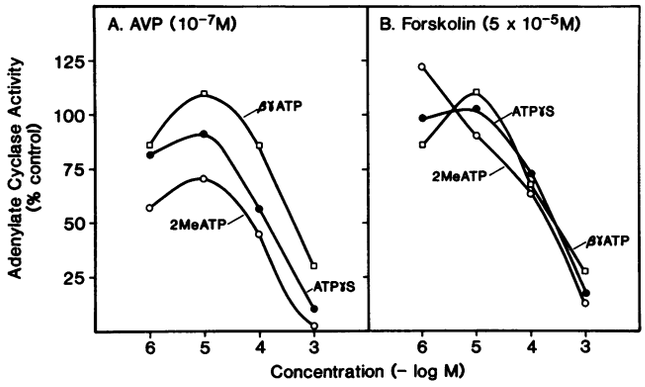

Figure 3. Dose-dependent effects of ATP analgogues on AVP- (*A*) and forskolin- (*B*) stimulated adenylate cyclase activity in LLC-PK_1 membranes. The values represent the mean of four separate assays done in triplicate. The SEM value for individual data points ranged from 1 to 6%.

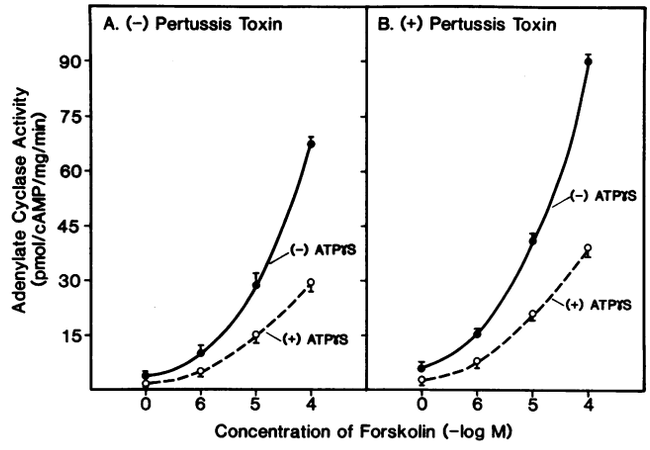

Figure 5. Effect of ATPγS (10^{-4} M) on forskolin-stimulated adenylate cyclase activity in LLC-PK_1 membranes in the absence (*A*) and presence (*B*) of pertussis toxin pretreatment (1 μg/ml for 18 h). The values represent the mean±SEM of four paired assays done in triplicate.

adenylate cyclase activity. To determine if ATPγS inhibition of adenylate cyclase activity is mediated via phospholipase A_2, the effects of 10^{-4} M quinacrine, a phospholipase A_2 inhibitor, were studied (Fig. 6 *A*). ATPγS inhibition of AVP-stimulated adenylate cyclase activity is the same in the presence and absence of quinacrine suggesting a phospholipase A_2 independent effect.

Role of G proteins in ATPγS inhibition of agonist-stimulated adenylate cyclase activity in LLC-PK_1 membranes. Previous studies suggest that P_{2y} receptors may be coupled to inhibition of adenylate cyclase activity via a GTP binding protein (5, 7, 28). We therefore examined the effect of ATPγS on AVP- and forskolin-stimulated adenylate cyclase activity in the presence and absence of pretreatment with 1.0 μg/ml pertussis toxin for 18 h (Figs. 4 and 5). Pertussis toxin ADP ribosylates the α subunit thereby inactivating a G protein linked to inhibition of adenylate cyclase activity. Pertussis toxin significantly potentiates AVP- and forskolin-stimulated adenylate cyclase activity. However, the effect of ATPγS to inhibit AVP- and forskolin-stimulated adenylate cyclase activity was the same in the presence and absence of pertussis toxin. Similar results were observed with 10.0 and 100.0 μg/ml of pertussis toxin.

Further studies were done to evaluate the possibility that the effect of ATPγS to inhibit adenylate cyclase activity is mediated by a pertussis toxin-insensitive G protein. In these studies, we used GDPβS, a stable analogue of GDP which prevents G protein activation by preventing dissociation of the α subunit. Pretreatment of LLC-PK_1 membranes with GDPβS consistently and significantly reduced (by ~ 50%) the effect of ATPγS to inhibit AVP- and forskolin-stimulated adenylate cyclase activity (Table III). Together, these results suggest that the inhibitory effect of ATPγS on AVP- and forskolin-stimulated adenylate cyclase activity may be mediated in part by a pertussis toxin-insensitive G protein.

Role of protein kinase C in ATPγS inhibition of agonist-stimulated adenylate cyclase activity in LLC-PK_1 cells. In many cells, P_{2y} purinergic receptors stimulate phospholipase C-catalyzed phosphoinositide hydrolysis and/or calcium mobilization (5–7, 28). To our knowledge, an effect of P_{2y} receptor

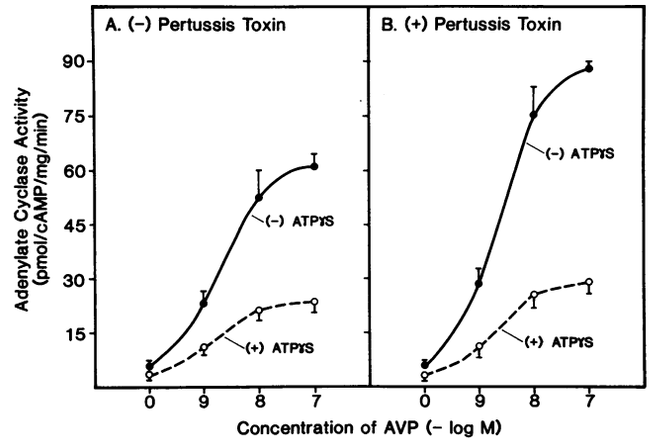

Figure 4. Effects of ATPγS (10^{-4} M) on AVP-stimulated adenylate cyclase activity in LLC-PK_1 membranes in the absence (*A*) and presence (*B*) of pertussis toxin pretreatment (1 μg/ml for 18 h). The values represent the mean±SEM of three to four paired assays done in triplicate.

Table II. Effect of Lubrol PX on Adenylate Cyclase Activity in LLC-PK_1 Membranes

Condition	Adenylate cyclase activity: Intact membrane	Adenylate cyclase activity: Solubilized fraction
	pmol/mg per min	
Basal	3.2±0.3	1.1±0.3
Arginine vasopressin (10^{-7} M)	43.8±2.9	1.7±0.5
GTP (10^{-5} M)	11.4±0.9	5.0±0.5
ATPγS (10^{-4} M)	1.9±0.1	1.8±0.1
Forskolin (5×10^{-5} M)	65.3±5.1	6.4±0.7
Forskolin (5×10^{-5} M) + ATPγS (10^{-4} M)	28.5±2.0	3.1±0.6

Values are mean±SE of four separate paired assays each done in triplicate.

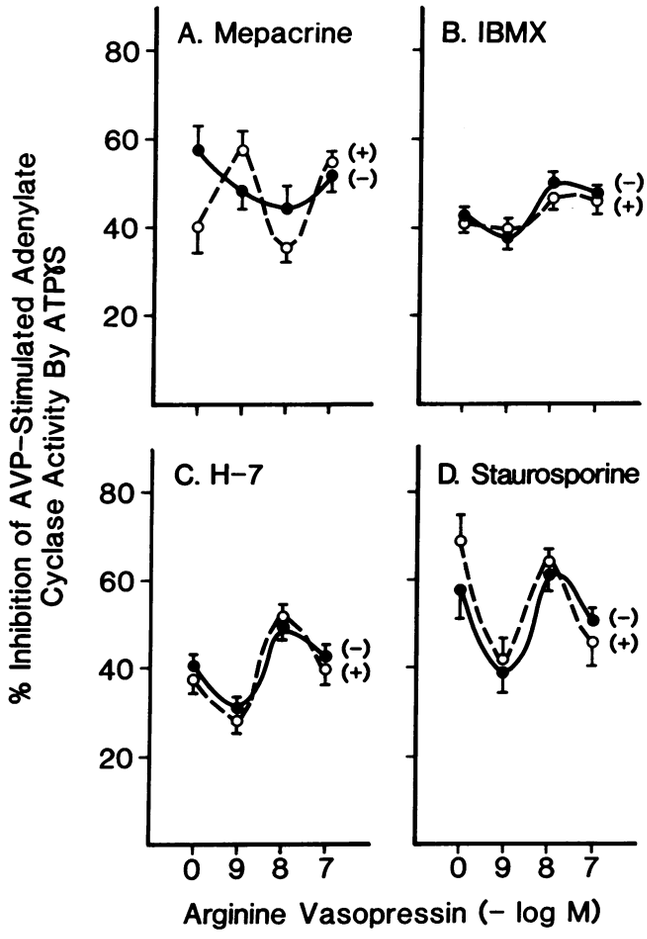

Figure 6. Effect of quinacrine (mepacrine, 10^{-4} M) (*A*), IBMX (10^{-3} M) (*B*), H-7 (10^{-6} M) (*C*), and staurosporine (10^{-8} M) (*D*) on ATPγS (10^{-4} M) inhibition of AVP-stimulated adenylate cyclase activity in LLC-PK_1 membranes. The broken and solid lines represent ATPγS inhibition of AVP-stimulated adenylate cyclase activity in the presence and absence, respectively, of the test agents. Individual data points represent the mean±SEM of percentage inhibition of AVP-stimulated adenylate cyclase activity by ATPγS in three to four paired experiments performed in triplicate.

agonists to activate protein kinase C has not been directly demonstrated. We first determined that a calcium- and phospholipid-dependent protein kinase, which can be activated by the phorbol ester PMA and by the diacylglycerol diolein, is present in LLC-PK_1 cells (Table IV). In these cells, PMA (10^{-7} M), a known activator of protein kinase C (22, 23), increased total protein kinase C from a control value of 1.039±0.2 to 1.590±0.19 nmol ^{32}P mg^{-1} min^{-1} ($P < 0.05$), and translocated protein kinase C from a soluble to a particulate cell fraction (78±7 to 32±5% soluble, $n = 7$, $P < 0.05$). To determine if ATPγS activates protein kinase C in these cells, the studies depicted in Fig. 7 were carried out in intact LLC-PK_1 cells. ATPγS exerted dose-dependent effects of protein kinase C activity with concentrations of 10^{-10} through 10^{-5} M significantly increasing activity over basal values. In these studies, basal protein kinase C activity averaged 1.056±0.13 nmol ^{32}P mg^{-1} min^{-1}. At 10^{-10} through 10^{-3} M, ATPγS decreased the soluble and increased the particulate form of total protein kinase C activity by 10±2%.

Table III. Effect of GDPβS on ATPγS-induced Inhibition of Adenylate Cyclase Activity in LLC-PK_1 Membranes

Treatment	Percentage inhibition of adenylate cyclase by 10^{-4} M ATPγS	
	GDPβS (−)	GDPβS (+)
Forskolin (*M*)		
0	30±1	0±2*
10^{-7}	61±3	22±2*
10^{-6}	53±3	29±10*
10^{-5}	56±3	29±9*
10^{-4}	52±4	27±7*
Arginine vasopressin (*M*)		
0	75±2	33±10*
10^{-9}	45±10	27±6*
10^{-8}	74±5	36±7*
10^{-7}	53±4	42±2*

Values are the percentage inhibition by 10^{-4} ATPγS of LLC-PK_1 crude membrane adenylate cyclase activity measured under basal and under forskolin- and arginine vasopressin-stimulated conditions. The values represent the mean±SE of percentage inhibition obtained from four to six separate paired assays, each done in triplicate. * $P < 0.05$ when compared with corresponding untreated values.

Previously, we have shown that activation and translocation of protein kinase C activity can induce a heterologous desensitization of adenylate cyclase activity in cultured renal epithelial cells (22, 23). To determine if ATPγS inhibition of adenylate cyclase activity is due to activation of protein kinase C, we examined the effect of two dissimilar protein kinase C inhibitors, H-7 (10^{-7} M) and staurosporine (10^{-8} M) on ATPγS (10^{-4} M) inhibition of AVP-stimulated adenylate cyclase (Fig. 6, *C* and *D*). Neither H-7 nor staurosporine altered the effect of 10^{-4} M ATPγS to inhibit AVP-stimulated adenylate cyclase activity suggesting a protein kinase C-independent effect. Since these studies were not performed with a concentration of ATPγS that maximally stimulated protein kinase C activity, additional experiments were performed. In five paired studies, ATPγS at 10^{-7} M inhibited the effect of 10^{-8} and 10^{-7} M AVP to stimulate adenylate cyclase activity by 22±2% in the absence and 20±2% in the presence of 10^{-8} M staurosporine. In another five paired studies, 10^{-7} M ATPγS inhibited the effect of 10^{-8} and 10^{-7} M AVP to stimulate adenylate cyclase activity

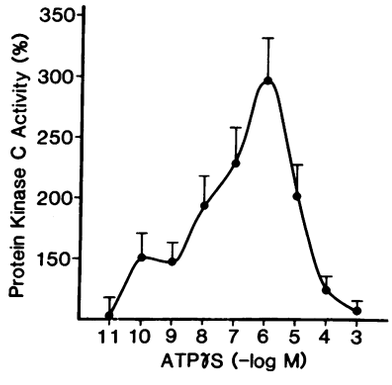

Figure 7. Dose-dependent effects of ATPγS exposure in intact LLC-PK_1 cells on total protein kinase C activity. Individual data points represent the mean±SEM of six experiments.

Table IV. Evidence for Protein Kinase C Activity in Intact LLC-PK$_1$ Cells

Assay addition	Protein kinase C activity	
	Cytosol	Particulate
	nmol $^{32}P \cdot mg^{-1} \cdot min^{-1}$	
None	0.120±0.07	0.177±0.07
$CaCl_2$	0.144±0.04	0.091±0.04
Phosphatidylserine + diolein	0.473±0.12*	0.299±0.08*
$CaCl_2$ + phosphatidylserine + diolein	0.704±0.17*	0.388±0.07*

Values are the mean±SE of five determinations. Assays were performed in the presence and absence of 1 mM $CaCl_2$, 60 μM phosphatidylserine and 8 μM diolein. * $P < 0.05$ when compared with preceding entry in Table.

by 26±3% in the absence and 22±2% in the presence of 10^{-7} M H-7.

Effect of ATPγS on LLC-PK$_1$ cellular cAMP phosphodiesterase activity. There is evidence that ATP can regulate cAMP hydrolysis (29, 30). We therefore examined the effect of ATPγS on cAMP phosphodiesterase activity in particulate and soluble fractions from LLC-PK$_1$ cells (Table V). ATPγS at 10^{-3} M reduced total and high affinity forms of cAMP phosphodiesterase activity by 35 and 26% respectively in a particulate fraction. In soluble fraction, ATPγS at 10^{-5} and 10^{-4} M decreased total cAMP phosphodiesterase activity by 32%.

Discussion

There is limited information on the presence and functional significance of ATP-responsive receptors on renal epithelial cells. In these studies, we find that the nonhydrolyzable ATP analogue, ATPγS, exerts a significant effect to inhibit AVP-stimulated cAMP formation in intact LLC-PK$_1$ cells. Since these cells were pretreated with a cAMP phosphodiesterase inhibitor, the results suggest that ATPγS inhibits AVP-stimulated adenylate cyclase activity. We confirmed this by directly measuring adenylate cyclase activity in crude LLC-PK$_1$ membranes. In these studies, ATP and several analogues exert dose-dependent effects to inhibit basal, GTP-, AVP-, and forskolin-stimulated enzyme activity. The ATP analogue 2 MeATP exerted the greatest inhibitory effect. Together, these results suggest the presence of functional P_{2y} receptors which act to inhibit AVP-stimulated adenylate cyclase activity in LLC-PK$_1$ membranes. An effect of P_{2y} receptor agonists to inhibit cAMP accumulation in hepatocytes and FRTL thyroid cells has recently been reported (5, 7). Our results supporting a P_{2y} type of ATP receptor in mediating ATP effects are solely dependent upon the rank order potency of several ATP analogues studied in other tissues (1, 3, 5, 7). Thus, further studies will be necessary to document the exact nature of the ATP receptor present in renal epithelial cells (31).

Table V. Effect of ATPγS on cAMP Phosphodiesterase Activity from LLC-PK$_1$ Cells

	cAMP phosphodiesterase activity (pmol/mg per 20 min)			
	Membrane		Soluble	
	Total	High affinity	Total	High affinity
Basal	9.3±3.6	0.33±0.08	33.5±3.6	1.04±0.09
IBMX, 10^{-3} M	0.69±0.04*	0.03±0.007*	1.29±0.6*	0.11±0.05*
ATPγS (M)				
10^{-5}	8.7±2.3	0.32±0.006	25.1±6.2*	0.99±0.09
10^{-4}	8.0±1.9	0.31±0.007	22.7±4.0*	0.88±0.15
10^{-3}	6.0±2.1*	0.25±0.05*	29.4±4.1	0.90±0.13

The data represent the mean±SE of five separate experiments.
* $P < 0.05$ when compared with basal value.

It is possible that some of the effect of ATPγS and other ATP analogues to inhibit agonist-stimulated adenylate cyclase activity in LLC-PK$_1$ membranes could be related to an action of the ATP analogues to act as a pseudosubstrate for adenylate cyclase. Indeed in plasma membranes, we observed a biphasic effect of ATPγS to inhibit AVP-stimulated adenylate cyclase activity with modest (25–40%) inhibition occurring at 10^{-8} through 10^{-4} M and a striking further inhibition occurring at 10^{-3} M. Although some of ATPγS inhibition of AVP-stimulated adenylate cyclase activity may be due to pseudosubstrate effect, particularly at high concentrations of ATPγS, there are several factors that suggest the presence of functional external ATP receptors on LLC-PK$_1$ cells. Thus, at 10^{-4} M, ATPγS significantly inhibits AVP-stimulated cAMP accumulation in intact LLC-PK$_1$ cells. Also, GDPβS significantly prevents ATPγS inhibition of agonist-stimulated adenylate cyclase activity in LLC-PK$_1$ membranes, an effect not readily explained if ATPγS were acting solely as a pseudosubstrate. Finally, exposure of intact LLC-PK$_1$ cells to concentrations of ATPγS from 10^{-10} through 10^{-6} M significantly increases protein kinase C activity in these cells. Together, these observations support the presence of functional ATP-responsive receptors on LLC-PK$_1$ cells.

We undertook several studies to clarify the mechanism whereby ATP-responsive receptors inhibit agonist-stimulated adenylate cyclase activity. Solubilization of LLC-PK$_1$ membranes resulted in loss of adenylate cyclase response to AVP. However, in these solubilized membranes, both GTP and forskolin significantly stimulated adenylate cyclase activity and ATPγS inhibited forskolin-stimulated enzyme activity. These findings demonstrate that ATPγS can act independent of an AVP receptor to inhibit adenylate cyclase activity. Pretreatment of LLC-PK$_1$ membranes with either IBMX or quinacrine did not prevent ATPγS inhibition of adenylate cyclase activity, suggesting that neither A_1 adenosine receptors nor phospholipase A_2 are involved in this process.

The effect of P_{2y} receptor agonists to inhibit adenylate cyclase activity in hepatocytes and in FRTL-5 thyroid cells has been reported to be pertussis toxin sensitive (5, 7). We did not find that exposure to very high concentrations of pertussis toxin attenuated the effect of ATPγS to inhibit either AVP- or forskolin-stimulated adenylate cyclase activity. Previously, in physiologic studies, we have found that pertussis toxin can reverse the effect of α_2 adrenergic agonists to inhibit AVP action in mammalian collecting tubular epithelia (32). The reasons for the discrepancy between our results in LLC-PK$_1$ cells and the results of others in hepatocytes and dedifferentiated thyroid cells are not clear. In other studies in LLC-PK$_1$ cells, Weinberg and collaborators did not find that pertussis toxin pretreatment attenuates the effect of exogenous ATP to increase free cyto-

solic calcium activity (19). Also, recent preliminary studies by Lederer et al., in AVP-responsive mammalian collecting tubular cells, found that pertussis toxin does not reverse the inhibitory effect of ATP on AVP-stimulated hydroosmotic response (21). We performed additional studies to explore the possibility that a pertussis toxin-insensitive G protein mediates the effect of ATPγS to inhibit AVP-stimulated adenylate cyclase activity. In these studies, we used GDPβS, a non-hydrolyzable GTP analogue that prevents G protein activation. In paired studies, GDPβS consistently attenuated the effect of ATPγS to inhibit AVP- and forskolin-stimulated adenylate cyclase activity. Our results suggest the possibility that ATPγS inhibition of adenylate cyclase activity involves a pertussis toxin-insensitive G protein. However, to date, a pertussis toxin-insensitive G protein linked to inhibition of adenylate cyclase activity has not been demonstrated. Thus, further studies are clearly needed to substantiate and clarify the role of G proteins in ATP inhibition of adenylate cyclase activity in LLC-PK_1 cells.

In virtually all cell types studied to date, including AVP-responsive LLC-PK_1 and mammalian collecting tubular cells, ATP and its analogues activate a phosphoinositide-specific phospholipase C with subsequent formation of IP_3 and mobilization of intracellular calcium stores (5–7, 19, 21, 27, 28). An effect of ATP to activate protein kinase C has not been directly demonstrated. We found that LLC-PK_1 cells contain a calcium and diacylglycerol activated protein kinase. The specific activity of this enzyme was significantly greater and the basal cellular location of the enzyme different than we observed previously in primary cultures of collecting tubular cells (22, 23). In these studies, exposure of intact LLC-PK_1 cells to the phorbol ester PMA and to ATPγS consistently and significantly increases total protein kinase C activity. Activation of protein kinase C has been reported to both potentiate and inhibit agonist-stimulated adenylate cyclase activity in AVP-responsive renal epithelial cells (22, 23, 33). To determine if activation of protein kinase C is responsible for the observed ATPγS inhibition of adenylate cyclase activity, cells were pretreated with high concentrations of two structurally dissimilar inhibitors of protein kinase C. Neither of these agents attenuate the effect of ATPγS to inhibit AVP-stimulated adenylate cyclase activity, suggesting a protein kinase C-independent effect.

In view of suggestions that the process of cAMP hydrolysis may be regulated by ATP, we measured cAMP phosphodiesterase activity in LLC-PK_1 cell fractions. ATPγS at high concentrations exerts a modest inhibitory effect on cAMP phosphodiesterase activity comparable to that seen previously in homogenized brain and rat kidney (29, 30). These observations suggest the potential for ATP to act at more than one site to regulate cAMP metabolism.

The results of our studies suggest that ATP responsive receptors are linked to two signal transduction systems in LLC-PK_1 cells. Other purinergic receptors, particularly the A_1 adenosine receptor, appear coupled to two signal transduction systems in renal epithelial cells (9, 13, 19, 34, 35). In this regard, A_1 receptor agonists inhibit cyclic AMP formation, increase cytosolic calcium activity, and stimulate phosphoinositide turnover (9, 13, 19, 34, 35). In other renal epithelial cells, other hormones such as parathyroid hormone and AVP may also be linked to both the protein kinase A and protein kinase C signal transduction pathways (36, 37). Our results do not establish if ATP receptors are coupled to the two signal transduction pathways by two separate receptors or by a single receptor with coupling through different G proteins. Although further studies will be required to clarify this issue, the work of Okajima et al. in rat hepatocytes suggests that distinct ATP receptors are linked to each signal transduction system (5).

In summary, our studies suggest the possibility that functional P_{2y} purinoceptors are present in LLC-PK_1 cells and are coupled to inhibition of adenylate cyclase and stimulation of protein kinase C activity. ATP receptor inhibition of adenylate cyclase activity appears independent of AVP and A_1 adenosine receptors and phospholipase A_2 and protein kinase C activity. The effects of ATP receptors to inhibit adenylate cyclase activity may be transduced by a pertussis toxin-insensitive G protein. The presence of functional ATP receptors in renal epithelial cells suggests the potential for these receptors to modulate cellular transport processes. Studies in various renal epithelia suggest that ATP receptors may regulate sodium, chloride, and potassium transport (17, 18, 20). Finally, there are multiple potential sites of origin for extracellular ATP including release during neurotransmission and cellular exocytosis (1).

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