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P_2 purinoceptor-mediated stimulation of adenylyl cyclase in PC12 cells

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Abstract

PC12 pheochromocytoma cells have P_2 purinoceptors which are activated by ATP and coupled to Ca^{2+} influx and catecholamine release. Also PC12 cells have adenosine receptors coupled positively to adenylyl cyclase, and cyclic AMP regulates cell functions such as catecholamine release. The effects of ATP and ATP analogs on cyclic AMP accumulation in PC12 cells were investigated in this study. ATP and adenosine 5′-*O*-(3-thiotriphosphate) stimulated cyclic AMP accumulation at low concentrations up to 300 μM but showed inhibitory effects above this concentration. 2′,3′-*O*-(4-Benzoyl)benzoyl ATP and 2-methylthio ATP showed similar effects, although the responses were very limited. Addition of adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) or β,γ-methylene ATP, but not α,β-methylene ATP, stimulated cyclic AMP accumulation markedly without causing an inhibitory phase. The effects of ATP, ADPβS and β,γ-methylene ATP were not inhibited by adenosine deaminase or specific antagonists to A_1 and A_2 adenosine receptors. Neither ADPβS nor β,γ-methylene ATP showed any effect on Ca^{2+} influx or noradrenaline release. Suramin, a P_2 receptors antagonist, had no inhibitory effect against ATP analog-stimulated cyclic AMP accumulation, although reactive blue 2 inhibited the β,γ-methylene ATP-stimulated reaction but not that up-regulated by ADPβS. These findings suggest that the pharmacological characteristics of these ATP receptors coupled to adenylyl cyclase are clearly different from those of ligand-gated ion channels defined by P_{2X} purinoceptors, which have been cloned and shown to be coupled to Ca^{2+} influx and catecholamine release in PC12 cells. The existence of a new type of P_2 purinoceptor-mediating stimulation of adenylyl cyclase is proposed in PC12 cells.

Keywords: PC12 cell; ATP receptor; cAMP; Reactive blue 2

1. Introduction

Extracellular ATP (and/or ADP) has hormone- and neurotransmitter-like effects in a variety of mammalian tissues. For example, ATP modulates platelet aggregation, immune responses, neurotransmission, plasma membrane permeability and cell growth (for reviews see Gordon, 1986; El-Moatassim et al., 1992; Dalziel and Westfall, 1994). It has been postulated that cellular responses to extracellular ATP are mediated by cell-surface receptors (P_2 purinoceptors). Based primarily on the pharmacological potencies of nucleotides, four different classes of P_2 purinoceptors (P_{2X}, P_{2Y}, P_{2T} and P_{2Z}) have been proposed (El-Moatassim et al., 1992). In addition, P_{2U} receptors for UTP and other classes have also been proposed (Dalziel and Westfall, 1994). Of the suggested subclasses of P_2 purinoceptors, the P_{2Y}, P_{2T}, and P_{2U} receptors are thought to be coupled to GTP-binding proteins (Dalziel and Westfall, 1994), and complementary DNAs encoding P_{2Y} (Webb et al., 1993) and P_{2U} (Lusting et al., 1993) receptors have been cloned. Recently, a new class of ligand-gated ion channel defined as an ionotropic P_{2X} receptor was cloned from the smooth muscle of the vas deferens (Valera et al., 1994) and from PC12 pheochromocytoma cells (Brake et al., 1994). However, these receptors in the smooth muscle are sensitive to α,β-methylene ATP, whereas those in PC12 cells are insensitive. The existence of additional new types or different subtypes of ATP receptors has also been proposed (El-Moatassim et al., 1992; Dalziel and Westfall, 1994).

Addition of ATP increases the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and catecholamine secretion in PC12 cells (Inoue et al., 1989; Sela et al., 1991; Majid et al., 1992; Rhoads et al., 1993; Oda et al., 1995). It seems that the responses evoked by ATP in PC12 cells are mediated by P_{2X} receptors, because the pharmacological characteristics of cloned ionotropic P_{2X} receptors from PC12 cells, when expressed in *Xenopus oocytes*, resemble those of ATP receptors coupled to the responses in PC12

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cells (Brake et al., 1994). Elevation of intracellular cyclic AMP levels by incubation with adenosine or forskolin increases noradrenaline secretion in response to KCl from PC12 cells by a pathway distinct from that governing $[Ca^{2+}]_i$ level (Martin and Koshland, 1992). In addition, adenosine stimulates noradrenaline release, and enhances ATP- and phorbol myristate acetate-stimulated noradrenaline release (Oda et al., 1995). These findings suggest that cyclic AMP-dependent mechanisms positively regulate noradrenaline release independently of Ca^{2+} and protein kinase C in PC12 cells. Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is also subject to regulation by cyclic AMP (Tischler et al., 1985; Roskoski et al., 1989; Wessels-Reiker et al., 1991). There have been no reports of the effects of ATP on cyclic AMP content in PC12 cells, although it has been reported that the addition of ATP inhibits cyclic AMP accumulation in rat hepatocytes (Okajima et al., 1987) and in clonal line of functional rat thyroid (FRTL-5) cells (Okajima et al., 1989). Thus, in this study, we examined the effects of ATP on cyclic AMP content in PC12 cells. The existence of a new type of ATP receptor positively coupled to adenylyl cyclase is proposed.

2. Materials and methods

2.1. Materials

Adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) and adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) were purchased from Boehringer Mannheim (Germany). 2-Methylthio ATP, 1,3-diethyl-8-phenylxanthine, CGS-15943 and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were obtained from Research Biochemicals (Natick, MA, USA). ATP, α,β-methylene ATP, β,γ-methylene ATP, 2′,3′-*O*-(4-benzoyl)benzoyl ATP (BzATP), 8-bromo ATP, adenosine deaminase, trifluoperazine and reactive blue 2 were purchased from Sigma (St. Louis, MO, USA). L-[7,8-^{3}H]noradrenaline (30–40 Ci/mmol) and fura-2 acetoxymethyl ester were purchased from Amersham (Amersham, UK) and Wako Pure Chemical (Osaka, Japan), respectively. Suramin and cyclic AMP assay kits were gifts from Bayer Japan (Tokyo, Japan) and Yamasa-Shoyu Corp. (Chiba, Japan), respectively. Rolipram and 4-(butoxy-4-methoxybenzyl)-2-imidazolidione (RO-20-1724) were purchased from BIOMOL Res. Lab. (Plymouth Meeting, PA, USA).

2.2. Cell culture

PC12 (D type) cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% horse serum, as previously reported (Oda et al., 1995; Murayama et al., 1995). Cells were maintained at 37°C under a humidified atmosphere of 10% CO_2/90% air. For the experiments, subconfluent PC12 cells were used at 48 h after the final change of medium.

2.3. Measurement of cyclic AMP content

PC12 cells were detached by replacing the medium with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (pH 7.4). Cells were washed twice by centrifugation at $100 \times g$ for 2 min at 4°C and suspended in modified Tyrode's Hepes buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Hepes, pH 7.4). In some experiments, the cells were prepared with Ca^{2+}-free Tyrode's buffer. Detached PC12 cells (40–70 μg/tube) were incubated with various test compounds or agents for 10 min at 37°C in Tyrode's buffer supplemented with 0.2 mM RO-20-1724 and 0.2 mM rolipram, as phosphodiesterase inhibitors. Incubation was terminated by acidification with HCl to 0.2 M and then boiling for 1 min. The cellular cyclic AMP transferred to the supernatant was quantified by radioimmunoassay. In some experiments, the cells were incubated with agents for 2 min at 37°C. Data from both procedures were similar. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Measurement of $[Ca^{2+}]_i$

PC12 cells on dishes were incubated in 0.7 μM fura-2 acetoxymethyl ester for 20 min at 37°C in Tyrode's buffer containing 0.2% fatty acid-free bovine serum albumin. The PC12 cells were washed and detached from the dishes under a gentle stream of buffer. Detached cells were washed twice by centrifugation at 4°C and resuspended with the same buffer. An aliquot of $3–5 \times 10^6$ cells was immediately used for autofluorescence measurements at 37°C. Fluorescence readings were taken with a Hitachi F-2000 spectrophotometer, as described previously (Kurozumi et al., 1990; Murayama et al., 1995).

2.5. Measurement of [^{3}H]noradrenaline secretion

[^{3}H]Noradrenaline secretion from PC12 cells labeled with 1 μCi/ml of [^{3}H]noradrenaline was estimated as described previously (Oda et al., 1995).

3. Results

Fig. 1 shows ATP- and ATPγS-stimulated cyclic AMP accumulation in PC12 cells. In the absence of extracellular $CaCl_2$, the addition of ATP at low concentrations up to 300 μM stimulated cyclic AMP accumulation up to about 3-fold (Fig. 1A). However, ATP at high concentrations over 300 μM inhibited cyclic AMP accumulation. Similar results were obtained using ATPγS. Next, we investigated

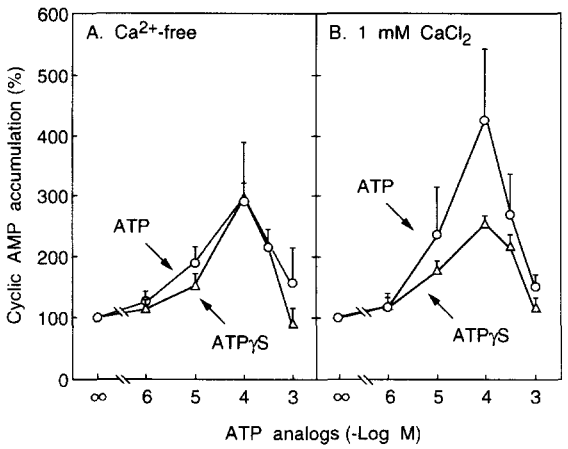

Fig. 1. ATP- and ATPγS-stimulated cyclic AMP accumulation in PC12 cells. PC12 cells were incubated with increasing concentrations of ATP (○) or ATPγS (△) in the absence (Panel A) or presence (Panel B) of 1 mM extracellular $CaCl_2$. 0.2 mM EGTA was further added to the Ca^{2+}-free buffer. Data are normalized as percentages of non-stimulated (basal) cyclic AMP accumulation and presented as means ± S.E.M. of 3–4 independent experiments. The absolute values of cyclic AMP accumulation were 91.4 ± 20.0 and 126 ± 33 pmol/mg protein per 10 min in the absence and presence of $CaCl_2$, respectively.

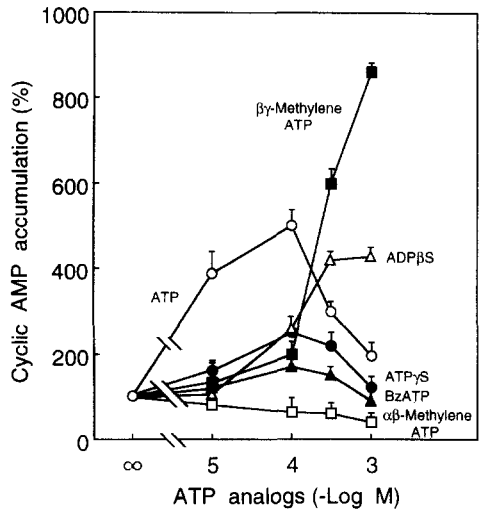

Fig. 2. Cyclic AMP accumulation in response to various nucleotides in PC12 cells. PC12 cells were incubated with increasing concentrations of ATP (○), ATPγS (●), α,β-methylene ATP (□), β,γ-methylene ATP (■), BzATP (▲) or ADPβS (△). Data are normalized as percentages of non-stimulated (basal) cyclic AMP accumulation and presented as means ± S.E.M. of 3–4 independent experiments.

the effects of extracellular $CaCl_2$ on ATP- and ATPγS-induced cyclic AMP accumulation (Fig. 1B). Basal (non-stimulated) cyclic AMP accumulation with phosphodiesterase inhibitors was 91.4 ± 20.0 and 126 ± 33 pmol/mg protein per 10 min (n = 3–4) in the absence and presence of 1 mM $CaCl_2$, respectively. The pattern of cyclic AMP accumulation evoked by ATP in the presence of $CaCl_2$ was also the same as that in the absence of $CaCl_2$. The response elicited by ATP in the presence of $CaCl_2$ was slightly higher than that without $CaCl_2$, although the difference was not significant because of large standard deviations. Similar patterns of cyclic AMP accumulation elicited by ATP and ATPγS were obtained in PC12 cells treated for 10 min with calmodulin antagonists, W-7 up to 50 μM or trifluoperazine up to 20 μM (Table 1).

Table 1
Effects of calmodulin antagonists on ATPγS-stimulated cyclic AMP accumulation

Addition	Cyclic AMP accumulation (%)		
	Vehicle	W-7	Trifluoperazine
None	100	98 ± 5	101 ± 8
10 μM ATPγS	202 ± 12	200 ± 12	198 ± 15
100 μM ATPγS	308 ± 20	298 ± 16	270 ± 24
1 mM ATPγS	89 ± 11	94 ± 11	96 ± 15

PC12 cells were incubated with 50 μM W-7, 20 μM trifluoperazine or vehicle for 10 min at 37°C in the Ca^{2+}-free buffer. The washed cells were stimulated with the indicated concentrations of ATPγS in the absence of extracellular $CaCl_2$. Calmodulin antagonists at the same concentrations were added to the assay mixture. Data are normalized as percentages of non-stimulated cyclic AMP accumulation in the control cells, and presented as means ± S.E.M. of 3 independent experiments. The absolute value of cyclic AMP accumulation was 105 ± 16 pmol/mg protein/10 min.

Fig. 2 shows the effects of various ATP analogs in the presence of 1 mM $CaCl_2$. The addition of ADPβS and β,γ-methylene ATP stimulated cyclic AMP accumulation in a dose-dependent manner. The addition of BzATP and 2-methylthio ATP (data not shown) induced biphasic effects on cyclic AMP accumulation similarly to ATP, although the stimulatory effect was very much less marked than that produced by ATP. In the cases of ADPβS and β,γ-methylene ATP, no inhibitory phase was observed. α,β-Methylene ATP inhibited cyclic AMP accumulation slightly in a dose-dependent manner. Addition of UTP and 8-bromo ATP had no effect up to 300 μM (data not shown).

PC12 cells, labeled with the Ca^{2+} indicator fura-2 acetoxymethyl ester, were challenged with various ATP analogs in the presence of 1 mM extracellular $CaCl_2$ (Fig. 3). Addition of 300 μM ATP and ATPγS evoked a rapid $[Ca^{2+}]_i$ rise with a peak at 5–10 s. The $[Ca^{2+}]_i$ rise evoked by ATP was almost entirely due to Ca^{2+} influx, as the peak $[Ca^{2+}]_i$ rise was reduced by removal of extracel-

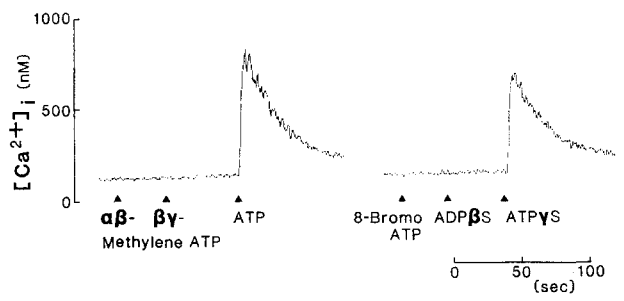

Fig. 3. $[Ca^{2+}]_i$ elevation evoked by ATP analogs in PC12 cells. Fura-2 AM-loaded PC12 cells were stimulated with ATP analogs (all at 300 μM) in the presence of 1 mM extracellular $CaCl_2$. Data shown are from a typical experiment and are representative of 3 independent experiments.

Table 2
Inhibitory effect of adenosine deaminase on adenosine-, AMP- or ADP- but not β,γ-methylene ATP- or ADPβS-stimulated cyclic AMP accumulation

Addition	Cyclic AMP accumulation (pmol/mg protein per 10 min)	
	Vehicle	Adenosine deaminase (5 units/ml)
None	73 ± 12 (1)	75 ± 8 (1)
β,γ-Methylene ATP (300 μM)	901 ± 33 (12.3 ± 0.4)	1165 ± 50 (15.5 ± 0.6)
ADPβS (300 μM)	392 ± 32 (5.3 ± 0.4)	462 ± 42 (6.1 ± 0.5)
Adenosine (100 μM)	3568 ± 840 (48.8 ± 11.5)	82 ± 15 (1.0 ± 0.2)
AMP (100 μM)	660 ± 82 (9.0 ± 1.1)	69 ± 10 (0.92 ± 0.13)
ADP (100 μM)	321 ± 45 (4.3 ± 0.6)	71 ± 11 (0.94 ± 0.14)

PC12 cells were incubated with the indicated concentrations of ATP analogs or adenosine in the presence or absence of adenosine deaminase. Data are presented as means ± S.D. of 3 determinations in a typical experiment. These results are representative of 3 independent experiments. In parentheses: data for magnitude of stimulation presented as means ± S.E.M. of 3 independent experiments.

lular $CaCl_2$ (Murayama et al., 1995). In contrast, ADPβS, α,β-methylene ATP and β,γ-methylene ATP had no effect. 8-Bromo ATP had no effect on $[Ca^{2+}]_i$ in PC12 cells, although it increased $[Ca^{2+}]_i$ in FRTL-5 thyroid cells (Okajima et al., 1989). In addition, they did not inhibit the effects of ATP or ATPγS on the $[Ca^{2+}]_i$. Addition of 300 μM ATP stimulated [^{3}H]noradrenaline release from prelabeled PC12 cells in an extracellular Ca^{2+}-dependent manner as described previously (Oda et al., 1995). ATPγS also showed this effect. However, α,β-methylene ATP, β,γ-methylene ATP, 8-bromo ATP and ADPβS had no or very limited effects on [^{3}H]noradrenaline release (data not shown, but see Oda et al., 1995). These findings suggest that the stimulatory effects of ATP, ADPβS and β,γ-methylene ATP on cyclic AMP accumulation were independent of the $[Ca^{2+}]_i$ rise and catecholamine release.

Further, we investigated the stimulatory effect of ADPβS and β,γ-methylene ATP, because both stimulated cyclic AMP markedly with no inhibitory phase. In PC12 cells, adenosine stimulated A_2 adenosine receptors and stimulated cyclic AMP accumulation markedly (Table 2). Addition of ADP and AMP also stimulated cyclic AMP accumulation. The effects of ADP, AMP and adenosine were dose-dependent and with no inhibitory phase (data not shown). The effect of ADPβS at 300 μM was 1/10 of the stimulatory effect of 100 μM adenosine. ATP analogs such as ATP and ATPγS are usually contaminated with trace amounts of ADP and AMP. Thus, it is possible that the stimulatory effects of ATP analogs such as ATP and ATPγS derived from the contamination with ADP, AMP or adenosine. Addition of the indicated concentration of adenosine deaminase to the assay mixture inhibited the effects of adenosine, AMP and ADP completely (Table 2). The effects of ADPβS and β,γ-methylene ATP, however, were slightly increased by addition of adenosine deaminase. Addition of an antagonist of A_2 adenosine receptors, 3-isobutyl-1-methylxanthine, did not inhibit the stimulatory effects of ADPβS or β,γ-methylene ATP (data not shown). Similarly, the stimulatory effect of ATP at low concentrations up to 300 μM was not modified by adenosine deaminase or the adenosine A_2 receptor antagonist (data not shown). Another potent adenosine A_1 and A_2 receptor antagonist, 1,3-diethyl-8-phenylxanthine, and a A_2-selective antagonist, CGS-15943, had no effect up to 100 μM on the dose-response curve of ADPβS (data not shown). These findings suggest that increases in cyclic AMP accumulation by ATP and ATP analogs were not caused by contaminating adenosine and were not adenosine receptor-mediated effects.

Table 3 shows the effect of reactive blue 2 on β,γ-methylene ATP- and ADPβS-stimulated increases in cyclic AMP accumulation. The addition of reactive blue 2 significantly inhibited the increase in cyclic AMP accumulation elicited by 0.3 mM β,γ-methylene ATP in a dose-dependent manner. The addition of 0.3 mM reactive blue 2 shifted the dose-response curve of β,γ-methylene ATP to the right without a change in the maximal response (data not shown). The addition of 1 mM reactive blue 2 inhibited the effect of β,γ-methylene ATP by 80%, although the effect of ADPβS was inhibited by only 15%. The effect of 300 μM ATP was inhibited by 30–40% in the presence of 1 mM reactive blue 2. Addition of reactive blue 2 to the assay mixture did not affect the basal cyclic AMP content (data not shown). Suramin, an antagonist of P_{2X} receptors in PC12 cells (Brake et al., 1994), at concentrations up to 300 μM did not inhibit ATP- or ATP analog-stimulated cyclic AMP accumulation in any con-

Table 3
Inhibition of β,γ-methylene ATP- but not ADPβS-stimulated cyclic AMP accumulation by reactive blue 2

Addition	Increase in cyclic AMP accumulation (%)	
	0.3 mM β,γ-Methylene ATP	0.3 mM ADPβS
None	100	100
Reactive Blue 2		
0.1 mM	73 ± 3	93 ± 1
0.3 mM	55 ± 5	85 ± 5
1 mM	22 ± 6	83 ± 6

PC12 cells were incubated with β,γ-methylene ATP or ADPβS in the presence of indicated concentrations of reactive blue 2. Data are shown as percentages of the response without reactive blue 2. Data are presented as means ± S.E.M. of 3 independent experiments.

centrations (data not shown). These findings suggest that (1) ATP receptors coupled to cyclic AMP accumulation have different pharmacological characteristics from those coupled to ion channels and noradrenaline release in PC12 cells, and (2) receptors of β,γ-methylene ATP and of ADPβS may be different.

4. Discussion

It was reported previously that the addition of ATP or ADP reduces the intracellular cyclic AMP content of a variety of cells (Okajima et al., 1987, 1989; Pianet et al., 1989; Colman, 1990, 1992). In PC12 cells, addition of ATP or ATPγS at low concentrations (≤ 100 μM) stimulated but at high concentrations (≥ 100 μM) inhibited cyclic AMP accumulation. We investigated the stimulatory effect of ATP in this experiment, because the stimulatory, not inhibitory, effect of ATP on cyclic AMP content has not been reported previously. PC12 cells contain A_2 adenosine receptors that activate cyclic AMP accumulation (Ukena et al., 1986; Oda et al., 1995), and adenosine is generated endogenously in PC12 cells during incubation (Roskoski et al., 1989). Thus, it seemed that ATP in the assay mixture was metabolized by ectonucleotidases to adenosine and that the effect of ATP was due to adenosine. However, the hydrolysis-resistant analog ATPγS also stimulated cyclic AMP accumulation (Fig. 1). ADPβS and β,γ-methylene ATP stimulated cyclic AMP content with no inhibitory phase (Fig. 2). The addition of adenosine deaminase did not inhibit the effects of ATP or its effective analogs, although it completely inhibited the effect of adenosine on cyclic AMP content (Table 2). Addition of adenosine A_2 receptor antagonist had no effect on ATP- or ATPγS-stimulated reactions (data not shown), and the addition of reactive blue 2, a P_2 purinoceptor antagonist, inhibited the effects of ATP and β,γ-methylene ATP (Table 3). Under our experimental conditions, when inhibitors of phosphodiesterase were added cyclic AMP accumulation increased over the assay period. These findings suggest that ATP, not adenosine, stimulated the observed increases in cyclic AMP content directly.

It has been reported that type I adenylate cyclase is activated by Ca^{2+} (for review see, Taussig and Gilman, 1995). The addition of ATP and ATPγS stimulates $[Ca^{2+}]_i$ rise via an influx of extracellular Ca^{2+} (Fig. 3, and Murayama et al., 1995). However, the effects of ATP and ATPγS on cyclic AMP content were independent of $[Ca^{2+}]_i$ (Fig. 1) and the calmodulin antagonists showed no effect on ATP analog-stimulated cyclic AMP accumulation (Table 1). β,γ-Methylene ATP and ADPβS, which stimulated cyclic AMP accumulation, had no effect on the mobilization of $[Ca^{2+}]_i$ (Fig. 3). Thus, the stimulatory effect of ATP seemed to be independent of $[Ca^{2+}]_i$. Some classes of P_2 purinoceptors are coupled to GTP-binding proteins (Dalziel and Westfall, 1994). Several forms of adenylyl cyclase are stimulated by β,γ-subunits of GTP-binding proteins such as G_i and G_o (Tang and Gilman, 1992). However, treatment with pertussis toxin (1 μg/ml, 24 h), which ADP ribosylates α-subunits of several GTP-binding proteins and inhibits the liberation of β,γ-subunits from GTP-binding proteins, had no effect on ATP-stimulated cyclic AMP accumulation (data not shown). Further studies are needed to explain the ATP receptor-mediated effect.

A new class of ligand-gated ion channels defined as ionotropic P_{2X} receptors has been cloned from PC12 pheochromocytoma cells (Brake et al., 1994). The pharmacological characteristics of these ATP receptors, when expressed in *Xenopus* oocytes, resemble those of ATP receptors coupled to Ca^{2+} influx and noradrenaline release in PC12 cells; ATPγS and 2-methyltio-ATP are equipotent as agonists, and neither α,β-methylene ATP nor β,γ-methylene ATP are active as agonists of noradrenaline release or Ca^{2+} influx in PC12 cells (Oda et al., 1995) or stimulation of ion channels in *Xenopus* oocytes (Brake et al., 1994). However, 2-methylthio ATP and BzATP, which are as effective as ATP in stimulating $[Ca^{2+}]_i$ rise and noradrenaline release (Oda et al., 1995), showed very limited effects on cyclic AMP accumulation (Fig. 2). Addition of ADPβS or β,γ-methylene ATP, which showed no effect on the $[Ca^{2+}]_i$ rise or on noradrenaline release, stimulated cyclic AMP accumulation markedly (Fig. 2). Ca^{2+} influx and catecholamine release in PC12 cells (Nakazawa et al., 1990; Inoue et al., 1989, 1991; Oda et al., 1995) and ion channels in oocytes (Brake et al., 1994) are inhibited by the addition of suramin. However, suramin showed no antagonistic effect on ATP- or ADPβS-stimulated cyclic AMP accumulation. UTP had no effect on cyclic AMP accumulation, although the addition of UTP stimulates Ca^{2+} influx and noradrenaline release in PC12 cells (Rhoads et al., 1993; Majid et al., 1993). These findings suggest that there is a new type of ATP receptor coupled to adenylyl cyclase positively and which has pharmacological characteristics different from those of P_{2X} receptors coupled to $[Ca^{2+}]_i$ rise and noradrenaline release in PC12 cells. In addition, receptor subtypes responsive to β,γ-methylene ATP and to ADPβS may be different, because the effects of the former but not of the latter were inhibited by reactive blue 2.

At concentrations over 300 μM, ATP and ATPγS inhibited cyclic AMP accumulation (Fig. 1). ATP or ATPγS at these concentrations also inhibited forskolin- and adenosine-stimulated cyclic AMP accumulation (data not shown). 2-Methylthio ATP showed a similar inhibitory effect. The inhibitory effects of ATP analogs were not modified by pertussis toxin treatment, which ADP-ribosylates inhibitory GTP-binding proteins and diminishes their function (Ui, 1984). The observed inhibitory effect of high concentrations of ATP and ATPγS was not related to Ca^{2+} influx (Fig. 1). The stimulatory and inhibitory effects were not modified by treatment with calmodulin antago-

nists (Table 1). The characteristics of another type of ATP receptor coupled to adenylyl cyclase negatively in PC12 cells have to be determined.

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