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P2 receptor-mediated inhibition of adenylyl cyclase in PC12 cells

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Abstract

PC12 pheochromocytoma cells have P2 receptors which are coupled to Ca^{2+} influx and catecholamine release. Previously we reported that ATP stimulated cyclic AMP accumulation at low concentrations up to 100 μM but showed inhibitory effects above this concentration [Yakushi, Y., Watanabe, A., Murayama, T., Nomura, Y., 1996. Eur. J. Pharmacol. (314) 243–248]. In this study we investigated the characteristics of the inhibitory effects of ATP analogs. In the presence of 10 μM forskolin, an activator of adenylyl cyclase, ATP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), 2′,3′-*O*-(4-benzoyl) benzoyl ATP, 2-methylthio ATP and adenosine 5′-*O*-(2-thiodiphosphate) inhibited cyclic AMP accumulation in a dose-dependent manner from 100 μM. UTP, $\alpha\beta$ and $\beta\gamma$-methylene ATP had no or very limited effects. The relative order of ATP analogs suggests that the ATP receptor appears to be P2Y-like. However, suramin, an antagonist of P2X and P2Y receptors, and reactive blue-2, which inhibited $\beta\gamma$-methylene ATP-induced cyclic AMP accumulation, did not modify the inhibitory effect of ATPγS. Treatment with pertussis toxin, which completely abolished the effect of carbachol, had no effect on the action of ATP over 300 μM. The existence of a new type of ATP receptor-mediated inhibition of adenylyl cyclase is proposed in PC12 cells. © 1998 Elsevier Science B.V.

Keywords: PC12 cell; ATP; cAMP; P2Y receptor

1. Introduction

Extracellular ATP (and/or ADP) has hormone- and neurotransmitter-like effects in a variety of mammalian tissues (for reviews, see Gordon, 1986; El-Moatassim et al., 1992; Dalziel and Westfall, 1994). It has been established that cellular responses to extracellular ATP are mediated by cell-surface receptors (P2 receptors). Based primarily on the pharmacological potencies of nucleotides, several classes of P2 receptors (P_{2X}, P_{2Y}, P_{2T}, P_{2U} and P_{2Z}, etc.) have been defined. Although the pharmacological phenotypes are not associated with several clones, a lot of P2 receptor subtypes in cloning studies have been further expanded. It is proposed in general that ATP-gated channels and GTP-binding protein (G protein)-coupled receptors are termed P2X and P2Y receptors, respectively (Dalziel and Westfall, 1994; Fredholm et al., 1994). The P2X receptors, which are ligand-gated ion channels, were cloned from the smooth muscle of the vas deferens (Valera et al., 1994) and from PC12 cells (Brake et al., 1994). A number of G protein coupled ATP receptors have been characterized by DNA cloning and they are all known as P2Y receptors (for review, see Fredholm et al., 1994; Akbar et al., 1996), $P2Y_1$ (P_{2Y}) receptors (where 2-methylthio ATP has greater activity than ATP, and UTP is inactive), $P2Y_2$ (P_{2U}) receptors (ATP and UTP are equally active), $P2Y_3$ receptors (UDP > ADP > ATP), and $P2Y_4$ receptors (UTP > > ATP). Further novel P2Y receptors have recently been identified from their cDNAs (Webb et al., 1996; Chang et al., 1995; Akbar et al., 1996).

Stimulation of P2Y receptors seems to be associated with an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (for review, see El-Moatassim et al., 1992; Dalziel and Westfall, 1994). The $[Ca^{2+}]_i$ rise is caused by the activation of phospholipase C in some cases, and by stimulated Ca^{2+} influxes or channel systems in other cases. On the other hand, it was also reported that changes in cyclic AMP accumulation are associated with the actions of ATP (and UTP) in many cells (Okajima et al., 1987, 1989; Pianet et al., 1989; Keppens et al., 1992; Valeins et al., 1992; Munshi et al., 1993; Boyer et al., 1993). Although UTP has a potent inhibitory effect on cyclic AMP accumulation in many cell types, it remains to be determined which subtypes of P2Y receptors are involved in the inhibition of adenylyl cyclase. Additionally, the G proteins coupled

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with the P2Y receptors seemed to be different depending on signal transduction systems, because the inhibitory effects of P2Y receptor stimulation on cyclic AMP were abolished by treatment with the pertussis toxin in many of the cells described above, but activation of phospholipase C and $[Ca^{2+}]_i$ increases were not modified in many cells. It is important to determine the G proteins coupled with P2Y receptors which inhibit adenylyl cyclase.

Previously we reported the existence of a new type of ATP receptor positively coupled to adenylyl cyclase in PC12 cells (Yakushi et al., 1996). Also we found that ATP at high concentrations over 100 μM inhibited cyclic AMP accumulation in PC12 cells. In this study, we examined the characteristics of the inhibitory effects of ATP on cyclic AMP accumulation, and proposed the existence of a new, pertussis toxin-insensitive type of P2Y receptor-mediated inhibition of adenylyl cyclase in PC12 cells.

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 and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were obtained from Research Biochemicals (Natick, MA, USA). ATP, $\alpha\beta$-methylene ATP, $\beta\gamma$-methylene ATP, 2′,3′-*O*-(4-benzoyl)benzoyl ATP (BzATP), 8-bromo ATP, reactive blue-2, adenosine deaminase were purchased from Sigma (St. Louis, MO, USA). Suramin and cyclic AMP assay kits were donated by Bayer Japan (Tokyo, Japan) and Yamasa-Shoyu (Chiba, Japan), respectively. Rolipram and 4-(butoxy-4-methoxybenzyl)-2-imidazolidione (RO-20-1724) were purchased from BIOMOL Res. Lab. (Plymouth Meeting, PA, USA). Pertussis toxin was obtained from Wako Pure Chemical (Osaka, Japan).

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 (Murayama et al., 1995, 1996; Yakushi et al., 1996; Oda et al., 1995, 1996, 1997).

2.3. Measurement of the cyclic AMP content

The cyclic AMP content was measured as described previously (Murayama et al., 1996; Yakushi et al., 1996). Briefly, detached PC12 cells were incubated with various test compounds for 10 min at 37°C 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) supplemented with 0.2 mM Ro-20-1724 and 0.2 mM rolipram, as phosphodiesterase inhibitors. In some experiments, adenosine deaminase (5 unit/ml) was added in the assay mixture. Data from both procedures were similar. Protein was determined using a BioRad assay kit 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. Measurement of $[Ca^{+}]_i$ was estimated as described previously (Murayama et al., 1995; Oda et al., 1995; Yakushi et al., 1996).

2.5. Statistics

Data are means ± S.E. of three or four independent experiments performed in duplicate or triplicate. Data were analyzed using the Student's or unpaired *t*-test.

3. Results

As previously reported (Yakushi et al., 1996), the addition of ATP at low concentrations of up to 100 μM stimulated cyclic AMP accumulation up to about 3-fold in PC12 cells (Fig. 1). However, ATP at concentrations over 100 μM inhibited cyclic AMP accumulation. The addition of ATPγS also showed biphasic, stimulatory and inhibitory effects depending on the concentration. In this study we focused on the inhibitory effect of ATP. In the following experiments in which decreases in cyclic AMP accumulation were measured, the incubation medium was supplemented with 10 μM forskolin to increase cyclic AMP and thus to allow an accurate estimation of cyclic AMP suppression (Murayama and Ui, 1985, 1987; Mu-

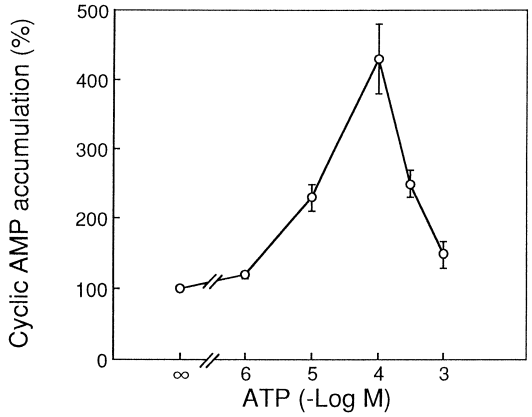

Fig. 1. Concentration-dependent effect of ATP on cyclic AMP accumulation in PC12 cells. PC12 cells were incubated with increasing concentrations of ATP. Data are normalized as percentages of cyclic AMP accumulation without ATP and represent the means ± S.E. of three to four independent experiments. The absolute value of cyclic AMP accumulation without ATP was 115 ± 24 pmol/mg protein per 10 min.

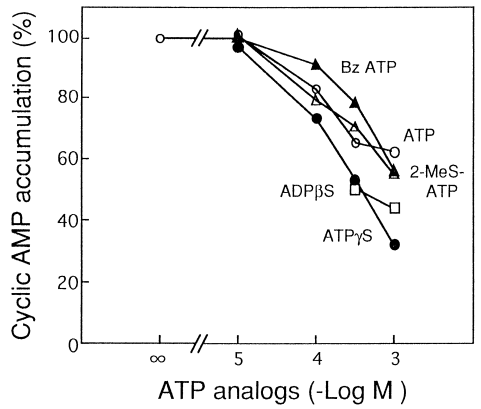

Fig. 2. The inhibitory effects of various ATP analogs on forskolin-stimulated cyclic AMP accumulation in PC12 cells. PC12 cells were incubated with increasing concentrations of ATP (○), ATPγS (●), 2-methylthio ATP (△), BzATP (▲) or ADPβS (□) in the presence of 10 μM forskolin. Data are normalized as percentages of cyclic AMP accumulation without ATP analogs and represent the means of three to four independent experiments. The S.E. of each point was within 5%. The absolute value of cyclic AMP accumulation in the presence of 10 μM forskolin was 11.3 ± 1.0 nmol/mg protein per 10 min.

rayama et al., 1996). The addition of 10 μM forskolin stimulated cyclic AMP accumulation remarkably. In the presence of forskolin, the addition of ATP and ATPγS at high concentrations inhibited cyclic AMP accumulation in a concentration dependent-manner (Fig. 2). The addition of ATP and ATPγS at low concentrations of up to 100 μM further enhanced forskolin-stimulated cyclic AMP accumulation in some cases by about 20–30%, but not in all cases. The addition of BzATP and 2-methylthio ATP also inhibited cyclic AMP accumulation. Neither αβ-methylene ATP nor βγ-methylene ATP had inhibitory effects at 300 μM (data not shown), although βγ-methylene ATP had stimulatory effects on cyclic AMP in PC12 cells (Yakushi et al., 1996). Addition of 300 μM and 1 mM ADPβS inhibited forskolin-stimulated cyclic AMP accumulation remarkably. UTP at 300 μM inhibited cyclic AMP accumulation slightly (5–10% at 300 μM and 10–15% at 1 mM). UDP

Table 1
ATPγS-induced inhibition of forskolin-stimulated cyclic AMP accumulation in PC12 cells

Addition	Cyclic AMP accumulation (%)	
	1 mM $CaCl_2$	$CaCl_2$-free
None	100	98 ± 3
300 μM ATPγS	61 ± 4[a]	64 ± 5[a]
1 mM ATPγS	25 ± 5[a]	26 ± 4[a]

PC12 cells were incubated with 10 μM forskolin and the indicated concentrations of ATPγS in the presence or absence of 1 mM $CaCl_2$. EGTA (0.1 mM) was further added to the assay buffer in both cases. Data are normalized as percentages of cyclic AMP accumulation without ATPγS in the presence of $CaCl_2$, and represent the means ± S.E. of three to four independent experiments. The absolute value of cyclic AMP accumulation in the presence of 10 μM forskolin was 11.1 ± 1.2 nmol/mg protein per 10 min. Significance; [a] $P < 0.05$, compared to none.

Table 2
Effects of reactive blue-2 and suramin on ATPγS-induced inhibition of cyclic AMP accumulation in PC12 cells

Addition	None	Cyclic AMP accumulation (%)	
		Reactive blue-2	Suramin
None	100	94 ± 2	96 ± 3
300 μM ATPγS	59 ± 5[a]	48 ± 2[a]	52 ± 2[a]
1 mM ATPγS	13 ± 1[a]	7 ± 1[a]	12 ± 1[a]

PC12 cells were incubated with 10 μM forskolin and the indicated concentrations of ATPγS in the presence of 100 μM reactive blue-2, 100 μM suramin or vehicle. Data are normalized as percentages of cyclic AMP accumulation without ATPγS in the absence of the antagonists, and represent the means ± S.E. of three to four independent experiments. The absolute value of cyclic AMP accumulation in the presence of 10 μM forskolin was 10.1 ± 1.0 nmol/mg protein per 10 min. Significance; [a] $P < 0.05$, compared to without ATPγS.

at 300 μM had no inhibitory effect. The IC_{50} values were 11 ± 3 μM (ATPγS), 12 ± 2 μM (2-methylthio ATP), 13 ± 2 μM (ATP) and 26 ± 3 μM (BzATP) ($n = 3–4$), although the values were obtained from estimation because ATP analogs at concentrations over 1 mM showed reverse (stimulatory) effects.

Table 1 shows the effect of extracellular $CaCl_2$ on ATP-induced inhibition of cyclic AMP accumulation. The addition of ATP inhibited cyclic AMP accumulation in the presence and absence of 1 mM $CaCl_2$ to the same degree. Similar inhibitory effects by ATP were obtained in PC12 cells treated for 10 min with calmodulin antagonists, 50 μM W-7 or 20 μM trifluoperazine (data not shown).

Table 2 shows the effects of reactive blue-2, a P2 receptor antagonist. The addition of 100 μM reactive blue-2 did not modify the inhibitory effect of ATPγS. The addition of 300 μM reactive blue-2, in which a concentration of 300 μM βγ-methylene ATP-stimulated cyclic AMP accumulation was inhibited by 50% in PC12 cells (Yakushi et al., 1996), did not have any effect. Suramin is known to inhibit P2Y and P2X receptor-mediated effects in PC12 cells (Hoyle et al., 1990; Nakazawa et al., 1990; Inoue et al., 1991; Brake et al., 1994). The addition of 100 μM suramin did not inhibit ATPγS-induced inhibition of cyclic AMP accumulation, although 100 μM suramin inhibited ATPγS-stimulated arachidonic acid release in PC12 cells (Murayama et al., 1995).

A class of P2Y receptors is coupled to G proteins (for review, see Dalziel and Westfall, 1994; Akbar et al., 1996). The inhibition of adenylyl cyclase by receptor stimulation is generally mediated by inhibitory G proteins (G_i), which are ADP-ribosylated and their functions are diminished by pertussis toxin (Murayama and Ui, 1983; Ui, 1984). Thus, we investigated treatment with pertussis toxin (1 μg/ml, 24 h) on ATPγS-induced inhibition of cyclic AMP accumulation (Table 3). Treatment with pertussis toxin reduced the inhibitory effect of 100 μM ATPγS significantly. However, the inhibitory effect of ATPγS at high concentrations (300 μM and 1 mM) was

Table 3
Effect of pertussis toxin treatment on ATPγS- and carbachol-induced inhibition of cyclic AMP accumulation in PC12 cells

Addition	Cyclic AMP accumulation (%)	
	Control	Pertussis toxin
None	100	105 ± 11
ATPγS (100 μM)	75 ± 3	90 ± 5[a]
(300 μM)	54 ± 3	54 ± 1
(1 mM)	27 ± 5	34 ± 3
Carbachol (300 μM)	55 ± 6	106 ± 4[a]

PC12 cells were cultured with or without 1 μg/ml of pertussis toxin for 24 h, and cyclic AMP accumulation in the washed cells was assayed. Control and pertussis toxin-treated cells were incubated with the indicated concentrations of ATPγS and 300 μM carbachol in the presence of 10 μM forskolin. Data are normalized as percentages of cyclic AMP accumulation induced by forskolin in the control cells, and represent the means ± S.E. of three to four independent experiments. The absolute value of cyclic AMP accumulation induced by 10 μM forskolin in the control cells was 11.7 ± 1.3 nmol/mg protein per 10 min. Significance; [a] $P < 0.05$, compared to that in the control (non-treated) cells.

not modified by this treatment. The addition of 300 μM carbachol inhibited cyclic AMP accumulation by about 50%, to a similar degree of the effect of 300 μM ATPγS. The inhibitory effect of carbachol via muscarinic receptors was abolished completely by this treatment.

4. Discussion

4.1. Inhibitory effect by ATP was not dependent on $[Ca^{2+}]_i$ or protein kinase C

Stimulation of P2 receptors by ATP stimulates an influx of extracellular Ca^{2+} in PC12 cells (Murayama et al., 1995; Yakushi et al., 1996). Type I phosphodiesterase is Ca^{2+}/calmodulin-dependent (Jin et al., 1992). All types of adenylyl cyclases are inhibited by high (over 100 μM) concentrations of Ca^{2+} as a result of competition for Mg^{2+}, which is required for catalysis, and types V and VI adenylyl cyclase are inhibited by low micromolar concentrations of Ca^{2+} (Tang and Gilman, 1992; Taussing and Gilman, 1995). The addition of Ca^{2+} ionophores inhibited cyclic AMP accumulation in other cells (Dorflinger et al., 1984; Murayama and Ui, 1985). These results suggest that the influx of Ca^{2+} stimulated by ATP may induce the inhibition of cyclic AMP accumulation in PC12 cells. However, the involvement of Ca^{2+}/calmodulin-dependent phosphodiesterase is excluded for several reasons: (1) the inhibitory effect by ATP was not dependent on extracellular $CaCl_2$ (Table 1); (2) treatment with calmodulin antagonists such as W-7 and trifluoperazine did not modify the inhibitory effect of ATP; (3) under our experimental conditions, inhibitors of phosphodiesterase were added to the assay mixture. Ca^{2+} mobilization stimulated by ATP did not seem to be involved in the inhibition of adenylyl cyclase, because in the absence of extracellular $CaCl_2$, where $[Ca^{2+}]_i$ increases were not stimulated by P2Y receptor stimulation (Murayama et al., 1995), ATPγS still inhibited cyclic AMP accumulation.

Stimulation of P2Y receptors seems to activate protein kinase C as a result of the accumulation of diacylglycerol induced by phospholipase C. In fact, the noradrenaline release induced by 300 μM ATP from PC12 cells was inhibited by calphostin C, a selective inhibitor of protein kinase C (Oda et al., 1995). Although potential regulation of adenylyl cyclase by protein kinase C has been proposed, treatment for 10 min and simultaneous addition of 100 nM phorbol 12-myristate 13-acetate, a direct activator of protein kinase C, had no effect on cyclic AMP accumulation with or without forskolin (data not shown). Thus, it is probable that an inhibition of cyclic AMP accumulation is derived from the P2Y receptor-mediated direct inhibition of adenylyl cyclase, not from Ca^{2+}- and protein kinase C-mediated process.

4.2. P2 receptor subtypes are coupled with the inhibition of adenylyl cyclase in PC12 cells.

In PC12 cells, the relative order of the agonist potency on inhibition of cyclic AMP accumulation (as judged in the presence of 10 μM forskolin) was: ATPγS = 2-methylthio ATP = ATP = ADPβS > BzATP > > UTP > αβ-methylene ATP = βγ-methylene ATP. Neither reactive blue-2 or suramin, antagonists of P2 receptors, were effective (Table 2). The inhibition of cyclic AMP by ATP analogs was not caused by contaminating adenosine and were not adenosine receptor-mediated effects, because the reaction was not modified by addition of adenosine deaminase (5 unit/ml).

Classically, P2Y receptors have been differentiated on the basis of the relative potencies of the ATP analogs; 2-methylthio ATP ≥ ATP > > αβ-methylene ATP = βγ-methylene ATP. 2-Methylthio ATP and BzATP are also active in noradrenaline (Oda et al., 1995) and arachidonic acid (Murayama et al., 1995) release from PC12 cells, which have P2Y-like receptors. In this study, ATP analogs which inhibit cyclic AMP accumulation had characteristics similar to P2Y receptors. There are many P2Y receptor subtypes, although the inclusion of some receptors (including $P2Y_5$ and $P2Y_7$) within the P2Y family remains controversial. In $P2Y_1$ cRNA-injected oocytes, both suramin (100 μM) and reactive blue-2 (10 μM) antagonized the responses to ATP and to 2-methylthio ATP (Webb et al., 1993). Because UTP and UDP had a limited inhibitory effect on cyclic AMP accumulation in PC12 cells, the involvement of $P2Y_4$, $P2Y_3$, $P2Y_4$ and $P2Y_6$ purinoceptors is excluded. In $P2Y_4$ cRNA-transfected 1321N1 cells, UTP and UDP had a maximal effect on phospholipase C activation, although ATP had a low potency and 2-methylthio ATP was completely inactive (Communi et al., 1995). ADP, ATP and UTP did not change forskolin-stimulated

cyclic AMP accumulation, and reactive blue-2 and suramin inhibited ADP-induced phospholipase C activation in cells expressing the cloned $P2Y_6$ receptor (Chang et al., 1995). Suramin ($K_i = 2900$ nM) effectively inhibited the ligand (4 nM [^{35}S]dATPγS) binding to the $P2Y_7$ receptor expressed in COS-7 cells (Akbar et al., 1996). Thus, the ATP receptors coupled to the inhibition of cyclic AMP in PC12 cells did not seem to be $P2Y_6$ and $P2Y_7$. The agonist potency on inhibition of cyclic AMP accumulation in PC12 cells resembles the pharmacological properties of $P2Y_5$, in such as ADP is active and UTP is inactive (Webb et al., 1996). However, the effect of ADP on the inhibition of cyclic AMP is difficult to estimate, because ADP, which was metabolized to adenosine, and adenosine stimulated cyclic AMP accumulation in PC12 cells (Yakushi et al., 1996). The effects of ADPβS and antagonists such as reactive blue-2 and suramin have not been shown in cells expressing $P2Y_5$ receptors. Recently, a new $P2Y_{11}$ receptor, which couples to the stimulation of adenylyl cyclase, was cloned (Communi et al., 1997). The characterization of P2Y-like receptors in PC12 cells which inhibit adenylyl cyclase is needed.

It was reported that PC12 cells have P2X receptors, which are ligand-gated ion channels and are activated by ATP, ATPγS, and 2-methylthio ATP (Brake et al., 1994). However, P2X receptor did not seem to be involved in the regulation of adenylyl cyclase in PC12 cells, because suramin and reactive blue-2, which showed an antagonistic effect on the reaction mediated by P2X receptor, did not have antagonistic effects on the inhibition of cyclic AMP by ATPγS (Table 2).

4.3. Effect of pertussis toxin on P2Y receptor-mediated inhibition in PC12 cells.

Stimulation of P2Y receptors inhibited cyclic AMP accumulation in many cell types. In C6 rat glioma cells, inhibition by UTP, ATP, 2-methylthio ATP, ADP, and ADPβS were abolished by pretreatment with pertussis toxin (Pianet et al., 1989; Boyer et al., 1993; Munshi et al., 1993). In the case of C6 cell membrane preparations, ADPβS did not compete with ATP, and inhibited adenylyl cyclase activity via pertussis toxin-sensitive G proteins (Valeins et al., 1992). In rat hepatocytes (Okajima et al., 1987) and in clonal line of functional rat thyroid (FRTL-5) cells (Okajima et al., 1989), treatment with pertussis toxin blocked the inhibition of cyclic AMP accumulation by P2Y receptor stimulation at least partially. These reports suggest that pertussis toxin-sensitive G protein(s), may be G_i, mediate the inhibitory action via P2Y receptors. In PC12 cells, however, the inhibition induced by ATPγS at high concentrations was not modified by pertussis toxin, although the inhibition by 100 μM ATPγS was diminished significantly (Table 3). Under these conditions, the pertussis toxin treatment caused the ADP-ribosylation of endogenous G_i and G_o almost completely (Oda et al., 1997). The effect by 300 μM carbachol, which inhibited the response to the same degree as 300 μM ATPγS, was completely abolished by pertussis toxin treatment.

Mastoparan, a peptide from wasp venom and interacts with proteins including G proteins, at 20 μM also inhibited cyclic AMP accumulation in a pertussis toxin-insensitive manner in PC12 cells, although the effect at 10 μM was partially but significantly diminished (Murayama et al., 1996). The pertussis toxin-insensitive and inhibitory effects caused by ATPγS at concentrations over 300 μM was not derived from cell toxicity, because the $[Ca^{2+}]_i$ elevation evoked by 300 μM ATP and ATPγS was transient, and the $[Ca^{2+}]_i$ level returned to the basal level after 50 s (Yakushi et al., 1996). These findings suggest that there are two routes to inhibit adenylyl cyclase in PC12 cells; one is mediated by pertussis toxin-sensitive G proteins which are activated by carbachol and by ATPγS at low concentrations, another is pertussis toxin-insensitive and activated by ATPγS at higher concentrations. Adenylyl cyclase is regulated not only by α subunits of G_i but by $\beta\gamma$ subunits (Tang and Gilman, 1992). P2Y receptors are shown to couple with pertussis toxin-insensitive G proteins and stimulate phospholipase C (Dalziel and Westfall, 1994; Chang et al., 1995). In PC12 cells, the involvement of pertussis toxin-insensitive G proteins in P2Y receptor system has been proposed (Majid et al., 1992, 1993). Thus, it is probable that stimulation of P2Y receptors couples with pertussis toxin-insensitive G protein(s), and then the $\beta\gamma$ subunits dissociate from the G protein(s) and directly inhibit adenylyl cyclase or regulate G_s and/or G_i activity.

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