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Effects of P_1 and P2 receptor antagonists on β,γ-methyleneATP- and CGS21680-induced cyclic AMP formation in NG108-15 cells

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1 We have previously shown that ATP increased cyclic AMP in NG108-15 cells, which was inhibited by P_1 receptor antagonist methylxanthines. In the present study, we examined the effects of P_1 and P2 receptor antagonists on cyclic AMP formation induced by β,γ-methyleneATP (β,γ-MeATP) and CGS21680, an A_{2A} adenosine receptor agonist, in NG108-15 cells.

2 β,γ-MeATP and CGS21680 increased intracellular cyclic AMP with EC_{50} values of 8.0 ± 0.98 μM ($n=4$) and 42 ± 7.5 nM ($n=4$), respectively.

3 Several P_1 receptor antagonists inhibited both β,γ-MeATP- and CGS21680-induced cyclic AMP increase with a similar rank order of potency; ZM241385 > CGS15943 > XAC > DPCPX. However, the pK_i values of these antagonists for β,γ-MeATP were larger than those for CGS21680.

4 Alloxazine, a P_1 receptor antagonist, and several P2 receptor antagonists (PPADS, *i*PPADS, reactive blue-2) inhibited β,γ-MeATP-induced response, while these antagonists little affected CGS21680-induced one. Suramin was effective only for β,γ-MeATP-induced response at 1 mM.

5 2-chloroadenosine (2CADO) and 2-chloroATP (2ClATP) increased cyclic AMP with similar potencies. The effects of these agonists were both inhibited by ZM241385, but only 2ClATP-induced response was inhibited by PPADS.

6 ATP- and β,γ-MeATP-induced responses were little affected by α,β-methyleneADP, a 5′-nucleotidase inhibitor.

7 These results clearly demonstrate that ATP-stimulated cyclic AMP formation can be distinguished from the A_{2A} receptor agonist-induced one by using the several P_1 and P2 receptor antagonists.

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Keywords: ATP; β,γ-methyleneATP; P_1 receptor antagonists; P2 receptor antagonists; P2 receptor; A_{2A} receptor; cyclic AMP; NG108-15 cells

Abbreviations: AC, adenylyl cyclase; alloxazine, benzo[g]pteridine-2,4 (1H, 3H)-dione; α,β-MeADP, α,β-methyleneADP; β,γ-MeATP, β,γ-methyleneATP; 2CADO, 2-chloroadenosine; 2ClATP, 2-chloroATP; CGS15943,9-chloro-2-(2-furyl)[1, 2, 4]triazolo[1,5-c]quinazolin-5-amine; CGS21680, 2-*p*-(carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine; DMEM, Dulbecco's modified Eagle's medium; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; *i*PPADS, pyridoxalphosphate-6-azophenyl-2′,5′-disulphonic acid; KRH, Krebs-Ringer-HEPES; 2MeSATP, 2-methylthioATP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; reactive blue-2 or RB-2, 1-amino-4-[[4-[[4-chloro-6-[[3(or 4)-sulfophenyl]amino]-1,3,5 - triazin - 2 - yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulphonic acid; Ro20-1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone; suramin, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulphonic acid; XAC, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; ZM241385, (4-(2-[7-amino-2-(2-furyl)[1, 2, 4]triazolo[2, 3-a][1, 3, 5]triazin-5-ylamino]ethyl)phenol)

Introduction

Extracellular purine compounds mediate a broad range of physiological responses by activation of purinoceptors at the cell membrane surfaces (Barnard *et al*., 1994; Fredholm *et al*., 1994; Harden *et al*., 1995; Olah & Stiles, 1995). P_1 receptors mainly recognize adenosine and have been divided into A_1 (Mahan *et al*., 1991), A_{2A} (Fink *et al*., 1992), A_{2B} (Stehle *et al*., 1992) and A_3 (Zhou *et al*., 1992) receptors by the molecular clonings. On the other hand, P2 receptors, which preferentially bind adenine and uridine nucleotides, have been classified into (1) ionotropic P2X subtypes ($P2X_{1-7}$) which form the non-selective cation channels (Brake *et al*., 1994; Chen *et al*., 1995; Collo *et al*., 1996; Lewis *et al*., 1995; Soto *et al*., 1996; Surprenant *et al*., 1996; Valera *et al*., 1994) and (2) G protein-coupled P2Y subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$), which mainly communicate with $G_{q/11}$ (Chang *et al*., 1995; Charlton *et al*., 1996; Lustig *et al*., 1993; Webb *et al*., 1993). Recently, the $P2Y_{11}$ receptor was identified to couple with both phospholipase C and adenylyl cyclase (AC) activation (Commumi *et al*., 1997).

Besides these cloned purinoceptors, we have previously reported an existence of a class of atypical purinoceptors in mouse neuroblastoma × rat glioma hybrid NG108-15 cells (Matsuoka *et al*., 1995). Pharmacological characteristics of this receptor were quite different from any cloned purinoceptors, because, firstly, the receptor stimulation was functionally coupled to AC activation without affecting intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). This property was a sharp contrast to the cloned P2 receptors which increase $[Ca^{2+}]_i$ through the intrinsic non-selective cation channels (P2X-type) or by activation of phospholipase C (P2Y-type). Secondly, the agonist selectivities did not match with any known P2 receptors, because both typical P2X and P2Y receptor agonists such as α,β-methyleneATP (α,β-MeATP), 2-methylthioATP (2MeSATP) and UTP were ineffective. In contrast, β,γ-methyleneATP (β,γ-MeATP) was found to be a useful agonist,

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since it stimulated cyclic AMP production without changing $[Ca^{2+}]_i$. Finally, the increase in cyclic AMP was inhibited by several P_1 receptor antagonists such as xanthine amine congener (XAC), isobutylmethylxanthine (IBMX) and 8-(*p*-sulfophenyl)theophylline (8SPT). We have recently found that 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) also inhibited ATP-stimulated cyclic AMP formation in a non-competitive manner (Ohkubo *et al*., 1998). From these results, we have speculated an existence of a novel receptor which stimulates cyclic AMP formation in NG108-15 cells.

P_1 receptor antagonist-sensitive ATP responses have been reported in various tissues or cells such as in rat caudal artery (Shinozuka *et al*., 1988), rat vas deferens (Forsyth *et al*., 1991), bovine vascular smooth muscle cells (Tada *et al*., 1992), FRTL thyroid cells (Sato *et al*., 1992) and follicular oocytes of *Xenopus Laevis* (King *et al*., 1996). It is interesting to note that β,γ-MeATP was shown to be an agonist in various P_1 receptor antagonist-sensitive ATP responses (Bailey & Hourani, 1990; Hourani *et al*., 1991; King *et al*., 1996; Piper & Hollingsworth, 1996). However, the true nature of the receptors involved in these responses is unclear, and the interpretations are varied in each report. For instance, some investigators regarded this phenomenon as the direct P_1 receptor-mediated responses (Hourani *et al*., 1991), while others proposed an existence of the third class of purinoceptors which recognized both ATP and adenosine in a P_1 receptor antagonist-sensitive manner (Shinozuka *et al*., 1988). These diverse interpretations may be due to the co-existence of functional P_1 receptors in the target organs. This is also the case in NG108-15 cells, because this cell line possesses functional A_2 adenosine receptors, and their activation also results in cyclic AMP formation (Gubits *et al*., 1990; Sapru *et al*., 1994). Therefore, more detailed pharmacological investigations are necessary to understand the mechanism of ATP-mediated cyclic AMP formation. In the present study, we attempted to separate the ATP-induced cyclic AMP response from A_2 receptor-mediated one using several P_1 and P2 receptor antagonists.

Methods

Cell culture

NG108-15 hybrid cells were a generous gift from Dr Haruhiro Higashida (Kanazawa University, Kanazawa, Japan). Cells were grown in high glucose DMEM supplemented with 7% foetal bovine serum, 100 μM hypoxanthine, 1 μM aminopterin and 16 μM thymidine and maintained in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. Cells were seeded in 24-well culture dishes at a density of $0.8-1.6\times10^4$ cells per well and cultured until they were confluent.

Analyses of cyclic AMP formation

Changes in intracellular cyclic AMP levels were measured according to the method described by Salomon (Salomon, 1991) with minor modifications. In brief, cells were labelled with 1 μCi ml^{-1} [^{3}H]-adenine in DMEM for 3–5 h. Labelled cells were washed twice with KRH buffer (in mM: NaCl 130, KCl 4.7, $NaHCO_3$ 4.0, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.5, HEPES 10, $CaCl_2$ 1.8, 0.1% BSA, pH 7.4), and preincubated with 1 U ml^{-1} adenosine deaminase in KRH buffer for 10 min at 37°C to eliminate the effects of adenosine. Cells were stimulated with various agonists in the presence of phosphodiesterase inhibitor Ro20-1724 (100 μM) for 10 min. Receptor antagonists were simultaneously added with each agonist. After aspirating the incubation buffer, the reactions were stopped by adding 0.4 ml of 2.5% perchloric acid containing 100 μM cyclic AMP and [^{14}C]-cyclic AMP (about 2300 d.p.m. per well). Acid-extracted [^{3}H]-cyclic AMP were mixed with one-tenth volume of 4.2 N KOH to deposit potassium perchlorate. The supernatant was applied to Dowex 50W-X8 column (200–400 mesh, hydrogen form, Bio-Rad), and the elution was subsequently passed through alumina column (90 active, neutral, Merck). [^{3}H]-cyclic AMP was eluted by 4 ml of 100 mM imidazole-HCl (pH 7.6). The recoveries of cyclic AMP from double columns were calculated by the ratio of ([^{14}C]-cyclic AMP eluted) / (total [^{14}C]-cyclic AMP added). [^{3}H]-cyclic AMP levels were expressed as the percentage of total [^{3}H]-adenine uptake.

Materials

High-glucose DMEM was purchased from GIBCO (Grand Island, NY, U.S.A.). Foetal bovine serum was obtained from CSL Ltd. (Victoria, Australia). Hypoxanthine, aminopterin and thymidine were purchased from Wako Pure Chemicals (Tokyo, Japan). Adenosine deaminase, BSA and α,β-MeADP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ro20-1724 was purchased from Calbiochem (La Jolla, CA, U.S.A.). β,γ-MeATP was purchased from Nakalai Tesque Inc. (Kyoto, Japan). CGS21680, CGS15943, XAC, DPCPX, alloxazine, reactive blue-2 (RB-2), suramin, 2CADO and 2ClATP were obtained from Research Biochemicals International (Natick, MA, U.S.A.). ZM241385, PPADS and *i*PPADS were purchased from Tocris Cookson Ltd. (Bristol, U.K.). [2–^{3}H]-adenine and [8-^{14}C]-cyclic AMP were obtained from Amersham Japan and Moravek Biochemicals Inc. (Brea, CA, U.S.A.), respectively. Other chemicals and drugs used were of reagent grade or the highest quality available.

Data analyses

Concentration-response curves were fitted by DeltaGraph Pro 3 (version 3. 0. 4 for Macintosh, Delta Point). The pK_i values of receptor antagonists were calculated by Cheng & Prusoff equation using the EC_{50} values of each agonist and the IC_{50} values of each antagonist (Cheng & Prusoff, 1973). Statistical comparisons were performed using unpaired Student's *t*-test, and $P<0.05$ was taken to indicate significance.

Results

Concentration-dependency of cyclic AMP formation by β,γ-MeATP or CGS21680

We first determined the concentration-dependency of β,γ-MeATP-induced cyclic AMP formation (Figure 1). β,γ-MeATP increased intracellular cyclic AMP by about 7.5-fold above the basal level with an EC_{50} value of 8.0 ± 0.98 μM ($n=4$). CGS21680, a selective A_{2A} receptor agonist, also raised cyclic AMP level in a concentration-dependent manner. The maximal response of CGS21680 was about 1.5-fold larger than that of β,γ-MeATP. The EC_{50} value of CGS21680 was 42 ± 7.5 nM ($n=4$), indicating that CGS21680 was about 200-fold more potent compared with β,γ-MeATP. Based on these results, we fixed the concentrations of β,γ-MeATP and CGS21680 at 100 and 1 μM, respectively in the following experiments to evaluate the inhibitory effects of several receptor antagonists.

Effects of various P_1 receptor antagonists on β,γ-MeATP- or CGS21680-induced cyclic AMP formation

The P_1 receptor antagonists examined were ZM241385, CGS15943, XAC, DPCPX and alloxazine. As shown in Figure 2a, β,γ-MeATP-induced cyclic AMP formation was concentration-dependently inhibited by all the antagonists tested, with the following rank order of inhibitory potency; ZM241385 > CGS15943 ≫ XAC > DPCPX ≫ alloxazine. Alloxazine antagonized the β,γ-MeATP-induced response by 45% at 100 μM, a maximum concentration used. On the other hand, CGS21680-induced cyclic AMP formation was also inhibited by these antagonists with the same rank order of inhibitory potencies except for alloxazine which did not have any inhibitory effects at the concentrations up to 100 μM (Figure 2b). We calculated the pK_i values of these antagonists against β,γ-MeATP or CGS21680, using the EC_{50} values of β,γ-MeATP and CGS21680, and the IC_{50} values obtained with each antagonist (Table 1). Interestingly, all the five P_1 receptor antagonists inhibited the β,γ-MeATP-induced response more potently than the CGS21680-stimulated response.

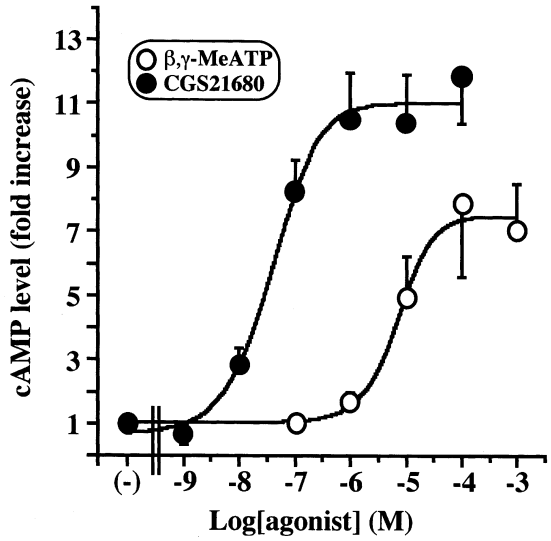

Figure 1 Concentration-response relationships of β,γ-MeATP and CGS21680. NG108-15 cells in 24-well culture dishes were preincubated with 1 U ml^{-1} adenosine deaminase for 10 min at 37°C. Cells were stimulated with increasing concentrations of β,γ-MeATP or CGS21680 in the presence of 100 μM Ro20-1724 for 10 min. [^{3}H]-cyclic AMP formed was measured as described in Methods. The experiments were undertaken in triplicate. The data are calculated as the fold increase above the basal cyclic AMP level, and are expressed as the mean ± s.e.mean from four independent experiments.

Table 1 The pK_i values of purinoceptor antagonists for β,γ-MeATP or CGS21680-induced response

	β,γ-MeATP	*CGS21680*
P_1 receptor antagonists		
ZM241385	10.03 ± 0.03	9.20 ± 0.05*
CGS15943	9.84 ± 0.01	8.97 ± 0.05*
XAC	8.05 ± 0.04	7.05 ± 0.04*
DPCPX	7.16 ± 0.04	partial inhibition
alloxazine	partial inhibition	NE
P2 receptor antagonists		
PPADS	5.52 ± 0.06	NE
*i*PPADS	4.94 ± 0.02	NE
suramin	partial inhibition	NE
RB-2	5.08 ± 0.03	partial inhibition

The pK_i values were calculated by Cheng & Prusoff (1973) equation from the EC_{50} values for β,γ-MeATP and CGS21680 and the IC_{50} values obtained from each inhibition curves shown in Figures 2 and 3. *$P < 0.05$ when compared with the pK_i values for β,γ-MeATP. NE; no effect.

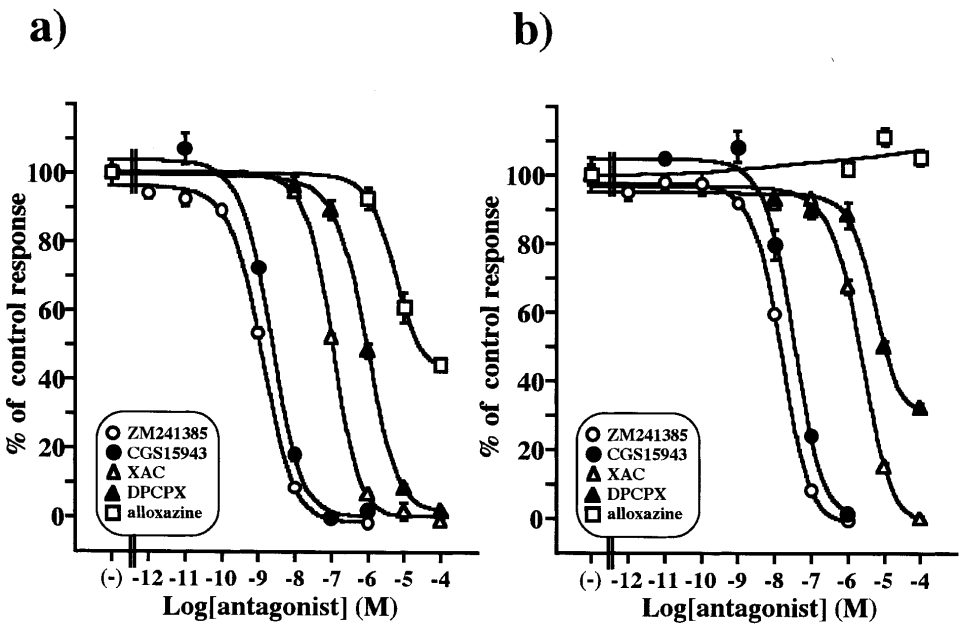

Figure 2 Effects of P_1 receptor antagonists on β,γ-MeATP- or CGS21680-induced cyclic AMP formation. Cells were stimulated with β,γ-MeATP (100 μM; a) or CGS21680 (1 μM; b) in the presence of increasing concentrations of ZM241385, CGS15943, XAC, DPCPX and alloxazine for 10 min. All experiments were performed in the presence of 1 U ml^{-1} adenosine deaminase and 100 μM Ro20-1724. Data are calculated as the percentage of the cyclic AMP production induced by each agonist in the absence of antagonists, and representative results are shown as the mean ± s.e.mean of triplicate determination. Similar results were obtained in two independent examinations.

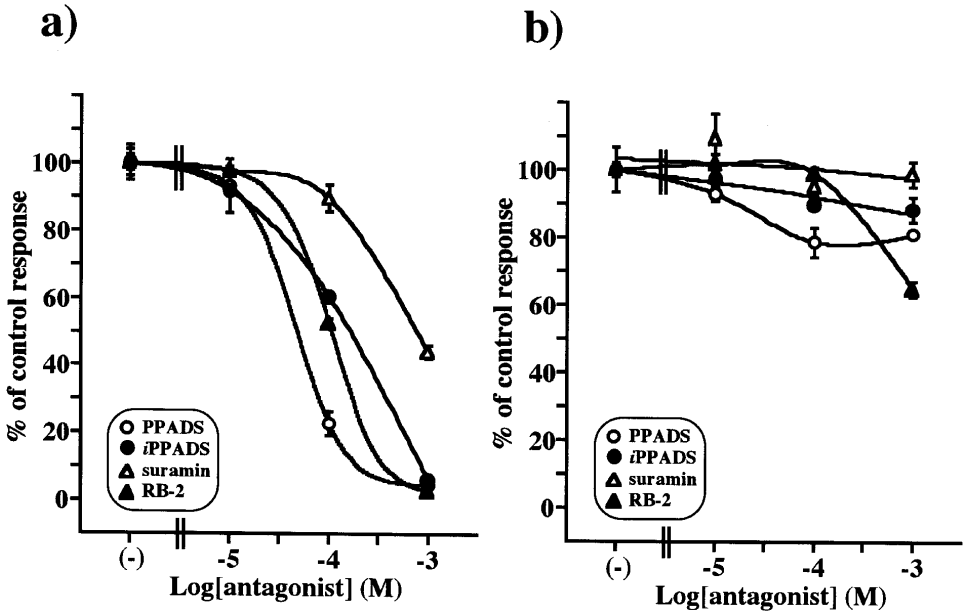

Figure 3 Effects of P2 receptor antagonists on β,γ-MeATP- or CGS21680-induced cyclic AMP formation. Cells were stimulated with β,γ-MeATP (100 μM; a) or CGS21680 (1 μM; b) in the presence of increasing concentrations of PPADS, *i*PPADS, suramin and RB-2 for 10 min. All experiments were performed in the presence of 1 U ml^{-1} adenosine deaminase and 100 μM Ro20-1724. Data are calculated as the percentage of the cyclic AMP production induced by each agonist in the absence of antagonists, and representative results are shown as the mean $\pm$ s.e.mean of triplicate determination. Similar results were obtained in two independent examinations.

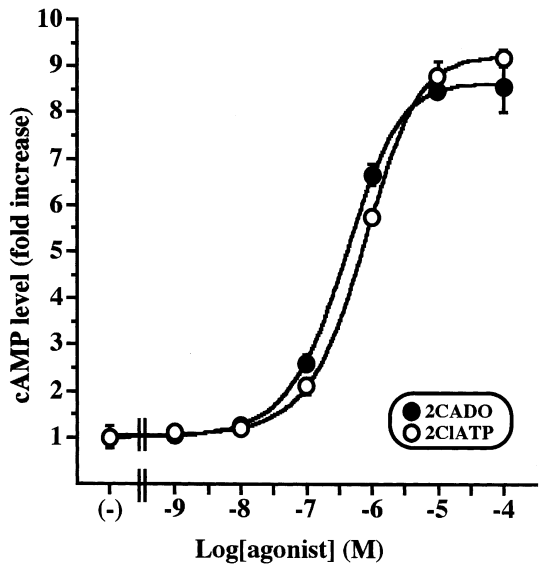

Figure 4 Concentration-response relationships of 2CADO and 2ClATP. Cells were stimulated with increasing concentrations of 2CADO or 2ClATP for 10 min. The experiments were undertaken in triplicate. Representative results are shown as the mean $\pm$ s.e.mean of triplicate determination. Similar results were obtained in three independent examinations.

Effects of P2 receptor antagonists on β,γ-MeATP- and CGS21680-induced responses

Next, we studied the effects of P2 receptor antagonists on β,γ-MeATP- and CGS21680-induced responses. β,γ-MeATP-induced cyclic AMP formation was inhibited by the P2 receptor antagonists, PPADS, *i*PPADS and RB-2 (Figure 3a and Table 1). Suramin was effective only at 1 mM. In contrast, these P2 receptor antagonists little affected CGS21680-induced response, except that RB-2 at 1 mM partially decreased cyclic AMP formation by 65% (Figure 3b). These results implicate that β,γ-MeATP- and CGS21680-stimulated cyclic AMP formations are caused by different mechanisms.

Effects of ZM241385 and PPADS on 2CADO- or 2ClATP-induced cyclic AMP formation

In the process of screening the effects of various purine compounds on cyclic AMP formation, we found that 2CADO and 2ClATP were equipotent for cyclic AMP production. Figure 4 shows the concentration-response curves of 2CADO- and 2ClATP-induced cyclic AMP elevation. Both agonists stimulated cyclic AMP formation with similar EC_{50} values (0.55 ± 0.08 μM for 2CADO, $n=4$; 0.60 ± 0.14 μM for 2ClATP, $n=3$). The maximal responses caused by two compounds were also similar (Figure 4). We examined the effects of ZM241385 and PPADS on the 2CADO- or 2ClATP-induced cyclic AMP formation. ZM241385 inhibited both agonists-induced increases in cyclic AMP in concentration-dependent manners, but it tended to antagonize 2ClATP more potently than 2CADO (Figure 5a). On the other hand, PPADS antagonized 2ClATP in a concentration-dependent manner, while it did not inhibit the effects of 2CADO (Figure 5b).

Effect of the 5′-nucleotidase inhibitor on nucleotides-induced cyclic AMP formation

We examined the effect of α,β-MeADP, a 5′-nucleotidase inhibitor (Bruns, 1980), on β,γ-MeATP- or ATP-induced cyclic AMP formation. β,γ-MeATP and ATP were used at 10 μM, near half-maximal concentrations of these agonists to induce cyclic AMP formation. These nucleotides-induced cyclic AMP formations were still observed even in the presence of 250 μM α,β-MeADP (Figure 6). α,β-MeADP did not affect prostaglandin E_1 (PGE_1)-induced cyclic AMP formation.

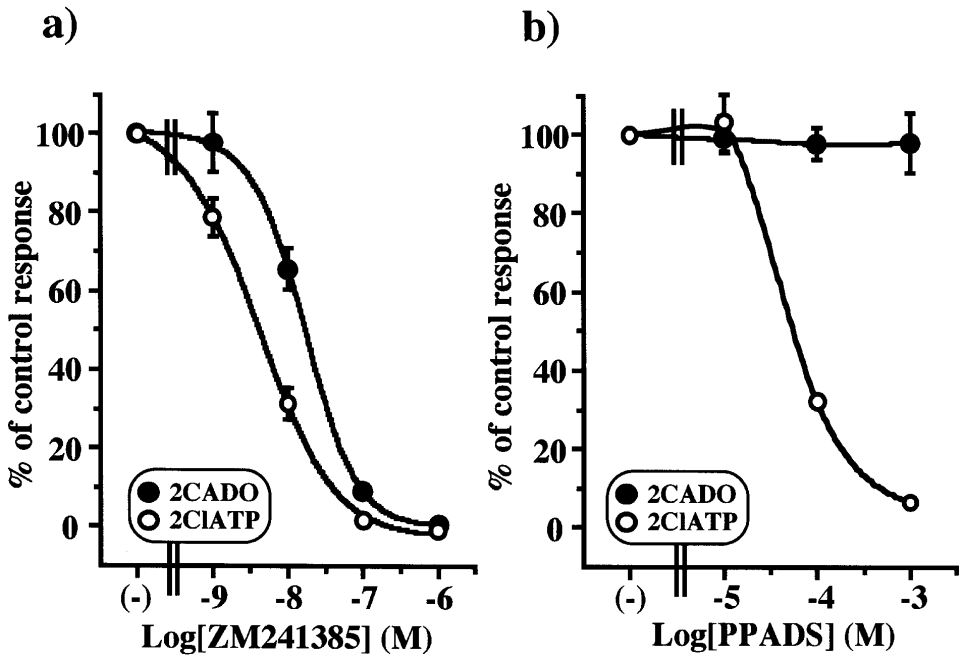

Figure 5 Effects of ZM241385 and PPADS on 2CADO- or 2ClATP-induced cyclic AMP formation. Cells were stimulated with 2CADO (10 μM) or 2ClATP (10 μM) in the presence of increasing concentrations of ZM241385 (a) or PPADS (b) for 10 min. The experiments were undertaken in triplicate. Data are calculated as the percentage of cyclic AMP production by each agonist in the absence of ZM241385 or PPADS, and are shown as the mean$\pm$s.e.mean of triplicate determinations. Similar results were obtained in three independent examinations.

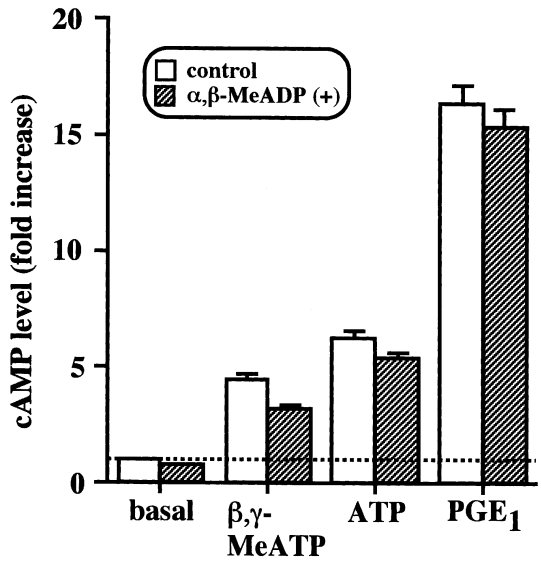

Figure 6 Effects of α,β-MeADP on β,γ-MeATP- and ATP-induced cyclic AMP formation. Cells were preincubated with 250 μM α,β-MeADP or vehicle for 10 min, and were stimulated with β,γ-MeATP (10 μM), ATP (10 μM) and prostaglandin E_1 (PGE_1, 0.03 μM) for 10 min. The data are calculated as the fold increase above basal, and representative results are shown as the mean$\pm$s.e.mean of triplicates from at least three independent examinations.

Discussion

Extracellular ATP induces a marked intracellular cyclic AMP accumulation in NG108-15 cells. According to the several lines of evidence obtained in our previous studies (Matsuoka *et al*., 1995; Ohkubo *et al*., 1998), we hypothesized that the ATP-induced cyclic AMP accumulation was mediated by a novel receptor linked to AC. In NG108-15 cells, a previously known P2 receptor agonists, α,β-MeATP, 2MeSATP, 2MeSADP, UTP and UDP are all ineffective, whereas ATP, ADP, AMP, adenosine 5′-O-(3-thiotriphosphate) (ATPγS) and adenosine 5′-O-(2-thiodiphosphate) (ADPβS) stimulate cyclic AMP formation. Interestingly, β,γ-MeATP, a previously known as a P2X-type agonist, rises in cyclic AMP level without affecting $[Ca^{2+}]_i$, suggesting that this compound is a selective agonist for cyclic AMP production in NG108-15 cells. These agonist selectivities for cyclic AMP formation are quite different from any previously described P2 receptors. Moreover, ATP-induced effects were inhibited by methylxanthines, well known P_1 receptor antagonists. Since NG108-15 cells possess the functional A_{2A} adenosine receptors, we attempted to separate the ATP-mediated response from the A_{2A} receptor-stimulated one. For this purpose, we compared the effects of several receptor antagonists on β,γ-MeATP- and CGS21680-induced cyclic AMP production in NG108-15 cells. β,γ-MeATP is a selective agonist for cyclic AMP formation as described above, and CGS21680 is known to be a highly selective agonist for the A_{2A} receptor (Jarvis *et al*., 1989). Results obtained in the present study revealed a clear difference between the responses induced by these two agonists.

β,γ-MeATP-induced cyclic AMP accumulation was inhibited by four different P_1 receptor antagonists in a concentration-dependent manner. The rank order of inhibitory potency of the P_1 receptor antagonists was ZM241385 > CGS15943 > XAC > DPCPX. CGS21680-induced cyclic AMP accumulation in NG108-15 cells was also inhibited by these antagonists with the similar rank order of potency, but each antagonist required higher concentrations for inhibiting the CGS21680-induced response. The pK_i values of these four antagonists calculated from the EC_{50} value of each agonist and IC_{50} values of each antagonist, were higher in the response to β,γ-MeATP than to CGS21680. Interestingly,

Hourani *et al*. (1991) reported that 8SPT, a P_1 receptor antagonist, inhibited the β,γ-MeATP-mediated relaxant effect more potently than the adenosine-mediated response in rat duodenum.

More prominent differences were obtained with P2 receptor antagonists. PPADS, *i*PPADS, RB-2 and suramin concentration-dependently inhibited the cyclic AMP accumulation induced by β,γ-MeATP, while these antagonists had no or only weak inhibitory effect on CGS21680-induced cyclic AMP accumulation. These results suggest that P2 receptor antagonists would have little inhibitory effects on the A_{2A} receptor directly, and that they would be able to discriminate the cyclic AMP responses induced by adenine nucleotides from the A_{2A} receptor-mediated one. It has been suggested that β,γ-MeATP directly stimulates adenosine receptor in rat duodenum (Hourani *et al*., 1991) or guinea-pig trachealis muscles (Piper & Hollingsworth, 1996). However, there has been no evidence to date in the literature indicating the ability of β,γ-MeATP to stimulate any P_1 receptor subtypes. To solve this discrepancy, further experiments using cells transfected with cDNA encoding each P_1 receptor subtype are required. Our present results demonstrate that β,γ-MeATP does not directly interact with A_{2A} receptors in NG108-15 cells.

Alloxazine, a P_1 receptor antagonist, also selectively inhibited β,γ-MeATP-induced cyclic AMP accumulation without affecting CGS21680-induced response. Alloxazine is an A_2 receptor antagonist, which is approximately 10-fold more selective for the A_{2B} receptor than for the A_{2A} receptor (Brackett & Daly, 1994). These results gave rise to a possibility that β,γ-MeATP cross-reacts with the A_{2B} receptor subtype to induce cyclic AMP accumulation. However, the rank order potency of several P_1 receptor antagonists did not fit to the A_{2B} receptor characteristics (Dionisotti, *et al*., 1997; Poucher *et al*., 1995). Furthermore, RT–PCR analysis of mRNA in NG108-15 cells revealed that this cell line little expressed the A_{2B} receptor transcript, whereas it had abundant mRNA for the A_{2A} receptor (unpublished data). Therefore, alloxazine would have another binding site beside the A_{2B} receptor in NG108-15 cells. The results of alloxazine also support the existence of a novel P2 receptor which recognizes β,γ-MeATP as an agonist.

The difference in the antagonist selectivity found in the responses to β,γ-MeATP and CGS21680 was also obtained with two structure-related compounds, 2CADO and 2ClATP. Since these compounds induced cyclic AMP accumulation with similar potency, the antagonist effects could be evaluated on the response induced by the same concentrations of agonists. Although ZM241385 inhibited cyclic AMP formation induced by these compounds, 2ClATP-induced response showed higher sensitivity to ZM241385 inhibition. In addition, P2 receptor antagonist PPADS blocked only 2ClATP-induced effect without affecting 2CADO-induced response. These results indicate that β,γ-MeATP and 2ClATP stimulate the same receptor population which are not activated by CGS21680 or 2CADO.

P_1 receptor antagonist-sensitive ATP responses have been described in several organs including submucosal neurons (Barajas-López *et al*., 1995), rabbit brain cortex slices (von Kügelgen *et al*., 1992) and guinea-pig trachealis muscles (Piper & Hollingsworth, 1996). Interestingly, β,γ-MeATP was found to be an agonist in various systems. Therefore, some of such ATP response may be mediated by similar receptor described in NG108-15 cells. For a P_1 receptor antagonist-sensitive ATP receptor, Shinozuka *et al*. (1988) proposed an existence of third class of purinoceptor, the P_3 receptor, in the presynaptic terminal of the sympathetic nerves. The P_3 receptor was characterized to be activated by both adenosine and ATP in a manner sensitive to methylxanthine inhibition. According to this category, the ATP receptor found in NG108-15 cells cannot be classified into the P_3 receptors, since cyclic AMP accumulation by adenosine analogues such as CGS21680 and 2CADO were hardly inhibited by PPADS. It is interesting to know the effects of various P2 receptor antagonists on the P_1 receptor antagonist-sensitive ATP responses in other organs.

Our present results show that the cyclic AMP response induced by ATP and its analogues can be separated from those mediated by the A_{2A} receptor using P2 receptor antagonists. However, P2 receptor antagonists including PPADS, RB-2 and suramin have been shown to inhibit ecto-nucleotidases in several different cells (Meghji & Burnstock, 1995; Ziganshin *et al*., 1996). Therefore, we have to take into account that P2 receptor antagonists may act as the inhibitor of the extracellular adenine nucleotide metabolism rather than receptor antagonists. Cunha *et al*. (1998) have recently indicated that extracellular ATP and adenine nucleotides are locally hydrolyzed to adenosine by ecto-nucleotidases and induce responses by activation of P_1 adenosine receptors. Recently, it has been reported that $gp130^{RB13\text{-}6}$, a member of ecto-phosphodiesterases/nucleotide pyrophosphatase family, is expressed in glioma cell lines (Deissler *et al*., 1999). PC-1, another member of the family, is also shown to be expressed and to degrade β,γ-MeATP into AMP in C6 glioma cells (Grobben *et al*., 1999). However, the conclusion that effects of ATP is mediated by adenosine is largely based on the lack of the effects of ATP either upon blockade of extracellular metabolism by the 5′-nucleotidase inhibitor α,β-MeADP, or upon removal of extracellular adenosine by adenosine deaminase (Cunha *et al*., 1998; Dunwiddie *et al*., 1997). In the present study, we showed that the cyclic AMP accumulation induced by ATP and β,γ-MeATP were resistant to both α,β-MeADP and adenosine deaminase. Furthermore, suramin was less effective than PPADS and RB-2 in the inhibition of β,γ-MeATP-induced cyclic AMP formation, whereas this antagonist was shown to inhibit ecto-nucleotidases just like PPADS or RB-2 (Ziganshin *et al*., 1996). It can be therefore concluded that cyclic AMP response induced by ATP and β,γ-MeATP are not mediated by ecto-nucleotidases.

In summary, ATP-stimulated cyclic AMP formation can be distinguished from the A_{2A} receptor agonist-induced response by using P2 receptor antagonists. From the present results, however, we cannot totally exclude the possible involvement of the A_{2A} receptor in β,γ-MeATP-induced cyclic AMP formation, because the rank order of inhibitory potencies of P_1 receptor antagonists are similar in spite of the differences of the pK_i values. It would be important to demonstrate an antagonism only on the A_{2A} receptor-mediated response without affecting the ATP-induced one. Further experiment is needed for understanding the mechanism of ATP-induced cyclic AMP accumulation in NG108-15 cells.

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