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Effects of AMP derivatives on cyclic AMP levels in NG108-15 cells

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1 In NG108-15 neuroblastoma × glioma hybrid cells, ATP stimulates intracellular cyclic AMP formation, which is inhibited by both adenosine (P_1) and P2 receptor antagonists. In the present study, we examined the effects of several AMP derivatives in NG108-15 cells and mouse neuroblastoma N18TG-2 cells.

2 Adenosine 2′-monophosphate (A2P), adenosine 3′-monophosphate (A3P) and adenosine 5′-phosphosulphate (A5PS) increased cyclic AMP levels with similar concentration-dependencies in NG108-15 cells.

3 Increases in cyclic AMP by AMP derivatives were inhibited by the P2 receptor antagonist PPADS, but not by suramin. Effects of AMP derivatives were also inhibited by P_1 receptor antagonists ZM241385, XAC, DPCPX and partially by alloxazine. The ecto-nucleotidase inhibitor α,β-methyleneADP was without effect.

4 In contrast, AMP derivatives did not change cyclic AMP levels in N18TG-2 cells. Accumulation of cyclic AMP in N18TG-2 cells was stimulated by adenosine A_2 receptor agonists CGS21680 and NECA, but not by ATP or β,γ-methyleneATP, agonists for cyclic AMP production in NG108-15 cells.

5 Reverse transcription-coupled polymerase chain reaction (RT–PCR) analyses revealed that N18TG-2 cells express both A_{2A} and A_{2B} receptors, while NG108-15 cells express mainly A_{2A} receptors.

6 AMP derivatives did not affect the P2X and P2Y receptors expressed in NG108-15 cells.

7 These results suggest that A2P, A3P and A5PS act as agonists for cyclic AMP production and that these compounds are valuable tools for determinating the mechanism of ATP-stimulated cyclic AMP response in NG108-15 cells.
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Keywords: Adenylyl cyclase; P2 receptor; cyclic AMP; adenosine monophosphate; NG108-15 cells

Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); alloxazine, benzo[g]pteridine-2, 4 (1H, 3H)-dione; A2P, adenosine 2′-monophospate; A3P, adenosine 3′-monophosphate; A5PS, adenosine 5′-phosphosulphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BzATP, benzoylbenzoylATP; CGS21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine; DMEM, Dulbecco's modified Eagle's medium; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; KRH, Krebs-Ringer-HEPES; α,β-MeADP, α,β-methyleneADP; β,γ-MeATP, β,γ-methyleneATP; 2MeSATP, 2-methylthioATP; NECA, 5′-(N-ethylcarboxamido)adenosine; PPADS, pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic 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; 8SPT, 8-(sulphophenyl)theophylline; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; 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/pyrimidine compounds mediate a broad range of physiological and pharmacological responses through purinoceptors (Ralevic & Burnstock, 1998). Purinoceptors are classified into nucleoside (P_1) receptors and nucleotide (P2) receptors. P_1 receptors have been divided into A_1, A_{2A}, A_{2B} and A_3 receptor subclasses, which mainly regulate adenylyl cyclase (AC) activities. On the other hand, P2 receptors, which bind adenine or uridine nucleotides preferentially, have been classified into P2X ($P2X_{1-7}$) and P2Y ($P2Y_{1,2,4,6,11}$) subfamilies. While the P2X receptors form non-selective cation channels, the P2Y receptors couple with $G_{q/11}$ followed by phosphoinositide hydrolysis. The $P2Y_{11}$ receptor is also linked to AC activation.

There are several reports which showed that the profiles of some ATP- or its analogue-mediated pharmacological responses are different from the characteristics of any known P2 receptors. For instance, in the rat caudal artery, Shinozuka *et al*. (1988) reported that β,γ-methyleneATP (β,γ-MeATP), a hydrolysis-resistant ATP analogue, and 2-chloroadenosine inhibited norepinephrine release from sympathetic nerve terminals, but both responses were inhibited by P_1 receptor antagonist 8-(*p*-sulphophenyl)theophylline (8SPT). Because the agonist/antagonist selectivities corresponded to neither P_1 nor P2 receptors, the above investigators have postulated a novel purinoceptor termed as the P_3 receptor which recognizes both ATP and adenosine as its ligands. Similarly, P_1 receptor antagonist-sensitive responses induced by adenine nucleotides have been reported from various tissues or cells, including *Xenopus Oocytes* (King *et al*., 1996), guinea-pig trachealis smooth muscle (Piper & Hollingsworth, 1996) and sympathetic nerves in the rat vas deferens (Forsyth *et al*., 1991).

We have found that ATP and its analogues raised intracellular cyclic AMP levels in NG108-15 cells (Matsuoka *et al*., 1995; Ohkubo *et al*., 1998a,b). ATP stimulates cyclic AMP formation *via* the activation of G_s, its response being

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inhibited both by the P2 receptor antagonist PPADS and P_1 receptor antagonists such as 8SPT and ZM241385. Agonist selectivities of cyclic AMP formation are also quite different from any other cloned P2 receptors. Adenine nucleotides and β,γ-MeATP act as agonists for cyclic AMP production, but not α,β-MeATP, 2-methylthioATP (2MeSATP) and uridine 5′-triphosphate (UTP). Moreover, β,γ-MeATP, which is shown to be a P2X-type receptor agonist, stimulates cyclic AMP formation without affecting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). From these ligand selectivities, we hypothesized that ATP-stimulated cyclic AMP formation might be caused by different purinoceptor from the previously known P2X- or P2Y-type.

To determine the characteristics of ATP-induced cyclic AMP formation in more detail, we tried to find selective ligands for cyclic AMP formation without affecting other purinoceptor subtypes. In this report, we demonstrate that three AMP derivatives, adenosine 2′-monophosphate (A2P), adenosine 3′-monophosphate (A3P) and adenosine 5′-phosphosulphate (A5PS), stimulate cyclic AMP production, and that their responses are sensitive to both P_1 and P2 receptor antagonists.

Methods

Cell culture

NG108-15 neuroblastoma × glioma hybrid cells and N18TG-2 mouse neuroblastoma cells were generous gifts from Dr Haruhiro Higashida (Kanazawa University, Kanazawa, Japan). NG108-15 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. In the case of N18TG-2 cells, hypoxanthine, aminopterin and thymidine were omitted from the growth medium. For the measurement of cyclic AMP levels, cells were seeded in a 24-well culture dish at a density of 1.6×10^4 cells per well and cultured for 3 days before the experiments.

Measurement of cyclic AMP levels

Changes in intracellular cyclic AMP levels were measured as described by Salomon (1991) with minor modifications. In brief, cells were labelled with [^{3}H]-adenine (1 μCi ml^{-1} for NG108-15 cells, 3 μCi ml^{-1} for N18TG-2 cells) in growth medium for 3–5 h. Labelled cells were washed twice with Krebs-Ringer-HEPES (KRH) buffer (in mM): NaCl 130, KCl 4.7, $NaHCO_3$ 4.0, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.8, glucose 11.5, BSA 0.1% and HEPES 10, pH 7.4. Cells were preincubated with 1 U ml^{-1} adenosine deaminase in KRH buffer for 10 min at 37°C to eliminate the effects of endogenous adenosine, and were stimulated with various agonists in the presence of the phosphodiesterase inhibitor Ro20-1724 (100 μM) for 10 min. 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). The acid-extracts were mixed with one-tenth volume of 4.2 N KOH to neutralize and deposit potassium perchlorate. The [^{3}H]-cyclic AMP in the clear supernatant were separated by Dowex 50W-X8/alumina double columns, and determined by liquid scintillation counting. The recoveries of cyclic AMP from each column were monitored by [^{14}C]-cyclic AMP. [^{3}H]-cyclic AMP levels were expressed as the percentage of total [^{3}H]-adenine uptake.

Reverse transcription-coupled polymerase chain reaction (RT–PCR)

Total RNA was extracted from rat and mouse brain, NG108-15 and N18TG-2 cells by the acid-guanidine thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). Poly $(A)^+$ RNA was then selected from each total RNA preparation by oligo(dT)-Latex (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). A first strand cDNA primed by random hexamers was prepared from poly $(A)^+$ RNA (1 μg) using Moloney murine leukemia virus reverse transcriptase in the presence of RNAsin ribonuclease inhibitor, and used as a template for the PCR analysis. Three pairs of sense and antisense primers specific for mouse A_{2A}, A_{2B} and rat A_{2B} receptors were designed based on the published cDNA sequences. The nucleotide sequence of each primer and the location in cDNA are: mouse A_{2A} (Marquardt *et al*., 1994); 5′-ACT GCT GGG TGG AAC AAC TGC A-3′ (sense, 648-669) and 5′-CTC AGA CGT GGG TTC GGA TGA T-3′ (antisense, 1147-1126), mouse A_{2B} (Marquardt *et al*., 1994); 5′-GGG TGG AAC AGT AAA GAC AG-3′ (sense, 459-479) and 5′-GGA TGG AAG AGG GTG ATA CA-3′ (antisense, 815-795) and rat A_{2B} (Stehle *et al*., 1992); 5′-CTG CTG CCC TGT GAA GTG TCT C-3′ (sense, 602-623) and 5′-GGG CCA CAT GCT TGA GAG GGT A-3′ (antisense, 1211-1190). The predicted length of PCR products for mouse A_{2A}, A_{2B} and rat A_{2B} are 500, 357 and 610 bp, respectively. PCR was carried out in a 10 μl solution containing (mM): Tris-HCl (pH 9.0) 10, KCl 50, $MgCl_2$ 1.5, 125 μM of each deoxynucleotide triphosphate, 0.5 μM primer mix, 25 U ml^{-1} Taq DNA polymerase. The PCR conditions were 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 72°C, and 1 cycle at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR products were confirmed by analysing the restriction sites or by direct sequencing using an automated DNA sequencer.

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

Increases in $[Ca^{2+}]_i$ were examined by monitoring the intensity of Fura-2 fluorescence as described previously (Ohkubo *et al*., 1998a). Cells were loaded with 1 μM Fura-2/acetoxymethyl ester (Fura-2/AM) for 15 min at 37°C. Cells were washed twice, and finally suspended at $0.5-1 \times 10^6$ cells ml^{-1}. Increases in $[Ca^{2+}]_i$ were measured in 1.5 ml of the cell suspension in the quartz cell with constant stirring at 37°C using a fluorescence spectrophotometer (F-2000, Hitachi Ltd, Tokyo, Japan). Fura-2 fluorescence at 510 nm was monitored with excitations at 340 and 380 nm. $[Ca^{2+}]_i$ was analysed by calculating the ratio of fluorescence intensity (I_{340}/I_{380}) by the method of Grynkiewicz *et al*. (1985).

Materials

High-glucose DMEM and Moloney murine leukemia virus reverse transcriptase were 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). Taq DNA polymerase and RNAsin ribonuclease inhibitor were obtained from Pharmacia Biotech (Tokyo, Japan) and Promega Co. (Madison, WI, U.S.A.), respectively. Adenosine deaminase, BSA, A2P, A3P, A5PS, ATP, ADP, AMP, UTP, UDP, BzATP, NECA and α,β-MeADP were obtained from Sigma Chemical Co. (St. Louis,

MO, U.S.A.). Ro20-1724 and Fura-2/AM were purchased from Calbiochem (La Jolla, CA, U.S.A.) and Dojindo Laboratories (Kumamoto, Japan), respectively. β,γ-MeATP was purchased from Nakalai Tesque Inc. (Kyoto, Japan). Suramin, XAC, DPCPX, alloxazine and CGS21680 were obtained from Research Biochemicals International (Natick, MA, U.S.A.). ZM241385 and 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. All other chemicals 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).

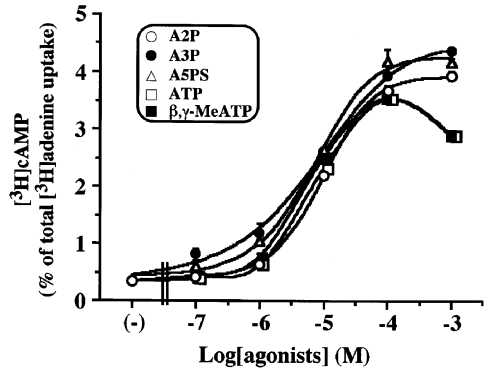

Figure 1 Concentration-dependencies of cyclic AMP formation induced by A2P, A3P or A5PS. [^{3}H]-adenine-prelabeled NG108-15 cells were stimulated with increasing concentrations of A2P, A3P, A5PS, ATP or β,γ-MeATP in the presence of 1 U ml^{-1} adenosine deaminase and 100 μM Ro20-1724 for 10 min. Produced [^{3}H]-cyclic AMP was separated by Dowex-alumina double column as described in Methods. [^{3}H]-cyclic AMP was calculated as the percentage of total [^{3}H]-adenine uptake. Representative data are shown from three independent results.

Results

AMP derivatives stimulate cyclic AMP formation in NG108-15 cells

We studied the effects of AMP derivatives on cyclic AMP levels in NG108-15 cells. A2P, A3P and A5PS as well as previously shown agonists for cyclic AMP production in NG108-15 cells such as ATP and β,γ-MeATP (Matsuoka *et al*., 1995) raised cyclic AMP levels with similar concentration-dependencies (Figure 1). The 50% effective concentrations (EC_{50}) were 5.7 ± 1.2, 6.5 ± 1.3 and 3.4 ± 0.9 μM ($n=3$) for A2P, A3P and A5PS, respectively. The EC_{50} values for ATP and β,γ-MeATP were similar to those for AMP derivatives, 5.3 μM ($n=2$) and 8.0 ± 1.0 μM ($n=4$), respectively. Maximal responses induced by three AMP derivatives were also similar to each other. On the other hand, maximal responses induced by ATP or β,γ-MeATP tended to be lesser.

Effects of P_1 and P2 receptor antagonists on cyclic AMP formation induced by AMP derivatives

We previously reported that the cyclic AMP formation stimulated by β,γ-MeATP was inhibited by both P_1 and P2 receptor antagonists in NG108-15 cells (Ohkubo *et al*., 1998b). To examine whether AMP derivatives also stimulate cyclic AMP formation in similar manner to β,γ-MeATP, we investigated the effects of P_1 and P2 receptor antagonists on the responses induced by A2P, A3P and A5PS. Figure 2 shows the effects of P2 receptor antagonists, PPADS and suramin, on cyclic AMP formation induced by the AMP derivatives. PPADS concentration-dependently inhibited the cyclic AMP formation induced by AMP derivatives (100 μM), with the 50% inhibitory concentrations (IC_{50}) of 280, 190 and 240 μM for A2P, A3P and A5PS, respectively. On the other hand, suramin was without effect over the concentration range up to 1 mM. Several P_1 receptor antagonists also inhibited cyclic AMP formation induced by AMP derivatives in a similar rank order of potencies (Figure 3). The rank order of the inhibitory potency was ZM241385 > XAC > DPCPX > alloxazine. The

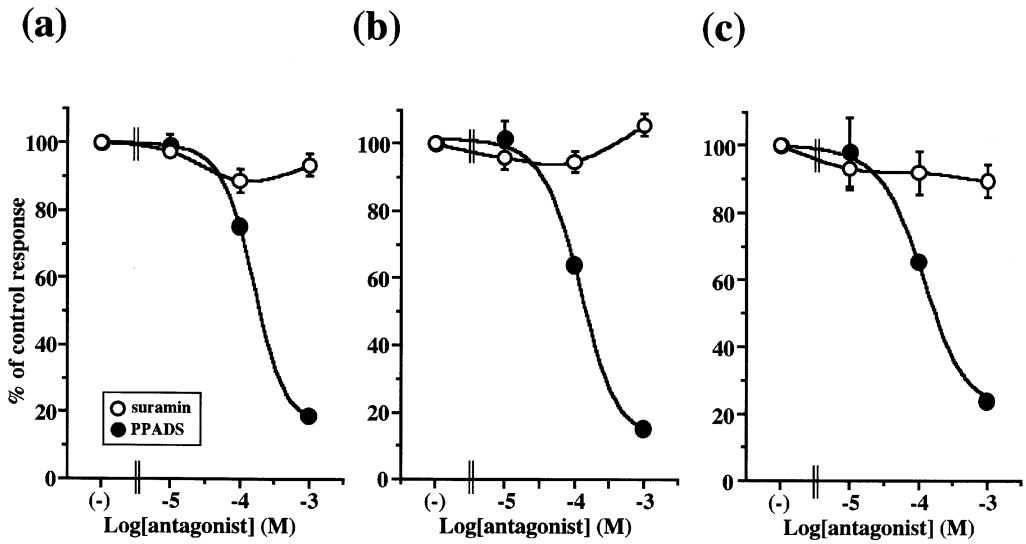

Figure 2 Effects of P2 receptor antagonists on cyclic AMP formation induced by AMP derivatives. Cells were stimulated with A2P (a; 100 μM), A3P (b; 100 μM) or A5PS (c; 100 μM) for 10 min in the presence of increasing concentration of PPADS or suramin. Each antagonist was added simultaneously with AMP derivatives. Data are calculated as the percentage of each agonist-induced control responses without antagonists, and expressed as the mean $\pm$ standard errors of triplicates. Representative results are shown from three independent results.

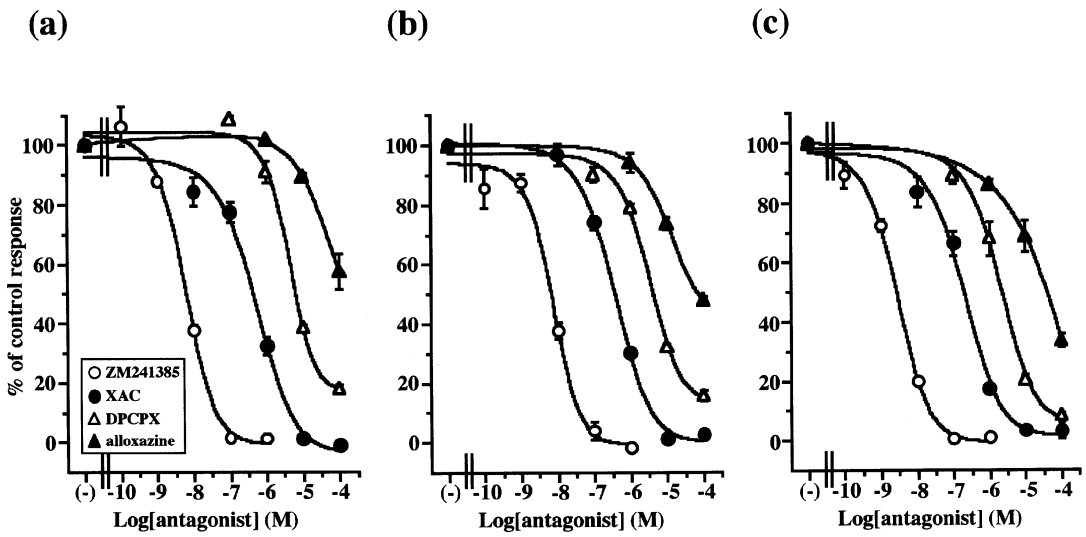

Figure 3 Effect of P_1 receptor antagonists on cyclic AMP formation induced by AMP derivatives. Cells were stimulated with A2P (a; 100 μM), A3P (b; 100 μM) or A5PS (c; 100 μM) for 10 min in the presence of increasing concentration of ZM241385, XAC, DPCPX and alloxazine. Each antagonist was added simultaneously with AMP derivatives. Data are calculated as the percentage of each agonist-induced control responses without antagonists, and expressed as the mean$\pm$standard errors of triplicates. Representative results are shown from three independent results.

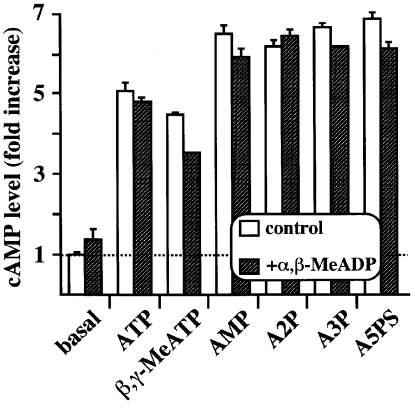

Figure 4 Effect of α,β-MeADP on various agonists-induced cyclic AMP formation. [^{3}H]-adenine-labelled NG108-15 cells were pre-incubated with 250 μM α,β-MeADP or vehicle for 10 min, and stimulated with ATP, β,γ-MeATP, AMP, A2P, A3P and A5PS (each 10 μM) for 10 min in the presence of 1 U ml^{-1} adenosine deaminase and 100 μM Ro20-1724. Data are calculated as the fold increase above basal cyclic AMP level, and expressed as the mean$\pm$standard error of triplicate examination. Representative results are shown from two independent experiments.

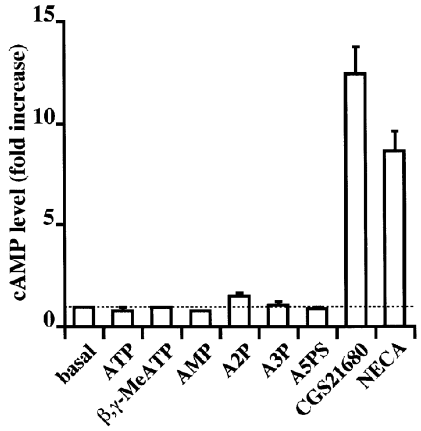

Figure 5 Effects of adenine nucleotides, AMP derivatives and P_1 receptor agonists on cyclic AMP level in mouse neuroblastoma cell line N18TG-2 cells. N18TG-2 cells were labelled with 3 μCi ml^{-1} [^{3}H]-adenine for 3–5 h. Cells were stimulated with ATP, β,γ-MeATP, AMP, A2P, A3P, A5PS (each 100 μM), CGS21680 (1 μM) or NECA (1 μM) for 10 min in the presence of 1 U ml^{-1} adenosine deaminase and 100 μM Ro20-1724. Data are calculated as the fold increase above basal cyclic AMP level, and expressed as the mean$\pm$standard error of triplicate examination. Representative results are shown from at least three independent experiments.

IC_{50} values for A2P, A3P and A5PS (calculated from Figure 3) were 5.8, 5.8, 2.7 nM (ZM241385); 410, 360, 210 nM (XAC); 6.1, 4.2, 2.4 μM (DPCPX), greater than >100, 85 and 34 μM (alloxazine) for A2P, A3P and A5PS, respectively.

Effect of ecto-nucleotidase inhibitor on cyclic AMP formation induced by AMP derivatives

NG108-15 cells possess functional adenosine A_{2A} receptors (Ohkubo *et al*., 1998b). Extracellular adenine nucleotides are known to be hydrolyzed by ecto-nucleotidases, resulting in the accumulation of adenosine. To examine the contribution of adenosine towards cyclic AMP formation, we examined the effect of α,β-methyleneADP (α,β-MeADP) on cyclic AMP formation induced by AMP derivatives. α,β-MeADP is known to be a potent inhibitor of ecto-nucleotidase (Bruns, 1980). All agonists were used at 10 μM, which was the concentration near the EC_{50} values (Figure 1). As shown in Figure 4, the pretreatment with 250 μM α,β-MeADP for 10 min did not change the basal cyclic AMP level. A2P, A3P and A5PS-induced cyclic AMP formation were not affected by α,β-MeADP treatment. Similarly, α,β-MeADP had little effect on the cyclic AMP formation induced by ATP, β,γ-MeATP and AMP.

Effects of AMP derivatives on cyclic AMP formation in N18TG-2 cells

In the preliminary experiments, we found that N18TG-2 cells, a parent cell line of NG108-15 cells, possessed a functional A_{2A} receptor, but not the ability to produce cyclic AMP by adenine nucleotides. To determine whether AMP derivatives directly

react with the A_{2A} receptor, we investigated the effects of A2P, A3P and A5PS on cyclic AMP formation in N18TG-2 cells (Figure 5). An A_{2A} receptor-selective agonist CGS21680 and a non-selective P_1 receptor agonist NECA raised cyclic AMP levels in N18TG-2 cells. However, A2P, A3P and A5PS as well as ATP, β,γ-MeATP and AMP did not affect cyclic AMP levels at concentrations up to 100 μM in N18TG-2 cells. These results suggest that AMP derivatives do not have agonistic actions at the A_{2A} receptor.

Adenosine A_2 receptor subtypes expressed in NG108-15 and N18TG-2 cells

Adenosine A_2 receptor subtypes expressed in NG108-15 and N18TG-2 cells were examined by RT–PCR (Figure 6). The primers recognizing mouse A_{2A} receptor generated PCR products (500 bp) in the mouse brain, NG108-15 and N18TG-2 cells. In contrast, PCR products (357 bp) derived from the mouse A_{2B} receptor was detected in mouse brain and N18TG-2 cells, but not in NG108-15 cells. Since NG108-15 cells are mouse neuroblastoma × rat glioma hybrids, the existence of rat A_{2B} receptor mRNAs was also examined. The rat A_{2B} receptor-derived PCR product (610 bp) was detected in rat brain, but not in NG108-15 cells. These results indicate that NG108-15 cells express mainly the A_{2A} receptor, while both A_{2A} and A_{2B} receptor exist in N18TG-2 cells.

Effects of A2P, A3P and A5PS on $[Ca^{2+}]_i$ in NG108-15 cells

NG108-15 cells also possess several other P2 receptor subtypes, such as $P2Y_2$, and $P2X_7$, each of which can increase $[Ca^{2+}]_i$ (Kaiho *et al*., 1996; Lustig *et al*., 1993). Therefore, the effects of AMP derivatives on $[Ca^{2+}]_i$ were examined in Fura-2-loaded NG108-15 cells. A3P alone did not change the basal $[Ca^{2+}]_i$ (Figure 7). Furthermore, A3P did not affect the

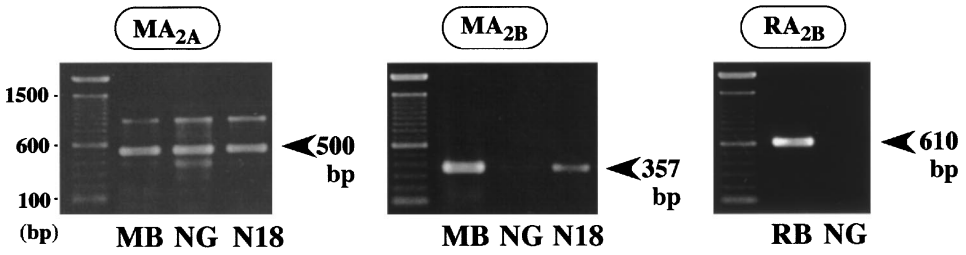

Figure 6 Expression of A_2 receptor subtypes in NG108-15 and N18TG-2 cells by RT–PCR analyses. Poly $(A)^+$ RNA from NG108-15 cells (NG), N18TG-2 cells (N18), mouse brain (MB) or rat brain (RB) were analysed by RT–PCR using each receptor subtype-specific primers. Representative results are shown from at least three independent experiments.

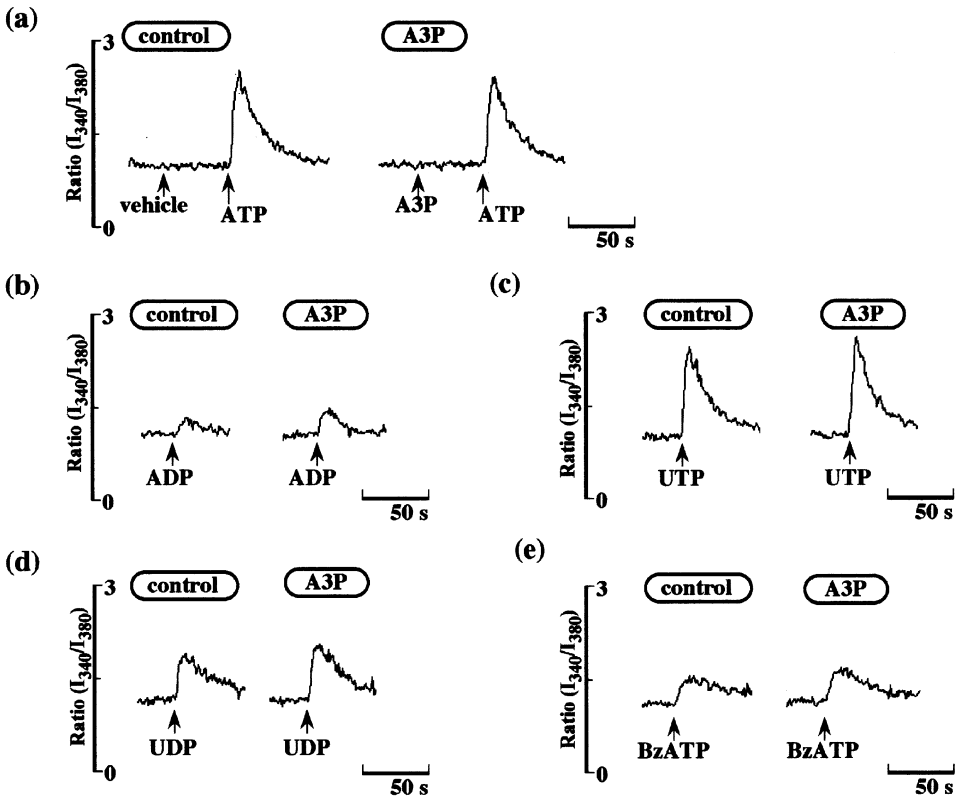

Figure 7 Effect of A3P on basal or the increase in $[Ca^{2+}]_i$ by P2 receptor agonists in NG108-15 cells. Fura-2-loaded NG108-15 cells were stimulated with 100 μM A3P or vehicle (a). Fifty seconds later, cells were stimulated with ATP (a; 100 μM), ADP (b; 10 μM), UTP (c; 100 μM), UDP (d; 10 μM) or BzATP (e; 100 μM). Representative results are shown from three independent results. Similar results were obtained in the case of A2P and A5PS (data not shown).

increase in $[Ca^{2+}]_i$ induced by additive application of other P2 receptor agonists such as ATP (Figure 7a, 100 μM), ADP (Figure 7b; 10 μM), UTP (Figure 7c; 100 μM), UDP (Figure 7d; 10 μM) and BzATP (Figure 7e; 100 μM). A2P and A5PS did not affect both the basal and the increase in $[Ca^{2+}]_i$ by P2 receptor agonists, a similar result observed with A3P (data not shown).

Discussion

The present study demonstrates that A2P, A3P and A5PS stimulate cyclic AMP formation in NG108-15 cells. The results obtained in this study indicate that cyclic AMP formation induced by AMP derivatives are mediated by the same purinoceptor which recognizes ATP or its analogue β,γ-MeATP as its ligands as previously reported (Matsuoka *et al*., 1995; Ohkubo *et al*., 1998a,b).

We have recently shown that cyclic AMP formation induced by β,γ-MeATP was inhibited by both P_1 and P2 receptor antagonists, whereas the response mediated by an A_{2A} receptor was not blocked by P2 receptor antagonists such as PPADS and RB-2 (Ohkubo *et al*., 1998b). The effects of A2P, A3P and A5PS on cyclic AMP formation were inhibited by several P_1 receptor antagonists including ZM241385, XAC, DPCPX and alloxazine. The rank order of inhibitory potencies of these P_1 receptor antagonists obtained against the three AMP derivatives were similar to each other, and compatible with our previous data obtained against the response to β,γ-MeATP. The partial inhibitory effect of alloxazine also suggested an involvement of the same purinoceptor with β,γ-MeATP rather than an A_{2A} receptor, because, like PPADS and RB-2, this compound inhibited cyclic AMP response induced by β,γ-MeATP, but not that by CGS21680. As expected, the effects of A2P, A3P and A5PS were concentration-dependently inhibited by PPADS. Lack of inhibition by suramin ($\leqslant$100 μM) was also consistent with our previous results obtained by the response to β,γ-MeATP. These results indicate that A2P, A3P and A5PS activate the same receptor which mediate β,γ-MeATP-induced cyclic AMP response.

It has been previously thought that AMP preferentially interacts with P_1 receptors rather than P2 receptors (Burnstock, 1978). Moreover, Ross *et al*. (1998) suggested recently that AMP, produced by ATP breakdown, directly stimulates P_1 receptors resulted in a depression of epileptiform activity in the CA3 region of the rat hippocampus. Since NG108-15 cells express functional adenosine A_{2A} receptor, it is necessary to consider whether AMP and its derivatives would be able to stimulate the A_{2A} receptor directly. However, the present study clearly demonstrates that AMP and its derivatives do not have any direct effect at least on the A_2 receptor subtypes, because they had no effects on cyclic AMP formation in N18TG-2 cells which have functional A_2 receptor and express both A_{2A} and A_{2B} receptor mRNA. Furthermore, ATP and β,γ-MeATP failed to stimulate cyclic AMP formation in N18TG-2 cells, suggesting that this cell line does not possess the sensitivities to adenine nucleotides.

P_1 receptor antagonist-sensitive ATP responses have been reported in variety of tissues and cells (Barajas-López *et al*., 1995; Côte *et al*., 1993; Hourani *et al*., 1991; King *et al*., 1996). However, recent data from two different groups cautioned against proposing the existence of a novel receptor, by suggesting that local ecto-nucleotidase activity at the plasma membrane plays an essential role for the P_1 receptor antagonist-sensitive ATP response. In rat hippocampus slices, Dunwiddie and colleagues (1997) demonstrated a rapid degradation of ATP, ADP and AMP to adenosine within 200 ms, resulting in an outward potassium current mediated by A_1 receptors. Cunha *et al*. (1998) suggested that a local ATP catabolism to adenosine by the ecto-nucleotidase might accumulate adenosine around the P_1 receptors and causes the receptor activation. This proposed mechanism could explain the phenomena observed in NG108-15 cells, in terms of the A_{2A} receptor-mediated effects, if the ecto-nucleotidase activity in this cell line was much higher than in N18TG-2 cells. However, we have shown that cyclic AMP formation induced by ATP, β,γ-MeATP, AMP and AMP derivatives was not inhibited by α,β-MeADP, a potent ecto-nucleotidase inhibitor. Taken together, the results of the present study support the existence of a P_1 receptor antagonist-sensitive purinoceptor, linked to AC, and suggest that A2P, A3P and A5PS directly stimulate this receptor.

NG108-15 cells possess at least two other P2 receptor subtypes, the $P2Y_2$ receptor (Lustig *et al*., 1993) and the $P2X_7$ receptor (Kaiho *et al*., 1996), both of which elicit increases in $[Ca^{2+}]_i$. As shown in Figure 7, the increases in $[Ca^{2+}]_i$ were observed by ATP, ADP, UTP, UDP and BzATP, suggesting that multiple P2 receptor subtypes are expressed in this cell line. The basal $[Ca^{2+}]_i$ or the P2 receptor agonist-induced Ca^{2+} mobilization was little affected by A3P, A2P or A5PS, suggesting that these compounds had little effects on the $P2Y_2$, $P2X_7$ and other Ca^{2+}-mobilizing P2 receptors.

The P2 receptor-mediated cyclic AMP formation have been described in several different cell lines such as HL-60 cells (Conigrave *et al*., 1998) and PC12 cells (Yakushi *et al*., 1996). However, the phenomenon observed in NG108-15 cells seems to be mediated different purinoceptor from those in HL-60 or PC12 cells, because (1) 2MeSATP, which is without effect in NG108-15 cells, has agonistic effect on HL-60 and PC12 cells, (2) ATP-mediated cyclic AMP formation in HL-60 cells was inhibited by suramin, but not by PPADS, and (3) P_1 receptor antagonists did not inhibit the responses in HL-60 or PC12 cells. These differences in agonist and antagonist sensitivities in each cell lines suggest that there are subtypes in the cyclic AMP-elevating P2 receptors.

The present study extends the agonist selectivity to cyclic AMP formation in NG108-15 cells. We previously showed that the cyclic AMP level in NG108-15 cells was increased by several adenine nucleotide analogues (Matsuoka *et al*., 1995). These include ATP, ADP, ATPγS, β,γ-MeATP, β,γ-imidoATP and ADPβS. In contrast, GTP, 2′-deoxyATP and several P2 receptor agonists such as 2MeSATP, α,β-MeATP, UTP, UDP were ineffective. These results indicate that the intact adenine structure is required for cyclic AMP elevation. The results obtained with A2P and A3P indicate that phosphate substitution at 2′- or 3′-position of adenosine results in loss of the agonist activity at the A_2 receptor subtypes, but gains the ability to stimulate cyclic AMP formation in similar manner to ATP or its analogues.

In conclusion, we showed that AMP derivatives stimulate cyclic AMP formation in NG108-15 cells. These compounds would be useful tools for the examination of the mechanisms of cyclic AMP formation in NG108-15 cells and other systems.

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