Correlation Between Adenine Nucleotide-Induced Cyclic AMP Elevation and Extracellular Adenosine Formation in NG108-15 Cells

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ABSTRACT—We previously demonstrated that extracellular adenine nucleotides induced cyclic AMP elevation in NG108-15 cells. This response was resistant to adenosine deaminase (ADA) and the ecto-5'-nucleotidase (CD73) inhibitor α,β -methylene ADP (α,β -MeADP), but was inhibited by both P1- and P2-receptor antagonists. In the present study, we investigated the relationship between adenine nucleotide-induced cyclic AMP elevation and extracellular adenosine formation. ATP, AMP and β,γ -methylene ATP (β,γ -MeATP) were time-dependently metabolized to adenosine in NG108-15 cells. Adenosine formations from ATP, AMP and β,γ -MeATP were not affected by α,β -MeADP, but suppressed by the P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). A close correlation between extracellular adenosine formation and cyclic AMP increasing effects were obtained with several adenine nucleotide agonists in NG108-15 cells as well as their parent cell line C6Bu-1 and N18TG-2 cells, all of which possess functional adenosine A₂ receptors. When NG108-15 cells were incubated with [³H]ATP or [³H]AMP in the presence of ADA, [³H]adenosine was found to distribute dominantly on the cell surface. NG108-15 cells expressed mRNA for the ecto-ATPase and nucleotide pyrophosphatase, but not for CD73. These results suggest that local adenosine formation by an ecto-enzyme distinct from CD73 is involved in adenine nucleotide-induced cyclic AMP formation in NG108-15 cells.

Keywords: Extracellular adenine nucleotide metabolism, Ecto-nucleotide pyrophosphatase, Ecto-5'-nucleotidase, Adenosine A_{2A} receptor, NG108-15 cell

Extracellular adenosine and purine/pyrimidine nucleotides stimulate the P1 and P2 receptors, respectively, resulting in diverse physiological effects in a variety of tissues. Recent molecular cloning studies revealed the existence of multiple subtypes in both P1- and P2-receptor families (1, 2). Pharmacological characteristics described in an individual recombinant receptor expression system clearly showed that P2-receptor antagonists, such as suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), widely inhibit several P2-receptor-mediated responses without affecting the effects mediated by P1 receptors, whereas several P1-receptor antagonists, including methylxanthine derivatives, do not modulate the P2-receptor functions (1, 2).

However, P1-receptor antagonists have been demonstrated to exhibit potent inhibitory effects on the responses induced by P2-receptor agonists in certain tissue prepara-

tions, including the rat caudal artery (3), rat duodenum smooth muscles (4), folliculated *Xenopus* oocytes (5) and brain capillary endothelial cells (6). We also previously demonstrated that cyclic AMP formations induced by ATP and its analogues were inhibited by P1-receptor antagonists in NG108-15 cells (7–9). Direct action of ATP without conversion to adenosine was suggested by observations that 1) removal of adenosine by adenosine deaminase (ADA) did not block the response; 2) β,γ -methylene ATP (β,γ -MeATP), a compound that is less rapidly hydrolyzed to adenosine than ATP, was equipotent to ATP; 3) the ecto-5'-nucleotidase (CD73) inhibitor α,β -methylene ADP (α,β -MeADP) did not affect the ATP-induced response; and 4) P2-receptor antagonists inhibited the ATP-induced cyclic AMP formation. The agonist selectivity for the cyclic AMP response in NG108-15 cells was quite different from those for cloned P2 receptors (7). Based on these results, we proposed the existence of a novel adenylyl cyclase-linked purinoceptor in NG108-15 cells, which may be relevant to the P3 purinoceptor (3, 10) or the nucleotide-

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sensitive P1 receptors (4, 5) supposed by observations in other tissues.

More recently, we found that ATP, β,γ -MeATP and AMP stimulated cyclic AMP formation in C6Bu-1 cells, a parent cell line of NG108-15 cells (11). The pharmacological characteristics of the cyclic AMP response in C6Bu-1 cells were similar to those in NG108-15 cells with respect to agonist and antagonist sensitivities and to the ADA resistance. Unlike in NG108-15 cells, however, the β,γ -MeATP-induced cyclic AMP response in C6Bu-1 cells was totally inhibited by α,β -MeADP (11). Analyses of extracellular adenine nucleotide metabolism in C6Bu-1 cells indicated that β,γ -MeATP-induced cyclic AMP formation in C6Bu-1 cells was mediated by hydrolysis to adenosine and subsequent activation of adenosine A_{2B} receptors (11).

The results obtained in C6Bu-1 cells indicated that 1) ADA can not eliminate the effects of metabolically generated adenosine; 2) under certain condition, the metabolically stable ATP analogue β,γ -MeATP produced its effects after conversion to adenosine and 3) PPADS acts as an ectonucleotide pyrophosphatase inhibitor in addition to the P2-receptor antagonist. These pharmacological tools were all key grounds for the adenylyl cyclase-linked purinoceptor theory in NG108-15 cells (7–9). Therefore, we investigated extracellular adenine nucleotide metabolism in NG108-15 cells to explore the possible involvement of metabolically generated adenosine in ATP-induced cyclic AMP formation, and the results are compared with those observed in C6Bu-1 and N18TG-2 cells, the parent cell lines of NG108-15 cells.

MATERIALS AND METHODS

Cell culture

NG108-15, C6Bu-1 and N18TG-2 cells were generous gift from Dr. Haruhiro Higashida (Kanazawa University, Kanazawa). NG108-15 cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 100 μ M hypoxanthine, 1 μ M aminopterin and 16 μ M thymidine and maintained in a humidified atmosphere of 10% CO₂ / 90% air at 37°C. C6Bu-1 and N18TG-2 cells were cultured in the same medium, but hypoxanthine, aminopterin and thymidine were omitted. For measurement of cyclic AMP, cells were seeded in a 24-well culture dish at a density of 1.6×10^4 cells per well and cultured for 2–3 days until confluent. For HPLC analysis, cells were cultured in a 15-cm diameter dish.

Measurement of cyclic AMP level

Intracellular cyclic AMP levels were measured as described previously (8). In brief, cells were labeled with [³H]adenine (3 μ Ci/ml) in the growth medium for 3–5 h. Labeled cells were washed twice with Krebs-Ringer HEPES

(KRH) buffer (130 mM NaCl, 4.7 mM KCl, 4.0 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.8 mM CaCl₂, 11.5 mM glucose and 10 mM HEPES, pH 7.4) and preincubated with 1 U/ml ADA for 10 min at 37°C. Cells were then stimulated by agonists in the presence of the phosphodiesterase inhibitor Ro20-1724 (100 μ M) and 1 U/ml ADA for 10 min. The reaction was terminated by aspirating the incubation buffer, followed by adding 0.4 ml of 2.5% perchloric acid. The acid-extracts were mixed with 0.10 volume of 4.2 M KOH to neutralize and deposit potassium perchlorate. The [³H]cyclic AMP in the supernatant was separated by Dowex 50W-X8/alumina double columns (Bio-Rad, Yokohama) (12) and determined by liquid scintillation counting.

HPLC analysis

Cells were harvested and washed twice with KRH buffer and finally suspended in fresh KRH buffer. Cells (2×10^6 cells per reaction in 0.2 ml of final volume) were preincubated in the presence or absence of α,β -MeADP (1 mM) or PPADS (1 mM) for 10 min. Cells were then incubated with several nucleotide substrates. After incubation for indicated times, cells were immediately centrifuged at 12000 rpm for 2–3 s, and the supernatant was collected. Samples were stored at –20°C until use. Separation of nucleotides and its metabolites were performed by reverse-phase HPLC equipped with an analytical C18 column (250 $\times$ 4.6 mm; YMC Co., Ltd., Kyoto) using 50 mM NaH₂PO₄ (pH 5.5) as solvent at a flow rate of 1 ml/min (7). Absorbance at 258 nm was monitored on-line with a UV detector (Nihon Bunko, Tokyo).

For measurement of [³H]adenosine formation from [³H]ATP or [³H]AMP, cells in a 24-well dish were washed twice and then pretreated with or without ADA (1 U/ml) for 10 min. [³H]ATP or [³H]AMP (10 μ Ci per well, finally adjusted to 5 μ M with unlabeled ATP or AMP in 300 μ l) was added and incubated for the indicated times. For determination of total [³H]adenosine formation (incubation medium plus cell fraction), reactions were stopped by adding 33 μ l of 25% PCA. To investigate the [³H]adenosine present on the cell surface, the reaction was stopped by aspirating the medium completely, followed immediately by adding 333 μ l of 2.5% PCA. Acid-extracts were mixed with one-tenth volume of 4.2 M KOH to neutralize and deposit potassium perchlorate, and then they were stored at –20°C until the analyses by HPLC. The samples were mixed with equal volume of 2 μ M adenosine as a standard marker, and the metabolites in 150 μ l of mixture was separated by HPLC. Adenosine was monitored by a UV detector, and the fractions containing [³H]adenosine were collected and measured by a liquid-scintillation counter.

Reverse transcriptase-coupled polymerase chain reaction (RT-PCR)

Total RNA was extracted from NG108-15, N18TG-2 cells and whole mouse brain by the acid-guanidine thiocyanate/phenol/chloroform method (13). A first stranded cDNA primed by random hexamer was pre-pared from total RNA (1 μ g) by Moloney murine leukemia virus reverse transcriptase and diluted with water by fivefold and this sample was used as a template for the PCR analysis. Transcripts analyzed were as follows: ecto-ATPase (CD39L1), ecto-ATP diphosphohydrolase (CD39), ecto-phosphodiesterase/nucleotidpyrophosphatase (PDNP) 1, PDNP2, PDNP3 and CD73. Each primer was designed on the basis of the published cDNA sequences. The nucleotide sequence and the location in cDNA are: CD39L1 (Accession No. Y11835); ACAGGATGAAGGGGTGTTTG (sense, 486–505), and ACCCCATTGAAGGAGCATTG (antisense, 998–979), CD39 (U81295), GAATGCCAAGTGAAAGGTCC (sense, 322–341) and GATCCAAAGCGCCAAAGGTT (antisense, 712–693), PDNP1 (AB017596); AGTGCTGTCGCTGGTTTTGT (sense 179–198) and AAGAGAGTAGGGGGTGATTC (antisense 592–573), PDNP2 (D28560); TTGAGAACTGCGGTCCTGT (sense 648–667) and GAAGGCATAAACTGAAGGCC (antisense 1000–981), PDNP3 (Z47987); TTTGTATCCAGAGTCGCACG (sense, 688–707) and GTTAGGGAAGGAGCCATTGA (antisense 895–876), CD73 (J05214); AGAGCAAACCAGCGATGACT (sense 514–533) and TCAATCAGTCCTCCACACC (antisense 777–758). The predicted length of PCR products for CD39L1, CD39, PDNP1, PDNP2, PDNP3 and CD73 are 513, 391, 414, 353, 208 and 264 bp, respectively. PCR was carried out as previously described (8) and PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR products were confirmed by analyzing the restriction sites or by direct sequencing using automated DNA sequencer.

Statistics

In each case, experiments were repeated at least three times, with similar results being obtained. Statistical analyses of the data were performed by one-way analysis of variance (ANOVA) with the Dunnett two-tailed test. For all evaluations, *P* values less than 0.05 were considered to be statistically significant.

Materials

High-glucose Dulbecco's modified Eagle's medium and Moloney murine leukemia virus reverse transcriptase were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum was obtained from CSL Ltd. (Victoria, Australia). Taq DNA polymerase was obtained from Pharmacia Biotech (Tokyo). ADA, AMP, A2P, A3P and

α,β -MeADP were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ro20-1724 was purchased from Calbiochem (La Jolla, CA, USA). β,γ -MeATP was from Nacalai Tesque Inc. (Kyoto). PPADS were obtained from Research Biochemicals International (Natick, MA, USA). [3 H]adenine and [3 H]-cyclic AMP were obtained from Amersham Japan and Moravsek Biochemicals Inc. (Brea, CA, USA), respectively. All other chemicals used were of reagent grade or the highest quality available.

RESULTS

Adenine nucleotide metabolism by ecto-nucleotidases in NG108-15 cells

We first examined the time course of degradation of ATP, AMP and β,γ -MeATP in NG108-15 cells. Figure 1 shows a typical HPLC chart recording of ATP metabolism in NG108-15 cells. ATP was rapidly hydrolyzed to ADP, AMP and adenosine. In addition to these adenylyl compounds, inosine and hypoxanthine were gradually increased during the incubation. Since inosine was formed from adenosine by ecto-ADA, followed by conversion into hypoxanthine (14), the amount of inosine and hypoxanthine were pooled to that of adenosine to evaluate the total adenosine formation (Fig. 2). ATP was decreased with *T*_{1/2} of 10 min, and more than 90% of ATP was hydrolyzed within 20 min. Conversely, adenosine was progressively increased and near 40% of the added ATP was metabolized to adenosine after a 20-min incubation (Fig.

Fig. 1. Typical HPLC chromatograms of ATP metabolism in NG108-15 cells. ATP (100 μ M) was incubated with NG108-15 cells (2×10^6 cells) for 5 and 20 min at 37°C, and ATP metabolites in the medium were analyzed by reverse-phase HPLC. Five metabolites including ADP, AMP, adenosine (Ade), inosine (Ino) and hypoxanthine (HX) were identified. "Time 0" shows the chromatogram of ATP substrate without incubation. Typical charts represent the results from 5–6 experiments.

Fig. 2. Time-dependent degradation of ATP, AMP and β,γ -MeATP and effects of PPADS and α,β -MeADP on the metabolisms in NG108-15 cells. ATP (a: 100 μ M), AMP (b: 100 μ M) or β,γ -MeATP (c: 100 μ M) were incubated with NG108-15 cells for the indicated times at 37°C. Metabolites in the supernatants were analyzed by HPLC. The metabolites evaluated were the substrates, ADP, AMP and adenosine (sum of adenosine, inosine and hypoxanthine). Data are expressed as a percentage of the initial amount of substrate, and typical results from 4-5 independent experiments are presented. d: Cells were preincubated with PPADS (1 mM) or α,β -MeADP (1 mM) for 10 min, and reactions were initiated by addition of ATP (100 μ M), β,γ -MeATP (100 μ M) or AMP (100 μ M) and incubated for 10 min. Adenosine formation was measured by HPLC. Data are expressed as a percentage of the control, and presented as the mean $\pm$ S.E.M. from three independent experiments.

2a). NG108-15 cells had a high AMP phosphohydrolase activity, as AMP was rapidly metabolized to adenosine (Fig. 2b). Adenosine formation was also observed from β,γ -MeATP (Fig. 2c). Although the rate of hydrolysis was much slower than ATP or AMP, a time-dependent decrease in β,γ -MeATP was accompanied by an increase in adenosine. After 20-min incubation periods, β,γ -MeATP was degraded by 27%, and 14% of the added β,γ -MeATP was converted into adenosine. During β,γ -MeATP hydrolysis, a slight increase in AMP (3–5%) was also observed, whereas no ADP formation was detected (Fig. 2c).

Effect of α,β -MeADP and PPADS on the nucleotide metabolism in NG108-15 cells

We previously showed that adenine nucleotide-induced cyclic AMP formation in NG108-15 cells was little affected by the CD73 inhibitor α,β -MeADP, but inhibited by the P2-receptor antagonist PPADS (8). We therefore examined the effects of α,β -MeADP and PPADS on adenosine for-

mation from ATP, β,γ -MeATP or AMP in NG108-15 cells (Fig. 2d). α,β -MeADP (1 mM) hardly affected the adenosine formation from all adenine nucleotides tested. In contrast, adenosine generations from ATP, β,γ -MeATP and AMP were all abolished by 1 mM PPADS (Fig. 2d).

Time course of ATP metabolism in C6Bu-1 cells and N18TG-2 cells

C6Bu-1 and N18TG-2 cells, the parent cell lines of NG108-15 cells, exhibited different cyclic AMP responses to extracellular ATP; namely ATP stimulated cyclic AMP formation in C6Bu-1 cells (11) but not in N18TG-2 cells (8). As shown in Fig. 3a, C6Bu-1 cells time-dependently hydrolyzed ATP accompanied by a significant increase in adenosine. As compared to adenosine formation, the increase in the intermediate metabolites, especially in AMP, were not pronounced, suggesting that C6Bu-1 cells rapidly convert AMP to adenosine (Fig. 3a). N18TG-2 cells also rapidly degraded ATP (Fig. 3b). The decrease in ATP was

Fig. 3. Time-dependent degradation of ATP in C6Bu-1 or N18TG-2 cells. ATP (100 μ M) was incubated with C6Bu-1 cells (a) or N18TG-2 cells (b) for 10 and 20 min at 37°C. The metabolites [ADP, AMP and adenosine (sum of adenosine, inosine and hypoxanthine)] are expressed as a percentage of the initial amount of ATP, and typical results from 3 independent experiments are presented.

much faster than in NG108-15 and C6Bu-1 cells. However, N18TG-2 cells did not produce adenosine at all, and ATP hydrolysis was accompanied by a large increase in AMP (Fig. 3b). These results suggest that N18TG-2 cells lack enzymes that catalyze conversion of AMP to adenosine.

Correlation between AMP isomer-induced cyclic AMP formation and extracellular adenosine production

We have recently found that AMP isomers such as adenosine 2'-monophosphate (A2P) and adenosine 3'-monophosphate (A3P) stimulate cyclic AMP formation in NG108-15 cells but not in N18TG-2 cells (9). Figure 4 shows the effects of AMP isomers on cyclic AMP production in C6Bu-1 cells. As previously reported, AMP in-

Fig. 4. Effects of A2P and A3P on intracellular cyclic AMP level in C6Bu-1 cells. [3 H]Adenine-prelabeled C6Bu-1 cells were preincubated with ADA (1 U/ml) for 10 min and then stimulated with A2P, A3P or AMP (each 100 μ M) in the presence of 100 μ M Ro20-1724 and 1 μ M forskolin for 10 min at 37°C. Data are expressed as the fold of control cyclic AMP level and shown as the mean $\pm$ S.E.M. from three determinations. *Significantly different from the control value at $P < 0.05$.

creased forskolin-stimulated cyclic AMP production in C6Bu-1 cells. Unlike in NG108-15 cells, A2P and A3P did not induce cyclic AMP accumulation in C6Bu-1 cells (Fig. 4). We examined metabolisms of these AMP isomers in NG108-15, C6Bu-1 and N18-TG-2 cells. A3P was rapidly converted to adenosine in NG108-15 cells (Fig. 5a). More than 80% of A3P was metabolized and converted to adenosine within 10 min. In contrast, C6Bu-1 and N18TG-2 cells failed to metabolize A3P, and adenosine was hardly detected even after 20-min incubation periods (data not shown). Similar results were obtained in these three cell lines with A2P (data not shown). The results obtained in C6Bu-1 cells sharply contrasted to our previous observation with 5'-AMP, which was effectively converted to adenosine. N18TG-2 cells could not degrade AMP at all (data not shown).

Since A3P-induced cyclic AMP formation in NG108-15 cells was inhibited by PPADS but not by α,β -MeADP (9), the effects of α,β -MeADP and PPADS on adenosine formation from A3P were examined. Consistent with the effect on the cyclic AMP response, adenosine formation from A3P was blocked by PPADS but not by α,β -MeADP (Fig. 5b).

Local adenosine formation from ATP and AMP on membrane surface

Although adenine nucleotide-induced cyclic AMP formation in NG108-15 cells was observed in the presence of ADA, adenosine in the extracellular medium was not detected in the presence of ADA by HPLC analysis with a UV monitor. We therefore hypothesized that adenosine would locally accumulate on the cell surface even in the presence of ADA. To examine this possibility, [3 H]ATP was incubated with NG108-15 cells and [3 H]adenosine preserved in the cell fraction was measured. In the absence

Fig. 5. Metabolism of A3P in NG108-15 cells. a: A3P (100 μ M) was incubated with NG108-15 cells for the indicated times at 37°C. Data are expressed as the percentage of total added A3P. b: Cells were preincubated with PPADS (1 mM) or α,β -MeADP (1 mM) for 10 min, and reactions were initiated by addition of A3P (100 μ M). After a 10-min incubation, the generated adenosine was measured by HPLC. Data are expressed as a percentage of the control and presented as the mean $\pm$ S.E.M. from three independent experiments.

Fig. 6. Detection of locally-produced [3 H]adenosine from [3 H]ATP in NG108-15 cells. Cells in a 24-well dish were preincubated with (b) or without (a) ADA (1 U/ml) for 10 min. [3 H]ATP was applied and incubated for the indicated times at 37°C. [3 H]Adenosine level in the cells fraction (closed symbol) or in the whole sample (open symbol) was determined and expressed as a percentage of the total [3 H]ATP added. Note that the values of cell fraction in the absence of ADA (closed circles in panel a) are the actual values multiplied by 10. Representative results are shown from three independent experiments.

of ADA, [3 H]adenosine in the total sample (contains both the incubation medium and cell fraction) was raised linearly, and nearly 20% of [3 H]ATP was converted to [3 H]adenosine at 10 min (Fig. 6a). In contrast, [3 H]adenosine levels in the cell fraction (acid-cell extracts after aspirating the incubation medium) was rapidly increased to a plateau level (about 0.5% of total [3 H]ATP) at 2 min, and this level was maintained until 10 min (Fig. 6a). The difference in the time course patterns suggest that [3 H]adenosine detected in the cell fraction is not due to a simple contamination of the residual amount of incubation medium. In the presence of ADA, [3 H]adenosine levels were decreased, but still observed in both the total and cell fractions (Fig. 6b). Furthermore, [3 H]adenosine in the cell fraction seem-

ed to be more resistant to ADA than that in the incubation medium, and 35–45% of adenosine in the total sample distributed in the cell fraction (Fig. 6b). These changes in [3 H]adenosine distribution in the presence and absence of ADA also suggest that [3 H]adenosine in the cell fraction does not relate to that in the incubation medium. Similar results were obtained with [3 H]AMP (data not shown).

[3 H]Adenosine in cell fraction detected here should contain both on cell surface and in intracellular adenosine taken up through adenosine transporter. To evaluate the intracellular adenosine levels, cells were incubated with [3 H]ATP for 5 min and washed with fresh buffer three times before acid-extraction. These treatments decreased [3 H]adenosine levels to 32.4%, indicating that [3 H]adeno-

Fig. 7. RT-PCR analyses of gene expression for ecto-nucleotidase enzymes. Total RNA from NG108-15 (NG), N18TG-2 (N18) cells and mouse brain (MB) were analyzed by RT-PCR using specific primers for six ecto-nucleotidases. M: 100 base pair (bp) DNA ladder. Representative results with 30-cycle amplification are shown from at least four independent experiments.

sine was dominantly localized on cell surface.

Analysis of ecto-nucleotidase mRNA expression by RT-PCR

Recently, several ecto-enzymes involved in adenine nucleotide metabolism have been identified and characterized (15). These include 1) the ecto-nucleotide triphosphatase family: CD39L1 (16) and CD39 (17); 2) PDNP family: PDNP1 (18), PDNP2 (19) and PDNP3 (20) and 3) CD73 (21). We previously showed that C6Bu-1 cells dominantly expressed mRNAs for PDNP1 and CD73 (11). The expression of ecto-nucleotidase families in NG108-15 and N18TG-2 cells were investigated by the RT-PCR (Fig. 7). The primers used in this experiment successfully amplified DNA fragments corresponding to mRNA for each ecto-enzyme in mouse brain cDNA. In both NG108-15 and N18TG-2 cells, PDNP1 was most abundantly expressed, and a lesser amount of CD39L1 and PDNP3 was also detected in NG108-15 cells. In contrast, CD73 was absent in both cell lines.

DISCUSSION

The aim of the present study is to evaluate the relationship between adenine nucleotide-induced cyclic AMP accumulation and extracellular adenosine formation in NG108-15 cells. Our previous studies using several purino-

ceptor agonists and antagonists together with purine metabolism inhibitors suggested that adenine nucleotides including ATP, β,γ -MeATP and AMP induced the cyclic AMP response without conversion to adenosine (7–9, 22). However, the results in this study revealed that such pharmacological tools used in previous studies did not exert their expected effects in NG108-15 cells. Firstly, the CD73 inhibitor α,β -MeADP failed to block the adenosine formation, meaning that results obtained with this inhibitor can not exclude possible involvement of adenosine in NG108-15 cells. Secondly, the P2-receptor antagonist PPADS potentially inhibited adenosine formation from ATP. Therefore, the inhibition of ATP-induced cyclic AMP formation by PPADS does not indicate a P2-receptor-mediated effect. Instead this suggests a crucial role of adenosine in the ATP-induced response. Thirdly, adenosine was produced even from the metabolically stable ATP analogue β,γ -MeATP, and this metabolism was also inhibited by PPADS but not by α,β -MeADP. Finally, the metabolism examined with [3 H]ATP or [3 H]AMP revealed that metabolically generated adenosine was preserved on the cell surface even in the presence of ADA. It is therefore suggested that ATP-induced cyclic AMP response in NG108-15 cells depends on the adenosine formation.

Comparison of the ATP-induced effect on cyclic AMP levels in NG108-15 cells and their parent cell line N18TG-2 and C6Bu-1 cells also suggest the requirement of ATP hydrolysis to adenosine. We previously demonstrated that these cell lines possess functional adenylyl cyclase-linked adenosine receptors; namely, NG108-15 and N18TG-2 cells dominantly express the A_{2A} -receptor subtype (8), whereas C6Bu-1 cells has only the A_{2B} receptor (11). ATP and several adenine nucleotide agonists including β,γ -MeATP and AMP could stimulate cyclic AMP formation in NG108-15 and C6Bu-1 cells, but not in N18TG-2 cells (8, 11). Since both NG108-15 and N18TG-2 cells responded to several A_{2A} -receptor agonists with similar pharmacological profiles (8), the difference in the response to ATP in these two cell lines added strength to our suggestion that NG108-15 cells possess an ATP receptor linked to adenylyl cyclase in addition to the A_{2A} receptors (7). However, the present results clearly demonstrate that N18TG-2 cells lack an ecto-enzyme activity catalyzing the conversion of AMP to adenosine. On the other hand, an active conversion of AMP to adenosine was detected in both NG108-15 and C6Bu-1 cells, which exhibited cyclic AMP responses to ATP. An additional evidence for the requirement of adenosine formation was obtained with metabolism of AMP isomers such as AMP, A2P and A3P. Thus, NG108-15 cells rapidly converted all AMP isomers into adenosine, whereas C6Bu-1 cells generated adenosine from AMP but not from A2P or A3P. The cyclic AMP response to these AMP isomers in NG108-15 and C6Bu-1 cells were totally

correlated to the adenosine formation. Taken together, it is suggested that cyclic AMP accumulations induced by several adenine nucleotide agonists in NG108-15 cells are mediated by adenosine formation and subsequent activation of A_{2A} adenosine receptors.

Although the results discussed above show that the cyclic AMP accumulation induced by adenine nucleotide agonists requires adenosine production, the magnitude of the cyclic AMP response to several agonists did not correlate with the amount of adenosine production. For example, although β,γ -MeATP and AMP stimulated cyclic AMP formation to a similar extent over similar concentration ranges (9), adenosine formation from AMP was much larger than from β,γ -MeATP. This discrepancy might be due to that the local adenosine levels on the cell surface would not be reflected by the adenosine level in the incubation medium. Indeed, adenosine in the medium could not be detected in the presence of ADA, whereas cyclic AMP response was observed even in such ADA-containing medium. In our previous study using adherent cells on a culture dish, adenosine formation from ATP was much less than that shown in this study with suspended cells (7). These differences indicate that a considerable gradient of adenosine concentration is formed from the cell surface to the upper layer of the medium. Furthermore, [³H]adenosine derived from [³H]ATP or [³H]AMP was found to distribute to the cell surface in the presence of ADA. In an attempt to reconcile these observations, we now hypothesize that the adenosine receptors (the A_{2A} subtype in this case) would be co-localized with an ecto-enzyme catalyzing the adenosine formation in a specialized microdomain on the plasma membrane, where the accumulation of metabolically generated adenosine is sufficient to activate the A_{2A} receptor. Adenosine preserved in such a microdomain would be hardly eliminated by exogenously added ADA, while once diffusing into the external medium, it is rapidly degraded by ADA. The local adenosine formation has been suggested in the rat hippocampus (23, 24) and frog neuromuscular junction (25). In those studies, ATP was supposed to undergo rapid hydrolysis to adenosine and subsequently induce neuromodulatory effects via the adenosine A₁ receptor. Therefore, phenomena found in NG108-15 cells would be important to understand the physiological role of ATP in the nervous system.

Extracellular nucleotide metabolism is mediated by a cascade of membrane-bound enzymes, such as CD39L1, CD39, PDNP1, PDNP2, PDNP3 and CD73 (15). We previously demonstrated that C6Bu-1 cells expressed mRNAs for PDNP1 and CD73 and exhibited high adenosine generating activity in a manner sensitive to the CD73 inhibitor α,β -MeADP (11). On the other hand, PPADS was found to have no effect on CD73, but inhibits PDNP1 activity, thereby inhibiting cyclic AMP formation induced by ATP

and β,γ -MeATP in C6Bu-1 cells (11). The present results obtained by RT-PCR showed that both NG108-15 and N18TG-2 cells expressed mRNA for PDNP1 but not for CD73. PDNP1 hydrolyzes ATP directly to AMP, thus bypassing the formation of ADP (18). This enzyme activity might therefore account for the hydrolysis of β,γ -MeATP to AMP in NG108-15 cells. Indeed, PDNP1 has been shown to hydrolyze β,γ -MeATP to AMP (26). The absence of CD73 transcript in N18TG-2 cells is consistent with the lack of adenosine generation from AMP in this cell line. In contrast, lack of CD73 mRNA in NG108-15 cells suggest that this cell line possess an AMP phosphohydrolase other than CD73. One could discriminate this enzyme activity from CD73 by evaluating the sensitivity to PPADS and α,β -MeADP. The AMP phosphohydrolase in NG108-15 cells is characterized as a PPADS-sensitive and α,β -MeADP-insensitive enzyme. These results show that difference in the adenosine generating enzyme expressed in NG108-15, N18TG-2 and C6Bu-1 cells accounts for the diverse pharmacological characteristics of the ATP-induced response in each cell type.

In conclusion, this study shows that ATP-induced cyclic AMP formation in NG108-15 cells is mediated by hydrolysis of ATP to adenosine and subsequent activation of A_{2A} adenosine receptors. Our results indicate that the enzyme involved in conversion of AMP to adenosine in NG108-15 cells is clearly different from the well-characterized ubiquitous enzyme CD73. Identification of this enzyme expressed in NG108-15 cells would largely help to understand the P1-antagonist-sensitive ATP responses in different tissues.

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