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Ecto-alkaline phosphatase in NG108-15 cells : a key enzyme mediating P1 antagonist-sensitive ATP response

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1 We previously demonstrated that extracellular adenine nucleotides induced cyclic AMP elevation through local adenosine production at the membrane surface and subsequent activation of adenosine A_{2A} receptors in NG108-15 cells. Furthermore, the adenosine formation was found to be mediated by an ecto-enzyme distinct from the ecto-5′-nucleotidase (CD73). In this study, we investigated the properties of the ecto-AMP phosphohydrolase activity in NG108-15 cells.

2 NG108-15 cells hydrolyzed AMP to adenosine with the K_M value of 18.8 ± 2.2 μM and V_{max} of 5.3 ± 1.6 nmol min^{-1} 10^6 $cells^{-1}$. This activity was suppressed at pH 6.5, but markedly increased at pH 8.5.

3 The AMP hydrolysis was blocked by levamisole, an alkaline phosphatase (ALP) inhibitor. NG108-15 cells released orthophosphate from 2′- and 3′-AMP as well as from ribose-5-phosphate and β-glycerophosphate, indicating that NG108-15 cells express ecto-ALP.

4 The cyclic AMP accumulation induced by several adenine nucleotides was inhibited by levamisole, *p*-nitrophenylphosphate and β-glycerophosphate, with a parallel decrease in the extracellular adenosine formation.

5 Reverse transcriptase polymerase chain reaction analysis revealed that NG108-15 cells express mRNA for the tissue-nonspecific isozyme of ALP.

6 These results demonstrate that AMP phosphohydrolase activity in NG108-15 cells is due to ecto-ALP, and suggest that this enzyme plays an essential role for the P1 antagonist-sensitive ATP-induced cyclic AMP accumulation in NG108-15 cells.
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Keywords: Ecto-alkaline phosphatase; P1 antagonist-sensitive ATP response; ATP; AMP; PPADS; levamisole; β-glycerophosphate; NG108-15 cells

Abbreviations: A2P, 2′-AMP; A3P, 3′-AMP; ADA, adenosine deaminase; ALP, alkaline phosphatase; α,β-MeADP, α,β-methylene ADP; β,γ-MeATP, β,γ-methylene ATP; CD73, ecto-5′-nucleotidase; KRH, Krebs-Ringer-HEPES; PDNP1, ecto-phosphodiesterase/pyrophosphatase 1; PPADS, pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid; Ro20-1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone; RT–PCR, reverse transcriptase polymerase chain reaction

Introduction

Extracellular purine and pyrimidine nucleotides are now recognized as important signaling molecules inducing diverse physiological responses. ATP is stored in the synaptic vesicle with other transmitters and released into synaptic cleft upon nerve stimulation (Burnstock, 1997; 1999). In addition, ATP and UTP are released from non-neuronal cells by mechanical stimulation and causes physiological responses in neighbouring cells by a paracrine mechanism (Harden & Lazarowski, 1999). Nucleotides released into the extracellular space are rapidly degraded by the ecto-nucleotidases (Zimmermann, 1999). Such metabolism is quite important in control of nucleotide-mediated cellular responses, not only terminating the activation of P2 receptors but also generating another signaling molecule, adenosine. Since P2 receptors usually co-exist with P1 adenosine receptors, extracellular adenine nucleotide metabolism converts the signal imput derived from P2 receptors to P1 receptors.

Extracellular adenine nucleotide metabolism to adenosine proceeds by a cascade of several ecto-enzymes, such as ecto-ATPase, ecto-apyrase, ecto-nucleotide pyrophosphatase and ecto-5′-nucleotidase (CD73) (Zimmermann & Braun, 1999). These enzyme activities largely affect P2 receptor-mediated physiological responses. Indeed, the ecto-ATPase inhibitor ARL67156 was reported to potentiate the neurotransmitter action of ATP in the guinea-pig vas deferens (Westfall *et al*., 1996) and urinary bladder (Westfall *et al*., 1997). Furthermore, it has been shown that rapid conversion of ATP to adenosine results in P1 receptor-mediated responses. Thus, the inhibitory effects of ATP on acetylcholine release in the frog neuromuscular junction appeared to be mediated by adenosine (Sebastião *et al*., 1999). Electrophysiological observations in the rat hippocampus have also suggested that complete hydrolysis of ATP could occur within 200 ms in the synaptic cleft, leading to an adenosine A1 receptor-mediated post synaptic effect (Cunha *et al*., 1994; Dunwiddie *et al*., 1997). We have also recently demonstrated that ATP-induced cyclic AMP accumulation in NG108-15 and C6Bu-1 cells is dependent on extracellular adenosine formation, and subsequent activation of adenosine A_2 receptors. The ATP-induced increase in cyclic AMP in these cells exhibited unique pharmacological features (Matsuoka *et al*., 1995; Ohkubo *et al*., 2000b,d). Namely, the response was resistant to adenosine deaminase (ADA), inhibited by both P1 and P2 antagonists

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and was mimicked by metabolically stable ATP analogues, such as β,γ-MeATP or ATPγS. However, cyclic AMP formation examined under different conditions with several inhibitors revealed a close correlation with extracellular adenosine generation. Analysis of ecto-nucleotidase gene transcriptions by reverse transcriptase-coupled polymerase chain reaction (RT–PCR) revealed that NG108-15 and C6Bu-1 cells possess ecto-phosphodiesterase/pyrophosphatase 1 (PDNP1) (Ohkubo *et al*., 2000a,c), an enzyme that hydrolyses ATP to AMP directly (Grobben *et al*., 1999). With respect to adenosine generating enzymes, CD73 was detected in C6Bu-1 (Ohkubo *et al*., 2000c), but not in NG108-15 cells (Ohkubo *et al*., 2000a). In agreement with these results, the CD73 inhibitor α,β-methylene ADP (α,β-MeADP) inhibited ATP-induced cyclic AMP formation in C6Bu-1 cells (Ohkubo *et al*., 2000c), but not in NG108-15 cells (Ohkubo *et al*., 2000a). Although CD73 has been considered to be major enzyme catalyzing the conversion of AMP to adenosine (Zimmermann & Braun, 1999), our previous studies suggested that NG108-15 cells may express another AMP phosphohydrolase. In this study, we examined the characteristics of the AMP phosphohydrolase in NG108-15 cells. The results obtained in this study show that the ecto-alkaline phosphatase (ALP) acts as the key enzyme converting AMP to adenosine in NG108-15 cells, leading to the P1 antagonist-sensitive ATP response.

Methods

Cell culture

NG108-15 cells were grown in high-glucose Dulbecco's modified Eagle's medium 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_2/90% air at 37°C. 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 nucleotidase assay, cells were cultured in a 15 cm diameter dish.

Measurement of cyclic AMP level

Intracellular cyclic AMP levels were measured as described previously (Ohkubo *et al*., 2000b). In brief, cells were labelled with [^{3}H]-adenine (3 μCi ml^{-1}) in the growth medium for 3–5 h. Labelled cells were washed twice with Krebs-Ringer HEPES (KRH) buffer (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 and HEPES 10, pH 7.4) and preincubated with 1 u ml^{-1} ADA for 10 min at 37°C. Cells were then stimulated by agonists in the presence of phosphodiesterase inhibitor 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone (Ro20-1724, 100 μM) and 1 u ml^{-1} ADA for 10 min. The reaction was terminated by aspirating the incubation medium, followed by adding 0.4 ml of 2.5% perchloric acid. 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 supernatant was separated by Dowex 50W-X8/alumina double columns (Salomon, 1991), and determined by liquid scintillation counting.

Measurement of adenine nucleotide hydrolysis

Cells were harvested and washed twice with a phosphate-free KRH buffer (omitting KH_2PO_4), and finally suspended in fresh phosphate-free KRH buffer at 2×10^6 cells ml^{-1}. The pH of the incubation medium was adjusted to 7.4 unless otherwise indicated. Cells (0.2 ml) were preincubated in the presence or absence of inhibitors for 10 min. Cells were then mixed with equal volume of AMP or adenine nucleotide substrates and incubated at 37°C. The incubation was terminated by addition of EDTA to a final concentration of 10 mM and centrifuged at 12,000 r.p.m. for 10 s. The cell-free supernatant was collected and nucleotide hydrolysis was assayed by the release of inorganic phosphate or by HPLC. Inorganic phosphate was determined colorimetrically by the malachite green-based assay as described by Lanzetta *et al*., (1979). Briefly, 0.3 ml of cell supernatant was mixed with malachite green reagent and the absorbance at 630 nm was determined. Inorganic phosphate concentration was determined by comparison against a standard curve constructed with KH_2PO_4. In some experiments, adenine nucleotides and its metabolites were measured by reverse-phase HPLC on an analytical C18 column (250×4.6 mm; YMC Co. Ltd, Kyoto, Japan) using 50 mM NaH_2PO_4 (pH 5.5) as solvent at a flow rate of 1 ml min^{-1} (Matsuoka *et al*., 1995). Absorbance at 258 nm was monitored on-line with a UV detector (Nihon Bunko, Tokyo, Japan).

RT–PCR

Total RNA was extracted from NG108-15 cells and whole rat and mouse brain by acid-guanidine thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). A first stranded cDNA primed by random hexamer was prepared from total RNA (1 μg) by Moloney murine leukaemia virus reverse transcriptase, and diluted with water by five times, and used as a template for the PCR analysis. Transcripts analysed were tissue-nonspecific ALP, intestinal ALP and CD73. Each primer was designed based on the published cDNA sequences. The nucleotide sequence and the location in the cDNA are: tissue-nonspecific ALP (Accession No. J02980); GACA-CAAGCATTCCCACTAT (sense, 967–986) and ATCAG-CAGTAACCACAGTCA (antisense, 1316–1297), intestinal ALP (M61705); CTAAAGGGACAGTTGGAAGG (sense, 220–239) and TTCTTGGCACGGTACATCAC (470–451) and CD73 (J05214); AGAGCAAACCAGCGATGACT (sense 514–533) and TCAATCAGTCCTTCCACACC (antisense 777–758). The predicted length of PCR products for tissue-nonspecific ALP, intestinal ALP and CD73 are 350, 251 and 264 bp, respectively. PCR was carried out as previously described (Ohkubo *et al*., 2000d). 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 and by direct sequencing using automated DNA sequencer.

Materials

High-glucose Dulbecco's modified Eagle's medium and Moloney murine leukaemia virus reverse transcriptase were purchased from GIBCO (Grand Island, NY, U.S.A.). Foetal bovine serum was obtained from CSL Ltd. (Victoria, Australia). Taq DNA polymerase was obtained from Pharmacia Biotech (Tokyo, Japan). ATP, ADA, AMP, 2′-AMP (A2P), 3′-AMP (A3P), polyvinyl alcohol, ammonium molybdate, malachite green, levamisole, β-glycerophosphate and *p*-nitrophenylphosphate were from Sigma-Aldrich Japan (Tokyo, Japan). Ro20-1724 was purchased from Calbiochem (La Jolla, CA, U.S.A.). β,γ-MeATP was from Nakalai Tesque Inc. (Kyoto, Japan). Pyridoxalphosphate-6-azophe-

nyl-2′, 4′-disulphonic acid (PPADS) were obtained from Research Biochemicals International (Natick, MA, U.S.A.). [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.

Results

AMP hydrolysis in NG108-15 cells

AMP hydrolysis was monitored by measurement of inorganic phosphate in the incubation medium. NG108-15 cells hydrolyzed AMP in a time-dependent manner (Figure 1a). Inorganic phosphate released from AMP (50 μM) to the medium increased linearly over the first 10 min and more than 90% AMP was hydrolyzed after 20 min. In the absence of AMP, inorganic phosphate level was slightly increased, but by less than 10 μM after 30 min. AMP was not degraded at all in cell free condition. To obtain the apparent kinetic parameters for AMP hydrolysis, the initial rates of hydrolysis were measured at concentrations ranging from 3.9 to 500 μM (Figure 1b). The velocity of AMP hydrolysis was increased by increasing concentrations of AMP and reached to the maximum at 125–250 μM AMP. At 500 μM AMP hydrolysis velocity decreased slightly. Analysis using the Lineweaver-Burke equation gave a K_M of 18.8 ± 2.2 μM and a V_{max} of 5.3 ± 1.6 nmol min^{-1} 10^6 cells^{-1}.

Effects of pH on AMP hydrolysis in NG108-15 cells

AMP hydrolysis exhibited a marked pH dependence (Figure 2). It was very weak at pH 6.5, and increased considerably with increasing extracellular pH. The optimal pH seemed to be higher than pH 8.5, the highest pH examined. The spontaneous release of inorganic phosphate in the absence of AMP was constant over this pH range. These results indicate an involvement of ALP in AMP hydrolysis in NG108-15 cells.

Hydrolysis of different organic phosphate substrates in NG108-15 cells

ALP is known to hydrolyze a variety of organic phosphates. The extracellular phosphatase activity in NG108-15 cells was examined using A3P, ribose-5-phosphate and β-glycerophosphate as substrates (Figure 3). All of these were degraded by NG108-15 cells, and the velocity of inorganic phosphate release from each substrate was quite similar at concentrations lower than 100 μM. Similar to AMP hydrolysis, the A3P hydrolysis decreased at concentrations higher than 300 μM, whereas such reduction was not evident for hydrolysis of ribose-5-phosphate or β-glycerophosphate.

Effects of ALP inhibitors on adenosine production

Reactions mediated by ALP are known to be inhibited by levamisole or by excess amount of enzyme substrates such as β-glycerophosphate and *p*-nitrophenylphosphate. We have recently shown in NG108-15 cells that extracellular adenosine production was closely correlated to the cyclic AMP accumulation induced by several adenine nucleotides such as ATP, β,γ-MeATP, AMP, A3P and A2P, and the final step

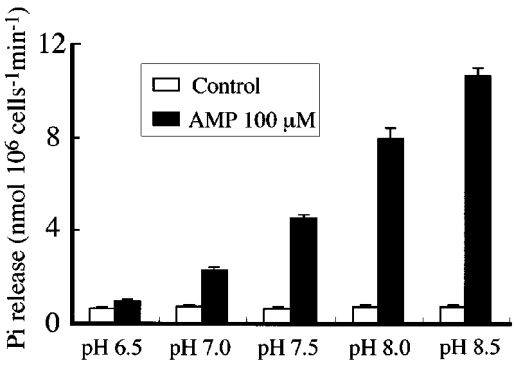

Figure 2 Effects of extracellular pH on AMP hydrolysis in NG108-15 cells. Aliquots of suspended cells were incubated with (dark column) or without (open column) AMP (100 μM) for 10 min at 37°C under different pH conditions, and free orthophosphate levels in the medium measured. Data are the mean±s.e.mean from three independent experiments.

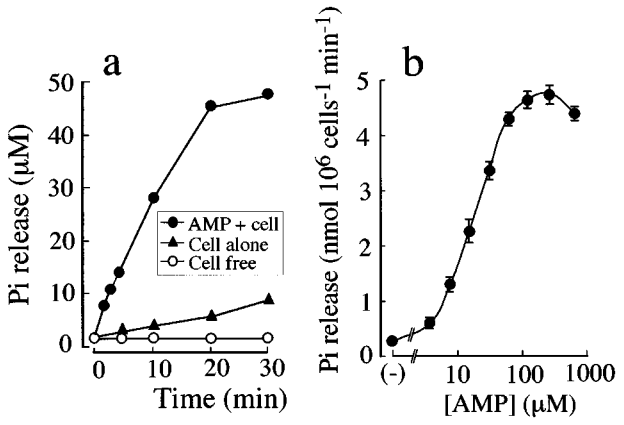

Figure 1 AMP hydrolysis in NG108-15 cells. (a) Aliquots of suspended cells were incubated with or without AMP (50 μM) for 5, 10, 20 and 30 min at 37°C, and inorganic phosphate levels in the medium measured. AMP was also incubated at 37°C in the absence of cells. Data are mean of duplicate determinations and are typical of 3–4 independent experiments. (b) Cells were incubated with different concentrations (3.9–500 μM) of AMP for 5 min at 37°C, and free inorganic phosphate levels in the medium were analysed. Data are the mean±s.e.mean from three independent experiments.

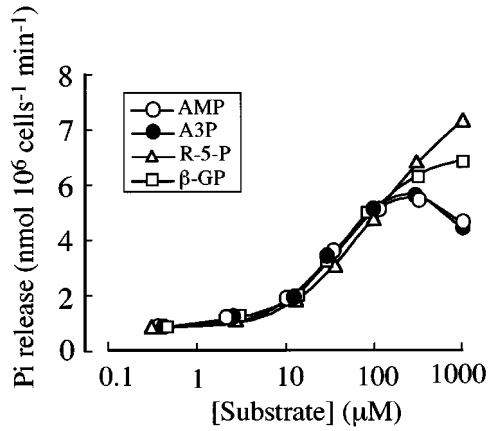

Figure 3 Hydrolysis of AMP, 3′-AMP (A3P), ribose-5-phosphate (R-5-P) and β-glycerophosphate (β-GP) in NG108-15 cells. Aliquots of suspended cells were incubated with different concentrations of AMP, A3P, R-5-P and β-GP for 10 min at 37°C, and free orthophosphate levels in the medium measured. Data are mean of duplicate determinations and are typical of three independent experiments.

of adenosine production was apparently mediated by an enzyme distinct from CD73 (Ohkubo *et al*., 2000a). Figure 4a shows effects of levamisole, β-glycerophosphate and PPADS on extracellular ATP metabolism in NG108-15 cells. PPADS is a nonselective P2 receptor antagonist, but acts as an inhibitor of AMP phosphohydrolase activity in NG108-15 cells (Ohkubo *et al*., 2000a). After 10 min incubation, ATP levels in the incubation medium were decreased to $28.6 \pm 2.3\%$ ($n=3$) and converted into ADP, AMP and adenosine. The adenosine level was increased to $9.7 \pm 0.3\%$ of ATP added ($n=3$). Levamisole (1 mM), β-glycerophosphate (10 mM) and PPADS (1 mM) all abolished adenosine production from ATP. ATP degradation was little affected by levamisole or β-glycerophosphate, but blocked by PPADS. To examine the involvement of ALP in adenosine production from other adenine nucleotides, the effects of levamisole and β-glycerophosphate on the adenosine production from β,γ-MeATP, AMP and A3P were examined (Figure 4b). After 10 min, the amounts of adenosine produced from 100 μM β,γ-MeATP, AMP and A3P were 2.8 ± 0.2, 26.9 ± 3.6 and $30.7 \pm 3.7\%$ of the initial substrate added, respectively ($n=3$). Both levamisole (1 mM) and β-glycerophosphate (10 mM) potently inhibited adenosine production from all adenine nucleotide substrates tested.

Effects of ALP inhibitors on adenine nucleotide induced cyclic AMP formation

The results shown above suggest that ecto-ALP acts as an AMP phosphohydrolase in NG108-15 cells, playing an essential role in adenosine production from different adenine nucleotide substrates, which act as agonist to induce cyclic AMP formation in NG108-15 cells. To explore the role of adenosine production in cyclic AMP response, effects of ALP inhibitors on adenine nucleotide-induced cyclic AMP formation were examined (Figure 5). The experiment was performed in the presence of 1 u ml^{-1} ADA. Under these conditions, adenosine derived from adenine nucleotides could not be detected by HPLC analysis. Stimulation by ATP, β,γ-MeATP, AMP and A3P (each 10 μM) resulted in cyclic AMP accumulation to a similar extent. These responses were all inhibited by levamisole, β-glycerophosphate and p-nitrophenyl phosphate (each 10 mM). In contrast, cyclic AMP accumulation induced by CGS21680 (1 μM) were hardly affected by these ALP inhibitors. These results suggest that the adenine nucleotide-induced cyclic AMP response was mediated by ecto-ALP-dependent adenosine production, and subsequent stimulation of adenosine A_{2A} receptors.

Analysis of ecto-ALP mRNA expression by RT–PCR

Currently, at least two different murine ALPs, the intestinal and tissue-nonspecific enzymes, have been cloned. We designed specific PCR primers for these isozymes on the basis of the mouse cDNA nucleotide sequence and the expression of mRNAs for these ALPs and CD73 in NG108-15 cells was investigated by RT–PCR. As shown in Figure 6a, NG108-15 highly expressed the tissue-nonspecific ALP. In contrast, the intestinal ALP and CD73 were absent. The performance of these primers were confirmed in mouse brain cDNA (for the tissue-nonspecific ALP and CD73) and in mouse ileum cDNA (for the intestinal ALP). These results indicate that AMP phosphohydrolase activity in NG108-15 cells is mediated by tissue-nonspecific ALP.

Discussion

The present study revealed that ecto-ALP acts as an AMP phosphohydrolase in NG108-15 cells. It is generally thought that CD73, the ecto-5′-nucleotidase, is the major enzyme

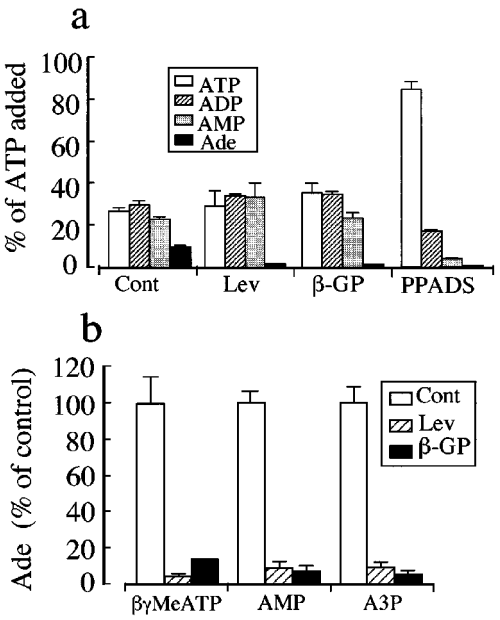

Figure 4 Effects of ALP inhibitors on adenine nucleotide hydrolysis in NG108-15 cells. (a) Aliquots of suspended cells were incubated with ATP (100 μM) in the absence (Cont) or presence of 1 mM levamisole (Lev), 10 mM β-glycerophosphate (β-GP) or 1 mM PPADS for 10 min at 37°C, and ATP and its metabolites (ADP, AMP and adenosine) in the medium were determined by HPLC. Data are expressed as percentage of ATP added, and presented as the mean ± s.e.mean from three independent experiments. (b) Aliquots of suspended cells were incubated with β, γ-MeATP, AMP or A3P (each 100 μM) in the absence (Cont) or presence of 1 mM levamisole (Lev) or 10 mM β-glycerophosphate (β-GP) for 10 min at 37°C, and adenosine levels in the medium were determined by HPLC. Data are expressed as percentage of control, and presented as the mean ± s.e.mean from three independent experiments.

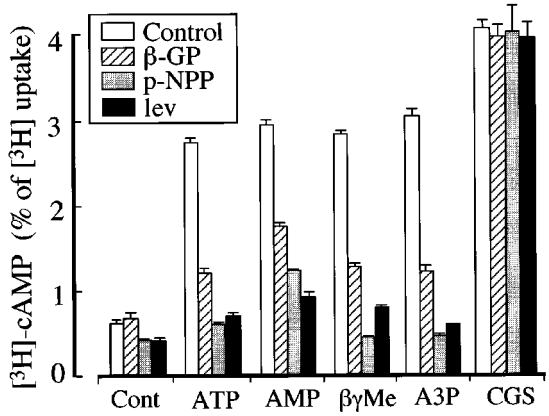

Figure 5 Effects of ALP inhibitors on adenine nucleotide-induced cyclic AMP formation in NG108-15 cells. [^{3}H]-adenine-prelabelled cells were preincubated with 10 mM levamisole (Lev), 10 mM β-glycerophosphate (β-GP) or 10 mM p-nitrophenylphosphate (p-NPP) in the presence of ADA (1 u ml^{-1}) for 10 min at 37°C. Cells were then stimulated with ATP (10 μM), AMP (10 μM), β, γ-MeATP (10 μM), A3P (10 μM) or CGS21680 (1 μM) in the presence of ADA (1 u ml^{-1}) and Ro20-1724 (100 μM) for 10 min. [^{3}H]-cyclic AMP levels are expressed as percentage of total [^{3}H]-adenine uptake and presented as the mean ± s.e.mean from three independent experiments.

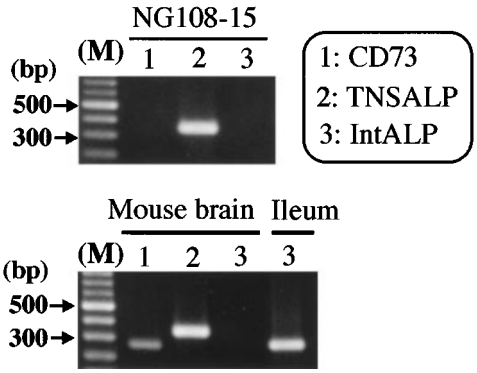

Figure 6 RT–PCR analysis of gene expression for CD73 and ALP isozymes in NG108-15 cells. Total RNA from NG108-15 cells and mouse brain were analysed by RT–PCR using specific primers for CD73, tissue nonspecific ALP (TNSALP) and intestinal ALP (IntALP). Since intestinal ALP was absent in brain, mouse ileum total RNA was subjected to RT–PCR with intestinal ALP primer as the positive control. M; 100 base pair (bp) DNA ladder. Representative results with 30 cycle-amplification from at least four independent experiments are shown.

catalyzing the hydrolysis of AMP to adenosine in many tissues (Zimmermann & Braun, 1999). We previously showed that AMP hydrolysis in NG108-15 cells was not inhibited by the CD73 inhibitor α,β-MeADP (Ohkubo *et al*., 2000a). The present results demonstrate that AMP hydrolysis is greatly affected by changing the extracellular pH. Thus, AMP hydrolysis activity was very weak at acidic condition (pH 6.5), and augmented with increasing pH. This alkaline-optimal AMP hydrolyzing activity suggests an involvement of ALP. ALP is known to dephosphorylate several organic phosphates. NG108-15 cells hydrolyzed β-glycerophosphate, ribose-5-phosphate and *p*-nitrophenyl phosphate and an excess amount of β-glycerophosphate inhibited the AMP hydrolysis, indicating that the same enzyme catalyzes the hydrolysis of AMP and β-glycerophosphate. Furthermore, AMP hydrolysis in NG108-15 cells was inhibited by the ALP inhibitor levamisole. Although the maximal enzyme activity seemed to occur at pH higher than 8.5, substantial AMP hydrolysis was observed even at the physiological pH range. In addition to these enzymatic properties, RT–PCR analysis revealed that NG108-15 cells express mRNA for tissue-nonspecific ALP, but not CD73. These results indicate that AMP hydrolysis is mediated by the ALP in NG108-15 cells.

The present study also demonstrate that the ALP in NG108-15 cells plays a critical role for adenine nucleotide-induced cyclic AMP accumulation. We have already shown that cyclic AMP accumulation induced by several adenine nucleotides is closely correlated with extracellular adenosine production (Ohkubo *et al*., 2000a). The failure of α,β-MeADP to inhibit the adenine nucleotide-induced cyclic AMP response, as well as extracellular adenosine production, suggested the existence of an AMP phosphohydrolase distinct from CD73 (Ohkubo *et al*., 2000a). In this study, we identified ALP as AMP phosphohydrolase in NG108-15 cells and further demonstrated that several ALP inhibitors prevented adenosine production and cyclic AMP accumulation induced by ATP, β,γ-MeATP and A3P, without affecting the response induced by the A_{2A} receptor agonist CGS21680. These results indicate that the ALP-mediated adenosine production is essential for the adenine nucleotide-induced cyclic AMP response. Although ALP has been implicated in extracellular nucleotide hydrolysis (Zimmermann & Braun, 1999), little is known about physiological significance of ALP-mediated nucleotide hydrolysis. To our knowledge, this is the first example of a physiological role of ALP in purinoceptor-mediated signaling systems.

We have recently demonstrated that adenine nucleotides also induce cyclic AMP accumulation in C6Bu-1 cells *via* adenosine formation and subsequent activation of adenosine A_{2B} receptors (Ohkubo *et al*., 2000c). However, the AMP hydrolysis to adenosine in C6Bu-1 cells is mediated by CD73 (Ohkubo *et al*., 2000c), being therefore different from that in NG108-15 cells. In both cell lines, ADA abolished adenosine-induced cyclic AMP formation, but failed to inhibit the adenine nucleotide-induced cyclic AMP responses (Matsuoka *et al*., 1995; Ohkubo *et al*., 2000d). We have previously shown in these cells that metabolically generated adenosine from ATP or AMP was preserved on the cell surface even in the presence of ADA (Ohkubo *et al*., 2000a, 2000c). On the basis of these results, we hypothesized that adenosine generating enzymes are co-localized with adenosine receptors on the membrane surface, where adenosine converted from AMP might efficiently stimulate adenosine receptors in an ADA-resistant manner. It is interesting to note that both CD73 and ALP are glycosylphosphatidilinositol-anchored proteins (Zimmermann & Braun, 1999), and are known to localize in the caveolae (Ide & Saito, 1980; Ludwig *et al*., 1999), a specialized microdomain of the plasma membrane which has been proposed to play a role in diverse cellular processes, including adenylyl cyclase-linked signal transduction system (Schwencke *et al*., 1999). Furthermore, Lasley *et al* (2000) have recently shown that adenosine A_1 receptors are also localized in the caveolae. Further experiments are required to demonstrate the co-localization of A_2 receptors with adenosine generating enzymes and their functional coupling.

Although the features of cyclic AMP responses to adenine nucleotides in NG108-15 and C6Bu-1 cells are very similar, a few, but significant, differences were found between the responses in these cell lines (Ohkubo *et al*., 2000a,c). Firstly, unlike the response in NG108-15 cells, the adenine nucleotide-induced cyclic AMP formation in C6Bu-1 cells was totally inhibited by α,β-MeADP. Secondly, the AMP isomers A2P and A3P increased cyclic AMP in NG108-15 cells, but were without effect in C6Bu-1 cells. Finally, PPADS inhibited cyclic AMP responses induced by ATP or β,γ-MeATP without affecting those to AMP in C6Bu-1 cells, whereas it inhibited the cyclic AMP responses induced by all adenine nucleotide agonists including AMP, A2P and A3P in NG108-15 cells. These differences can be explained by the difference in the AMP hydrolyzing enzymes. For example, CD73 expressed in C6Bu-1 cells can hydrolyze AMP, but not A2P or A3P, whereas ALP in NG108-15 cells can produce adenosine from both of these AMP isomers. The differential inhibitory effects of PPADS on AMP-induced response suggest that this compound blocks AMP hydrolysis by ALP, but not by CD73. These interpretations were supported by our previous studies that PPADS abolished adenosine formation from AMP, A2P and A3P in NG108-15 cells (Ohkubo *et al*., 2000a), but had no effect on AMP hydrolysis in C6Bu-1 cells (Ohkubo *et al*., 2000c). The inhibition by PPADS of ATP- and β,γ-MeATP-induced responses in C6Bu-1 cells is due to the inhibition of PDNP1-mediated conversion of ATP to AMP (Grobben *et al*., 1999). Since NG108-15 cells also abundantly express PDNP1 mRNA, it is considered that PPADS inhibits adenine nucleotide-induced cyclic AMP formation in NG108-15 cells by preventing both PDNP1- and ALP-mediated metabolism.

ALP is considered to hydrolyze a variety of organic phosphate compounds including ATP, ADP and AMP (Zimmermann & Braun, 1999). In NG108-15 cells, ALP inhibitors selectively blocked adenosine formation without affecting the decrease in ATP and the resulting increase in ADP and AMP. These effects of ALP inhibitors were clearly different from those of PPADS, which blocked the decrease in ATP and abolished adenosine formation. It is therefore suggested that ATP hydrolysis is mainly mediated by PDNP1, a PPADS-sensitive enzyme, and ALP predominantly mediates AMP hydrolysis in NG108-15 cells.

In summary, the present study demonstrate that in addition to CD73, ecto-ALP plays an important role for adenosine production from AMP. In NG108-15 cells, ALP-mediated adenosine production is critical for the P1 antagonist-sensitive adenine nucleotide response. Since ALP is widely distributed in many organs, the mechanism described here may be important for understanding the P_1 antagonist-sensitive responses to ATP in various tissues.

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