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SHORT COMMUNICATION

Extracellular ATP-Stimulated Increase of Cytosolic cAMP in HL-60 Cells

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ABSTRACT. Extracellular ATP increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through activation of P_{2U} purinoceptors in HL-60 cells. We found that extracellular ATP also increased the intracellular cAMP level in undifferentiated HL-60 cells. ATP and the hydrolysis-resistant ATP analogue adenosine 5′-O-(3-thiotriphosphate) triggered cAMP production in a concentration-dependent manner. The effects of ATP analogues on the $[Ca^{2+}]_i$ elevation was distinguished from their effects on cAMP generation. Reactive Blue 2, the antagonist of P_2 purinoceptors, inhibited ATP-induced cAMP generation in a concentration-dependent manner without inhibiting Ca^{2+} mobilization. The results suggest that a distinct P_2 purinoceptor, one different from P_{2U}, is linked to adenylyl cyclase in HL-60 cells. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:429–432, 1997.

KEY WORDS. P_2 purinoceptor; adenylyl cyclase; cAMP; HL-60 cells; nucleotides

Extracellular ATP serves as one of a number of important signals that evoke various cellular responses in many tissues (for a review, see [1]). Especially in the inflammatory and immune systems, extracellular ATP regulates the activity of macrophages, neutrophils and natural killer cells [2–4]. In platelets, extracellular ADP serves as a signal for the expression of the adhesion molecule to induce aggregation [5]. The various actions of extracellular ATP are mediated through purinoceptors that have been classified by Burnstock as P_2 purinoceptors [6]. Up to now, six subtypes of P_2 purinoceptors have been recognized: P_{2X}, $_{-Y}$, $_{-U}$, $_{-T}$, $_{-Z}$ and $_{-D}$ [7]. P_2 purinoceptors have also been classified into two major fields: P2X, the intrinsic cation channel family, and P2Y, the G-protein-linked receptor family [8].

HL-60 cells are a human promyelocytic leukemia cell line [9] and a good model system for extracellular ATP-mediated Ca^{2+} signaling. HL-60 cells express P_{2U} purinoceptors [10, 11], and extracellular ATP induces the activation of nonselective cation channels permeable to Ca^{2+} and Na^+ in differentiated HL-60 cells [12]. At present however, there has been no report on P_2 purinoceptors eliciting cAMP production. In this article, we report that ATP elevates cAMP in HL-60 cells in a manner not linked to P_{2U} purinoceptors, which are coupled to PLC.†

MATERIALS AND METHODS

Materials

RPMI 1640 and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). Bovine calf serum was obtained from HyClone Laboratories (Logan, UT, USA). ATP, ADP, AMP, adenosine, GTP, CTP, UTP, ITP, XTP, ATP-[-S, ADP-β-S, AMPPCP, AMPPNP, BzATP, bradykinin, TCA, IBMX, CTX, PTX, and sulfinpyrazone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ionomycin, AMPCPP, 2-Cl ATP, 2-MeS ATP, DPCPX, DMPX, XAC, 8-PT and PMA were obtained from Research Biochemicals Inc. (Natick, MA, USA). [^{3}H]adenine was purchased from NEN (Boston, MA, USA). Fura-2/AM and BAPTA/AM were obtained from Molecular Probes (Eugene, OR, USA).

Cell Culture

HL-60 cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% (v/v) of heat-inactivated bovine calf serum and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2.

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† *Abbreviations:* $[Ca^{2+}]_i$, intracellular-free calcium concentration; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetoxymethyl ester; PLA_2, phospholipase A_2, PLC, phospholipase C; PLD, phospholipase D; AMPCPP, adenosine 5′-(α,β-methylene) triphosphate; AMPPCP, adenosine 5′-(β,γmethylene) triphosphate; AMPPNP,5′-adenylylimidodiphosphate; BzATP, 3′-O-(4-benzoyl) benzoyl ATP; ATP-γ-S, adenosine 5′-O-(3-thiotriphosphate); ADP-β-S, adenosine 5′-O-(2-thiodiphosphate); 2-MeS ATP, 2-methylthio ATP; 2-Cl ATP, 2-chloro ATP; 8-PT, 8-phenyl theophylline; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amine congener, IBMX, 3-isobutyl-1-methyl xanthine; CTX, chlorea toxin; PTX, pertussis toxin; TCA, trichloroacetic acid; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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Measurement of cAMP

Intracellular cAMP was determined by measuring the formation of [^{3}H]cAMP from [^{3}H]adenine nucleotide pools as previously described [13]. Briefly, cells were loaded with [^{3}H]adenine (2 μCi/mL) in complete medium for 24 h. Then [^{3}H]cAMP produced by agonist stimulation was fractionated by sequential chromatography on Dowex AG50W-X4 (200–400 mesh) and an alumina column. In some sets of experiments, we used stimulants containing IBMX, the inhibitor of phosphodiesterase, to accumulate intracellular cAMP. The increase in intracellular cAMP concentration was calculated as [^{3}H]cAMP/[^{3}H]ATP + [^{3}H]cAMP) × 10^3.

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

The level of intracellular Ca^{2+} was determined using 3 μM fura-2 pentaacetoxymethyl ester (fura-2/AM) as previously described [13]. Changes in fluorescence ratio were measured at the dual excitation wavelengths of 340 nm and 380 nm and the emission wavelength of 500 nm. The calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed according to Grynkeiwicz *et al.* [14].

Analysis of Data

All quantitative data are expressed as the mean ± SEM. The results were analyzed for variation using one-way analysis of variance. We calculated EC_{50} and E_{max} with the AllFit program [15]. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In HL-60 cells, P_{2U} purinoceptors are coupled to PLC [10, 16], PLA_2 [17] and PLD [18]. The receptor is also coupled to PTX-sensitive G protein. We observed the generation of cAMP in HL-60 cells after treatment with extracellular ATP. Extracellular ATP and ATP-γ-S both triggered cAMP production in a concentration-dependent manner (Fig. 1A). EC_{50} of ATP and ATP-γ-S were 212.1 ± 11.9 μM (E_{max} = 110.6 ± 5.1 μM) and 67.9 ± 25.7 μM (E_{max} = 95.9 ± 12.4 μM), respectively. Maximum increase of intracellular cAMP concentration was obtained 3 min after stimulation (Fig. 1B). The data suggest that in HL-60 cells P_2 purinoceptors are linked to adenylyl cyclase in addition to phospholipases.

The extracellular ATP-mediated effect could be linked to the stimulation of adenosine receptors, the agonist of which is derived from hydrolysis of ATP [19]. With the results showing that ATP-γ-S was able to induce cAMP production (Fig. 1), the possibility of adenosine-receptor-mediated cAMP generation could be excluded and was also confirmed with adenosine receptor antagonists. When adenosine receptor antagonists such as DPCPX, 8-PT, DMPX, and XAC were used in more than sufficient concentrations (5 μM), the ATP-induced cAMP production was not affected. However, Reactive Blue 2 and suramin, antagonists of P_2 purinoceptors, did inhibit the ATP-induced cAMP production (Fig. 2A). Reactive Blue 2 markedly inhibited the cAMP production in a concentration-dependent manner (IC_{50} = 65.2 ± 0.7 μM), with the maximal inhibitory concentration being 1 mM (Fig. 2B).

In spite of its antagonism for cAMP generation, Reactive Blue 2 did not have an inhibitory effect on the rise in extracellular ATP-induced Ca^{2+}, even at the maximal inhibitory concentration for cAMP signaling. We propose that extracellular ATP enhances cAMP production by ac-

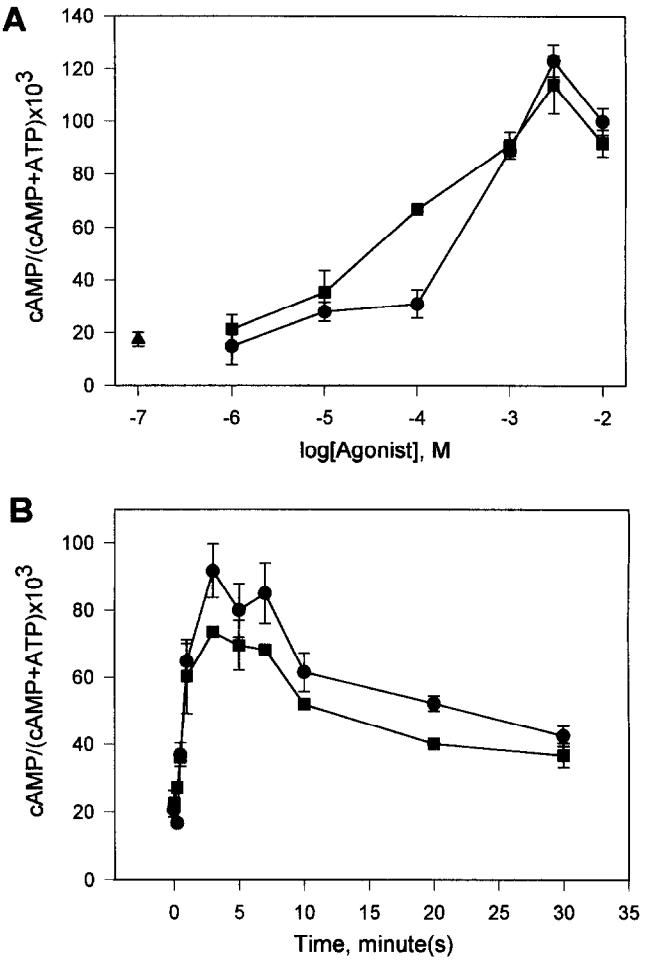

FIG. 1. Extracellular ATP-induced cAMP generation in undifferentiated HL-60 cells. (A) ATP-induced cAMP production exhibits a concentration-dependency. [^{3}H]adenine-loaded cells were stimulated with various concentrations of ATP (circle) or the hydrolysis-resistant ATP analogue ATP-γ-S (square) for 15 min in the presence of 1 mM IBMX. The basal level of cAMP obtained by treatment with vehicle only (without any agonist) is also indicated (triangle). (B) Time courses of ATP and ATP-γ-S-induced cAMP production. Cells were stimulated with 300 μM ATP (circle) or ATP-γ-S (square) for the indicated periods of time in the absence of IBMX. The experiments shown in A and B were carried out six and five times independently, respectively. The levels of cAMP produced were measured as described in Materials and Methods. The results were reproducible. Each point is the mean ± SEM of triplicate samples.

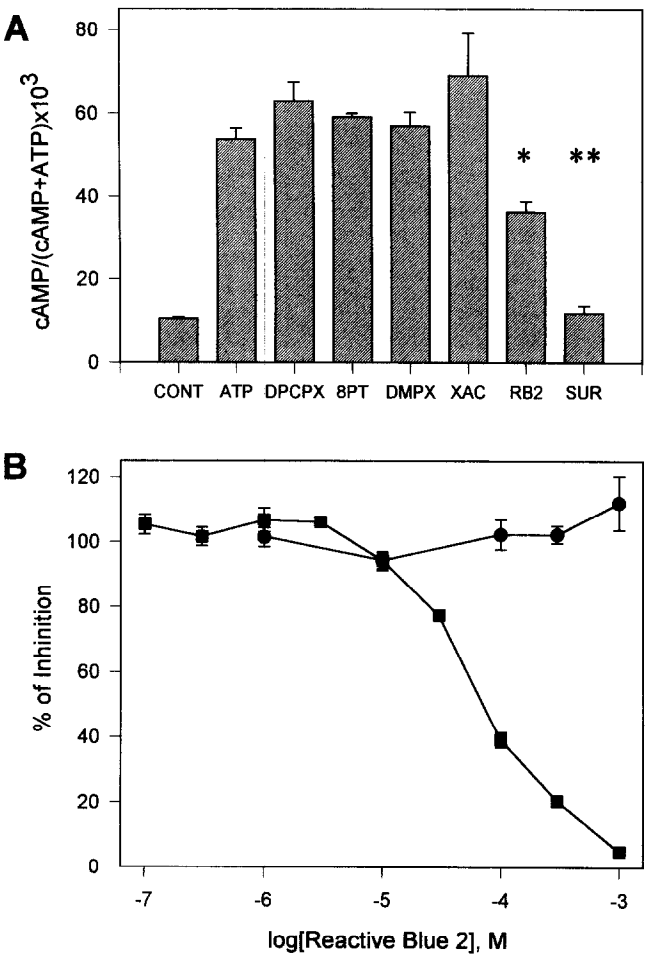

FIG. 2. Effects of P_1 and P_2 purinoceptor antagonists on extracellular ATP-induced cAMP production. (A) The inhibitory effects of P_1 and P_2 antagonists were measured. Cells were preincubated at indicated with 5 μM DPCPX, 5 μM 8-PT, 5 μM DMPX, 5 μM XAC, 100 μM Reactive Blue 2 (RB2), or 100 μM suramin (SUR) for 5 min and then stimulated with 300 μM ATP for 15 min in the absence of IBMX. Only the marked columns showed a significant difference in a comparison with the ATP effect: *$P < 0.01$, **$P < 0.005$. (B) Inhibition of extracellular ATP-induced cAMP generation by Reactive Blue 2. Cells were pretreated with Reactive Blue 2 for 5 min and then stimulated with 300 μM ATP for determination of the levels of cytosolic cAMP (square) and Ca^{2+} (circle). The levels of cAMP and elevated Ca^{2+} were measured as described in Materials and Methods. The experiments were performed five times independently and were reproducible. The results are the mean ± SEM of triplicate assays and are presented as a percentage of the control, the 300-μM ATP-evoked response without Reactive Blue 2 pretreatment.

tivating a distinct P_2 purinoceptor not involved in the cytosolic Ca^{2+} increase in HL-60 cells. Studies with nucleotides and ATP analogues are useful in distinguishing between the subtypes of P_2 purinoceptors [1, 6]. When we applied 300 μM of each nucleotide to the cells, the effect on the $[Ca^{2+}]_i$ elevation was ATP ≥ ITP = dATP ≥ ATP-γ-S = 2-Cl ATP = UTP ≥ ADP = XTP ≥ GTP > AMPPNP ≥ BzATP ≥ 2-MeS ATP > ADP-β-S > CTP (Table 1). The order of the analogues' effects on Ca^{2+} mobilization clearly demonstrates that the $[Ca^{2+}]_i$ increase is mediated by P_{2U} purinoceptors. However, the pattern of cAMP production induced by the ATP analogues was different from their effect on Ca^{2+} mobilization, the order of effectiveness being BzATP ≥ dATP ≥ ATP-γ-S ≥ ATP ≥ 2-MeS ATP > 2-Cl ATP > ADP > adenosine = AMPPCP ≥ ADP-β-S > AMPPNP (Table 1). Furthermore, this potency order for cAMP production did not match any of the known P_2 purinoceptor subtypes. This result suggests that there may be another type of P_2 purinoceptor involved in cAMP signaling.

However, extracellular ATP-mediated modulation of cAMP production could also be due to other factors, such as Ca^{2+} or PKC. We examined this possibility with ionomycin, a Ca^{2+}-specific ionophore, BAPTA/AM, a Ca^{2+} chelator, and PMA, a PKC activator. Both $[Ca^{2+}]_i$ modifiers had no influence on the ATP effect, although a marked increase in $[Ca^{2+}]_i$ by ionomycin was detected and could be completely blocked by BAPTA/AM (data not shown). Treatment with 5 μM PMA for 20 min also did not reduce the increment in the cAMP level induced by ATP, although PMA by itself caused a slight increase in the cAMP level (data not shown). Our results suggest that ATP-mediated activation of adenylyl cyclase in HL-60 cells is not sensitive to Ca^{2+} or PKC.

We examined the type of G protein involved in the ATP-mediated cAMP generation by treating the cells with CTX and PTX. Cells treated with CTX for 15 min responded with a 2.3-fold increase in the cAMP level (data

TABLE 1. Effects of nucleotides and ATP analogues on $[Ca^{2+}]_i$ elevation and cAMP generation

Agonist	Net increase in $[Ca^{2+}]_i$ (nM)	cAMP generation (cAMP/(cAMP + ATP) × 1000)
Control	0	22.4 ± 2.44
ATP	562 ± 32	194.8 ± 41.9
UTP	443 ± 15	25.2 ± 3.2
GTP	344 ± 13	22.1 ± 3.4
CTP	42 ± 12	18.7 ± 2.5
ITP	477 ± 16	19.7 ± 1.5
XTP	383 ± 7	21.6 ± 1.7
ADP	384 ± 17	82.1 ± 18.1
AMP	<10	25.0 ± 1.9
Adenosine	<10	73.5 ± 13.8
AMPPCP	<10	70.9 ± 12.8
AMPPNP	212 ± 19	32.3 ± 1.4
BzATP	204 ± 17	336.1 ± 9.3
ADP-β-S	97 ± 24	61.6 ± 6.6
ATP-γ-S	447 ± 4	255.5 ± 20.5
2-MeS ATP	189 ± 12	171.8 ± 28.0
2-Cl ATP	445 ± 14	135.1 ± 33.5
dATP	470 ± 19	296.9 ± 59.7

Aliquots of HL-609 cells (5×10^5 cells) were stimulated with 300 μM concentrations of the indicated nucleotides. The changes in the fluorescence ratios at the peak heights of $[Ca^{2+}]_i$ were monitored. For the measurement of cAMP generation, [^{3}H] adenine-loaded cells were preincubated with 1 mM IBMX for 15 min and then treated with analogues for 15 min in the presence of 1 mM IBMX. The results are given as the mean ± SEM of triplicate assays.

not shown). However, in the cells treated with CTX or PTX for 24 h to downregulate the responsible G proteins, ATP triggered an elevation in cAMP (data not shown). This result is surprising, because it is generally believed that the adenylyl-cyclase-linked receptors are associated with the CTX-sensitive G protein G_s. The results suggest that adenylyl cyclase may be activated by a $\beta\gamma$ subunit of CTX-, a PTX-insensitive G protein that is linked to a P_2 purinoceptor. Among the isozymes, there may be a type IV adenylyl cyclase that is positively regulated by the $\beta\gamma$ subunit and insensitive to Ca^{2+} and PKC [20]. However, PKC-sensitive adenylyl cyclases such as types II and VII might also contribute to cAMP signaling because PMA slightly elevated the basal level of cAMP in HL-60 cells.

The present study suggests that P_2 purinoceptors may play a role in immune functions because extracellular ATP induces cAMP production in HL-60 cells. Histamine and prostaglandin E_2 elevate the cAMP level and also induce the differentiation of HL-60 cells [21]. The ATP effect on cAMP production might also have a regulatory function in the differentiation process of immune cells.

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