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Extracellular ATP Stimulates Adenylyl Cyclase and Phospholipase C Through Distinct Purinoceptors in NG108–15 Cells

ISAO MATSUOKA, QIAN ZHOU, HIROMI ISHIMOTO, and HIRONORI NAKANISHI

Department of Pharmacology, Fukushima Medical College, Fukushima 960–12, Japan

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SUMMARY

In neuroblastoma × glioma hybrid NG108–15 cells, ATP induced a concentration-dependent increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), accompanied by inositol phosphate formation. Under the same conditions, we found a marked increase in cAMP levels produced by ATP at concentrations similar to those required to increase $[Ca^{2+}]_i$. The Ca^{2+} ionophore A23187 or bradykinin, which evoked inositol phosphate formation and increases in $[Ca^{2+}]_i$, did not increase, and instead slightly decreased, cAMP content, indicating that ATP-induced cAMP accumulation was not due to activation of Ca^{2+}-sensitive adenylyl cyclase. The effect of ATP on cAMP production was not dependent on generation of adenosine caused by ATP hydrolysis. Among several P_2 purinoceptor agonists, adenosine-5′-*O*-(3-thio)triphosphate, 5′-adenylylimidodiphosphate, and adenosine-5′-*O*-(2-thio)diphosphate evoked both cAMP accumulation and Ca^{2+} mobilization. In contrast, β,γ-methylene-ATP selectively elicited cAMP accumulation, whereas 2-methylthio-ATP and UTP induced only Ca^{2+} mobilization, without affecting cAMP levels. The potent P_{2x} purinoceptor agonist α,β-methylene-ATP did not induce cAMP accumulation or Ca^{2+} mobilization. The cAMP accumulation induced by ATP was not affected by the P_2 receptor antagonist suramin but was inhibited by P_1 receptor antagonists such as 8-(*p*-sulfophenyl)theophylline, 3-isobutyl-1-methylxanthine, and xanthine amine congener. However, the ATP-induced increase in $[Ca^{2+}]_i$ was not affected by suramin or xanthine amine congener. Taken together, these results indicate that ATP activates two distinct purinoceptors that are coupled to different signal transduction systems, one being adenylyl cyclase and the other phospholipase C, in NG108–15 cells. Furthermore, pharmacological profiles of the adenylyl cyclase-coupled receptor were quite different from those of any known purinoceptor subtypes, especially in the unusual sensitivity of the receptor to P_1 and P_2 receptor agonists and antagonists. It is therefore suggested that ATP-induced cAMP accumulation may be mediated by a novel subtype of purinoceptor in NG108–15 cells.

There is now increasing evidence that indicates that extracellular adenosine and adenine nucleotides such as ATP and ADP play important roles as chemical mediators to induce various cellular responses in several tissues (1, 2). These effects induced by adenosine and adenine nucleotides are mediated by specific receptors, termed P_1 and P_2 purinoceptors (3, 4). P_1 purinoceptors preferentially interact with adenosine and AMP and appear to regulate cAMP second messenger systems (5). In contrast, P_2 receptors are activated by ATP and ADP, but not by adenosine or AMP, and are further classified into several subtypes, such as P_{2x}, P_{2y}, P_{2z}, and P_{2u} (2–4). The post-receptor signal transduction mechanisms induced by stimulation of different P_2 purinoceptors have been extensively studied using turkey erythrocytes (6, 7), phagocytic leukocytes (8, 9), and various cell lines in culture (10–12). In most cells, P_{2y} and P_{2u} purinoceptors have been demonstrated to induce phospholipase C activation and inositol-1,4,5-trisphosphate-mediated increases in $[Ca^{2+}]_i$ (6–8), whereas P_{2x} purinoceptors lead to activation of intrinsic ligand-gated ion channels (13). The P_{2z} subtype is involved in ATP-induced permeabilization, due to reversible formation of nonselective pores on the plasma membrane (14).

In the central and peripheral nervous systems, adenosine is well known to act as an important neuromodulator by stimulating P_1 purinoceptors. In contrast, ATP is proposed as a transmitter (15, 16) or co-transmitter acting together with other well known neurotransmitters, such as noradrenaline

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ADPβS, adenosine-5′-*O*-(2-thio)diphosphate; App(NH)p, 5′-adenylylimidodiphosphate; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; BK, bradykinin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; α,β-MeATP, α,β-methylene-ATP; β,γ-MeATP, β,γ-methylene-ATP; 2MeSATP, 2-methylthio-ATP; NECA, 5′-(*N*-ethylcarboxamido)adenosine; PGE_1, prostaglandin E_1; 8SPT, 8-(*p*-sulfophenyl)theophylline; XAC, xanthine amine congener (8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxyl]-phenyl]1,3-dipropylxanthine).

(17). Recent studies have demonstrated that ATP modulates neurotransmitter release at presynaptic terminals (4) and triggers excitation of several neurons (13). These observations suggested that P_2 purinoceptors exist not only in postsynaptic organs but also in presynaptic nerve terminals or the cell body of neurons. These effects of ATP have been considered to be mediated by Ca^{2+} signaling pathways or by modulation of ion channel activity (13, 15). Although recent studies in cultured cells from peripheral tissues demonstrated that ATP affected cellular cAMP content (10, 12, 18, 19), little is known about the effect of ATP on the cAMP second messenger system in neuronal cells.

Neuroblastoma × glioma hybrid NG108–15 cells have been extensively used as a convenient model system for neuronal cells, to study receptor-operated transmembrane signaling mechanisms (20, 21). NG108–15 cells have been shown to possess P_2 purinoceptors coupled to phospholipase C, resulting in an increase in $[Ca^{2+}]_i$ (22). In the present study, we show that ATP induces marked cAMP accumulation in addition to an increase in $[Ca^{2+}]_i$. Our results suggest that cAMP accumulation and $[Ca^{2+}]_i$ mobilization produced by stimulation of NG108–15 cells with ATP occur through distinct purinoceptors.

Materials and Methods

Cell cultures. Neuroblastoma × glioma hybrid NG108–15 cells were kindly provided by Dr. H. Higashida (Kanazawa University, Kanazawa, Japan) and grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 7% (v/v) fetal calf serum, 0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine. Cells were grown in 100-mm tissue culture dishes in a humidified atmosphere of 90% air/10% CO_2 at 37°.

Measurement of cAMP levels. For cAMP determinations, cells were seeded in a 24-well culture plate at a density of ~5 × 10^4 cells/well and were used just after growing to confluence. Cells were washed twice with a HEPES buffer containing 130 mM NaCl, 4.7 mM KCl, 4.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11.5 mM dextrose, 0.1% bovine serum albumin, and 10 mM HEPES, pH 7.4, and were preincubated for 10 min at 37°. Reaction was initiated by addition of test reagents and was carried out for 10 min at 37° unless otherwise indicated. At the end of the incubation time, reaction was terminated by removal of the medium, followed immediately by addition of 0.3 ml of 5% trichloroacetic acid. The trichloroacetic acid extract was washed three times with 2–3 volumes of diethyl ether and assayed for cAMP by radioimmunoassay, as described previously (23).

Measurement of [^{3}H]inositol phosphate formation. Total [^{3}H]inositol phosphate formation was measured as described previously (24). NG108–15 cells were seeded in a 12-well culture plate at a density of ~10^5 cells/well. After growing to subconfluence, cells were incubated for 12–18 hr with *myo*-[2-^{3}H]inositol (2–3 μCi/ml) in the culture medium. Cells were washed twice with HEPES buffer and preincubated for 10 min at 37° in the presence of 10 mM LiCl. Reaction was initiated by addition of test reagents and carried out for 10 min. Inositol phosphate formation was terminated by addition of 0.3 ml of methanol after aspiration of the incubation medium. The methanol extract was collected into a glass tube and cells were rinsed with an additional 0.3 ml of methanol. Samples of the pooled methanol extract were mixed with 0.5 ml of chloroform and 0.45 ml of distilled water, vigorously vortex-mixed, and then centrifuged at 1500 × *g* for 10 min. Aliquots of the upper aqueous phase (0.75 ml) were diluted to 10 ml with distilled water and loaded onto AG1-X8 (100–200 mesh, formate form) columns (0.8-ml bed volume). After the columns were washed with 8 ml of distilled water and 8 ml of 50 mM ammonium formate, total [^{3}H]inositol phosphates were eluted with 6 ml of 1 M ammonium formate in 0.1 M formic acid. The eluate was mixed with 8 ml of scintillation cocktail and counted.

Measurement of $[Ca^{2+}]_i$. NG108–15 cells grown to confluence in 150-mm culture dishes were washed twice with phosphate-buffered saline and harvested with HEPES buffer containing 0.2% bovine serum albumin. Cells were pelleted by centrifugation at 150 × *g* for 2 min and resuspended in 10 ml of HEPES buffer. The cell suspension was incubated with 1 μM fura-2/acetoxymethyl ester in a shaking water bath at 37° for 20 min. Cells were then washed twice and finally resuspended at 2–4 × 10^6 cells/ml in the same buffer. These cells were kept at room temperature during experiments. Just before determinations, aliquots of cell suspension (1 ml) were centrifuged at 200 × *g* for 1 min in a microcentrifuge. The supernatant medium was discarded, and cells were resuspended in 2 ml of fresh HEPES buffer that had been prewarmed to 37°. The cell suspension was transferred into a 10- × 10-mm quartz cuvette placed in the thermostat-regulated sample chamber of a dual-excitation beam spectrofluorometer (Hitachi F-2000). The cell suspension was continuously stirred with a circular stirring bar. The excitation wavelengths were 340 and 380 nm, and fura-2 fluorescence emission was measured at 510 nm. At the end of the measurement, Triton X-100 was added to the cell suspension to obtain maximal fluorescence and then excess EGTA was added to obtain minimal fluorescence. The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at two excitation wavelengths, with a K_d value of 224 nM for the fura-2/Ca^{2+} equilibrium, as described by Grynkiewicz *et al.* (25).

Measurement of extracellular ATP metabolism. NG108–15 cells grown to confluence in a six-well culture plate were washed twice, preincubated for 10 min, and incubated with 2 ml of HEPES buffer containing 100 μM ATP. At several times, aliquots of incubation medium (0.25 ml) were collected in microcentrifuge tubes, followed immediately by centrifugation at 12,000 × *g* for 1 min to eliminate a trace amount of detached cells. The supernatants (0.2 ml) were stored at −20° until use. Adenine nucleotides (ATP, ADP, and AMP) and adenosine in the samples of incubation medium were analyzed using a Nihon Bunko HPLC system equipped with a Finepak SIL C_{18} column (Nihon Bunko) and were detected by UV absorption at 258 nm. The column was equilibrated with 50 mM NaH_2PO_4, pH 4.5, and maintained at a flow rate of 1 ml/min. After injection of 0.1 ml of sample, elution was performed with 50 mM NaH_2PO_4, pH 4.5, for the first 7 min. The elution buffer was then changed to 50 mM NaH_2PO_4, pH 2.5, and elution was continued for an additional 30 min. Before the next sample analysis, the column was re-equilibrated with 50 mM NaH_2PO_4, pH 4.5, for >20 min. The recoveries of adenine nucleotides and adenosine were >95%.

Materials. High-glucose Dulbecco's modified Eagle's medium was purchased from GIBCO. Fetal calf serum (Bioserum lot 10220–01) was from United Biotechnological Co. (Tokyo, Japan). ATP, ADP, App(NH)p, α,β-MeATP, β,γ-MeATP, ATPγS, ADPβS, GTP, adenosine deaminase, creatine phosphokinase, creatine phosphate, IBMX, and NECA were obtained from Sigma Chemical Co. (St. Louis, MO). Adenosine, AMP, fura-2/acetoxymethyl ester, and UTP were obtained from Wako Pure Chemicals (Osaka, Japan). XAC, 2MeSATP, and 8SPT were from Research Biochemicals (Natick, MA). Forskolin and Ro20–1724 were from Calbiochem (La Jolla, CA). Anti-succinyl-cAMP rabbit antiserum was kindly provided by Yamasa Shoyu Co. (Chousi, Japan). [5′,8-^{3}H]cAMP (38 Ci/mmol) was purchased from Amersham Japan (Tokyo, Japan). *myo*-[2-^{3}H]Inositol was from American Radiolabeled Chemicals (St. Louis, MO).

Results

Effects of ATP on $[Ca^{2+}]_i$, [^{3}H]inositol phosphate formation, and cAMP levels in NG108–15 cells. In fura-2-loaded NG108–15 cells, ATP (100 μM) caused a rapid and transient increase in $[Ca^{2+}]_i$; the maximum was reached

within 10 sec, followed by a decrease to nearly basal levels by 1 min (Fig. 1A). This effect of ATP was concentration dependent, with an EC_{50} value of approximately 10 μM, and could be observed in the absence of extracellular Ca^{2+} (data not shown). In [^{3}H]inositol-labeled cells, ATP (100 μM) increased total inositol phosphate formation >3-fold above the control value (Fig. 1C), indicating that ATP induces phospholipase C activation, resulting in a subsequent increase in $[Ca^{2+}]_i$ by inositol-1,4,5-trisphosphate-mediated Ca^{2+} mobilization from intracellular Ca^{2+} stores. These results are consistent with a recent study reported by Lin *et al.* (22). Similar effects were obtained upon stimulation with BK (10 nM), which resulted in a transient increase in $[Ca^{2+}]_i$ accompanied by total inositol phosphate accumulation (Fig. 1, B and C).

Under the same conditions, we found that extracellular ATP (100 μM) produced a significant increase in cAMP levels (about 10-fold above the basal value) (Fig. 1D). ATP-induced cAMP accumulation was not related to an increase in $[Ca^{2+}]_i$, because BK (10 nM), which was equipotent with 100 μM ATP in Ca^{2+} mobilization, as shown above, and the Ca^{2+} ionophore A23187 failed to increase, and instead decreased, cAMP levels in NG108–15 cells (Fig. 1D).

Lack of involvement of adenosine generation by ATP hydrolysis in ATP-induced cAMP accumulation. NG108–15 cells have been shown to possess an adenosine receptor coupled to adenylyl cyclase activation (26). We observed that adenosine (10 μM) indeed increased cAMP levels about 6-fold in these cells (Fig. 2A). ATP-induced increases in cAMP might therefore reflect ATP hydrolysis to, or contamination by, adenosine. However, ATP at the same concentration (10 μM) was as potent as adenosine (Fig. 2A), suggesting that a trace amount of adenosine, if present, cannot account for the effect of ATP on cAMP levels. Moreover, in the presence of adenosine deaminase (1 unit/ml), which catalyzes the deamination of adenosine to inosine (an inactive metabolite at adenosine receptors), adenosine-induced cAMP accumulation was abolished, whereas ATP could still elicit significant increases in cAMP levels (Fig. 2A). Inclusion of ATP-regenerating agents (25 mM creatine phosphate together with 25 units/ml creatine phosphokinase) did not affect the ATP-induced cAMP accumulation (Fig. 2B).

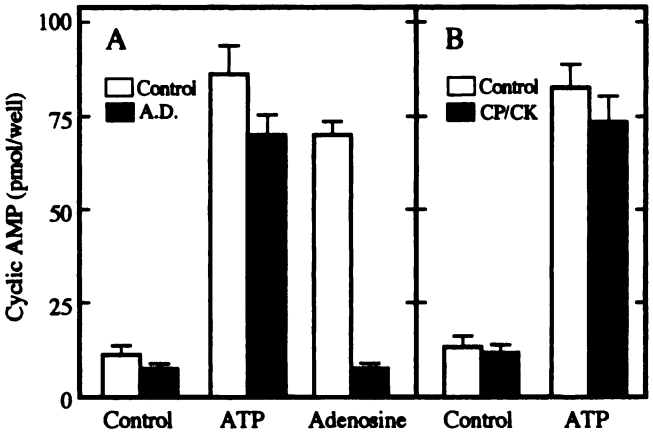

Fig. 2. Effects of adenosine deaminase and an ATP-regenerating system on ATP-induced cAMP accumulation. A, Cells and a 100 μM solution of ATP or adenosine were separately preincubated with or without adenosine deaminase (*A.D.*) (1 unit/ml) at 37° for 10 min before the incubation was started. Reaction was initiated by addition of ATP or adenosine at a final concentration of 10 μM, and the incubation was performed at 37° for 10 min. B, Experimental conditions were the same as in A, except that cells and the 100 μM ATP solution were preincubated with or without an ATP-regenerating system (10 mM creatine phosphate and 25 units/ml creatine phosphokinase) (*CP/CK*) for 10 min at 37°. Data are mean ± standard error from triplicate determinations, and the results are representative of two similar experiments.

We next examined ATP hydrolysis under our experimental conditions using HPLC analysis. Fig. 3, *upper*, shows an elution profile for ATP, ADP, AMP, and adenosine in the standard solution. No detectable amount of adenosine, AMP, or ADP was present in the initial incubation medium containing 100 μM ATP (data not shown). During the incubation of NG108–15 cells with 100 μM ATP for 45 min, the amount of extracellular ATP decreased linearly ($t_{1/2}$ = 28 ± 3 min, four experiments), with a parallel generation of its hydrolyzed metabolites, mainly ADP. Further hydrolysis of ADP to AMP occurred to a small extent after 25 min of incubation, but adenosine generation was not observed at this time point. After incubation for 45 min, almost all ATP added was recovered as ATP, ADP, AMP, and adenosine, at a ratio of 0.38:0.45:0.15:0.02, on average (Fig. 3). These results suggest that adenosine and AMP do not contribute to the ATP-induced cAMP accumulation, at least within the 10-min incubation period.

Time course of the effects of ATP and adenosine. The time courses for the effects of ATP and adenosine on cAMP levels in NG108–15 cells are shown in Fig. 4A. Upon stimulation with ATP (100 μM), the cAMP level increased gradually, reached the maximal level (which in this case was 9-fold greater than the basal level) by 10 min, and remained at this high level for at least an additional 30 min. Adenosine-induced cAMP accumulation was more rapid, reaching the maximum at 5 min, followed by a decrease to a plateau level (7-fold above the basal level) by 10 min. Interestingly, ATP-induced cAMP accumulation was significantly facilitated in the presence of adenosine deaminase (1 unit/ml). Under these conditions, the maximal increase was observed within 2 min, the earliest time point measured, followed by a slightly lower, steady state level (about 6-fold greater than

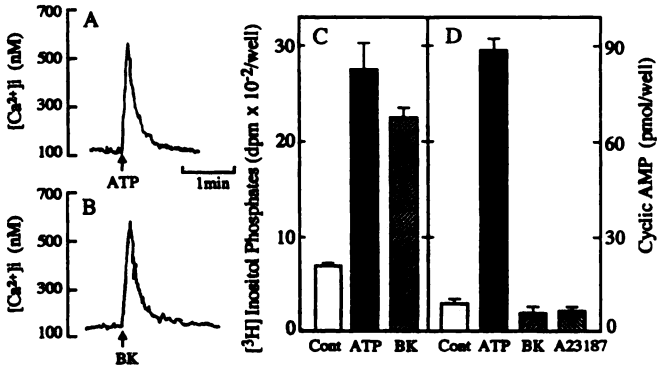

Fig. 1. Effects of ATP and BK on $[Ca^{2+}]_i$, inositol phosphate formation, and cAMP levels in NG108–15 cells. A and B, Fura-2-loaded cells were stimulated with ATP (100 μM) (A) or BK (10 nM) (B) as indicated (*arrows*), and $[Ca^{2+}]_i$ was measured as described in Materials and Methods. C, [^{3}H]inositol-labeled cells were preincubated with 10 mM LiCl at 37° for 10 min, and cAMP levels were measured by radioimmunoassay. The cells were then stimulated with ATP (100 μM) or BK (10 nM) for 10 min. *Cont*, control. D, Cells were stimulated with ATP (100 μM), BK (10 nM), or A23187 (1 μM) for 10 min. Data shown in C and D are mean ± standard error from triplicate determinations, and the results in A–D are representative of three or four experiments.

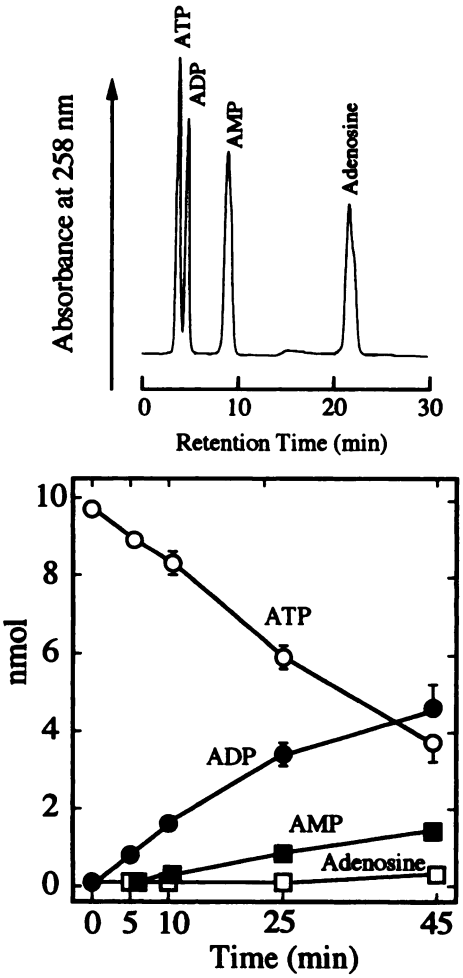

Fig. 3. *Lower*, hydrolysis of extracellular ATP by NG108–15 cells. Cells were incubated with 100 μM ATP at 37° for different time periods, and ATP (○), ADP (●), AMP (■), and adenosine (□) in the incubation medium were measured by HPLC as described in Materials and Methods. *Upper*, elution profile of 0.2 ml of standard solution containing 10 μM ATP, ADP, AMP, and adenosine. The fresh ATP solution did not contain any detectable ADP, AMP, or adenosine. Data are mean ± standard error from four experiments.

the basal level) (Fig. 4B). These results also argue against the involvement of adenosine generated by ATP hydrolysis in the cAMP response elicited by ATP.

Concentration-effect relationship for ATP. Effects of various concentrations of ATP (0.01–1000 μM) on cAMP levels were investigated after a 10-min incubation period (Fig. 5). ATP caused cAMP accumulation in a concentration-dependent manner, with an EC_{50} value of 11 ± 3 μM (eight experiments), and the maximal effect was usually obtained with 100 μM ATP. At concentrations higher than 100 μM, the ATP-induced cAMP response declined in some experiments. This concentration-dependent effect of ATP was greatly potentiated by Ro20–1724 (100 μM), a phosphodiesterase inhibitor, which increased the maximal response about 5-fold and shifted the EC_{50} value to the left about 3-fold (Fig. 5A). In some cell types, including hepatocytes (10) and FRTL-5 thyroid cells (18), ATP has been shown to inhibit forskolin-induced cAMP accumulation through activation of inhibitory G proteins. In NG108–15 cells, however, ATP produced only stimulatory effects in the presence of 10 μM forskolin, at all concentrations tested (Fig. 5B).

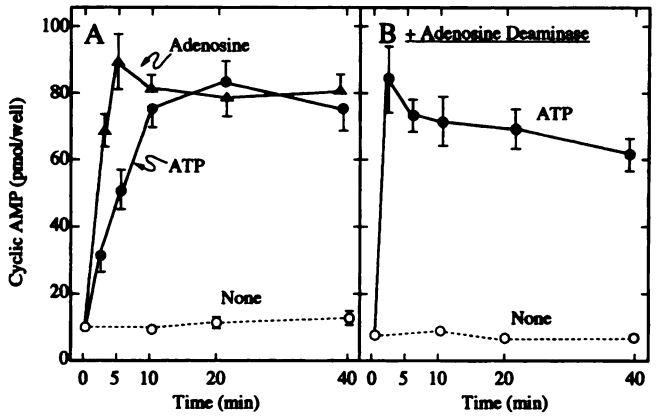

Fig. 4. Time course of cAMP accumulation induced by ATP or adenosine in NG108–15 cells. A, Cells were incubated with ATP (100 μM) (●), adenosine (100 μM) (▲), or HEPES buffer (○), at 37°, for different times as indicated. B, Cells were preincubated with adenosine deaminase (1 unit/ml) at 37° for 10 min and then stimulated with 100 μM ATP (●) or HEPES buffer (○). Data are mean ± standard error from triplicate determinations and are representative of results obtained in two similar experiments.

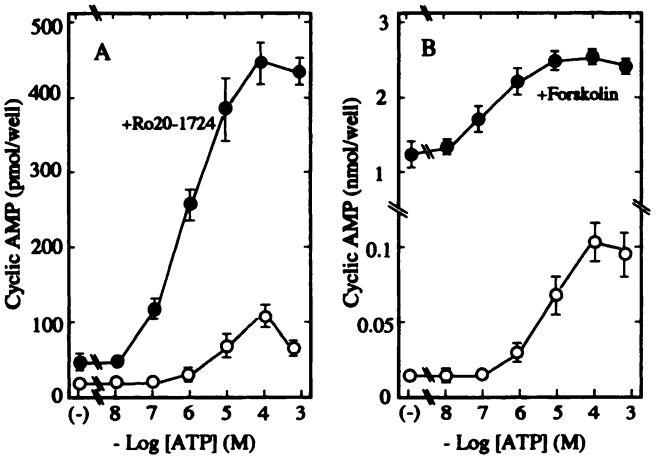

Fig. 5. Effects of different concentrations of ATP on cAMP levels in NG108–15 cells in the absence or presence of Ro20–1724 or forskolin. Cells were stimulated with increasing concentrations of ATP, in the absence (○) or presence of 100 μM Ro20–1724 (●) (A) or 10 μM forskolin (●) (B), at 37° for 10 min. Data are mean ± standard error from triplicate determinations and are representative of results obtained in three experiments.

Effects of purinoceptor agonists on cAMP levels and $[Ca^{2+}]_i$. The effects of various purinoceptor agonists (at 100 μM) on the cAMP level and $[Ca^{2+}]_i$ are summarized in Table 1. Similarly to ATP and adenosine, ADP and AMP induced cAMP accumulation. Like ATP, ADP also elicited an increase in $[Ca^{2+}]_i$, whereas AMP and adenosine had no effect on $[Ca^{2+}]_i$. Among several adenine nucleotide analogues, ATPγS, App(NH)p, and ADPβS induced both cAMP accumulation and a rise in $[Ca^{2+}]_i$. The P_{2y} purinoceptor agonist 2MeSATP and P_{2u} purinoceptor agonists such as UTP and GTP increased $[Ca^{2+}]_i$ without affecting the cellular cAMP content, whereas β,γ-MeATP induced only cAMP accumulation. In contrast, α,β-MeATP, a potent P_{2x} purinoceptor agonist, could not elicit any effect. dATP had a weak effect on $[Ca^{2+}]_i$ but not on the cellular cAMP level. These results suggest that NG108–15 cells possess at least two distinct ATP receptors; one produces the cAMP response and the

TABLE 1

Effects of several nucleotides on the cellular cAMP level and $[Ca^{2+}]_i$ in NG108–15 cells

Cells were incubated with the specified compounds (100 μM) for 10 min, and cAMP levels were measured by radioimmunoassay. For $[Ca^{2+}]_i$ assays, cells were loaded with fura-2 and stimulated with the specified compounds (100 μM). Data were taken from the peak $[Ca^{2+}]_i$. Results are presented as percentages of the response induced by ATP.

Nucleotides	cAMP level	$[Ca^{2+}]_i$
	%	%
ATP	100	100
ADP	112	85
AMP	68	NE[a]
Adenosine	93	NE
dATP	NE	<5
ATPγS	120	98
ADPβS	58	68
App(NH)p	95	42
α,β-MeATP	<5	NE
β,γ-MeATP	146	NE
2MeSATP	NE	57
UTP	NE	113
GTP	NE	32

[a] NE, no effect.

other induces the $[Ca^{2+}]_i$ response. The effects of several agonists on $[Ca^{2+}]_i$ determined in the present study are consistent with recent reports that suggested that ATP-stimulated Ca^{2+} mobilization was mediated by P_{2u} purinoceptors (22).

To examine the effects of purinoceptor agonists on cAMP generation in more detail, concentration-effect relationships were examined in the presence of 100 μM Ro20–1724 (Fig. 6). All adenine nucleotides, except α,β-MeATP and 2MeSATP, and adenosine induced a concentration-dependent increase in cAMP levels in NG108–15 cells. These agonists increased cAMP levels to similar extents at the maximally effective concentrations. The rank order of agonist potency, as determined from EC_{50} values, was β,γ-MeATP > ADP ≥ ATP = ATPγS = App(NH)p = adenosine > ADPβS > AMP. These

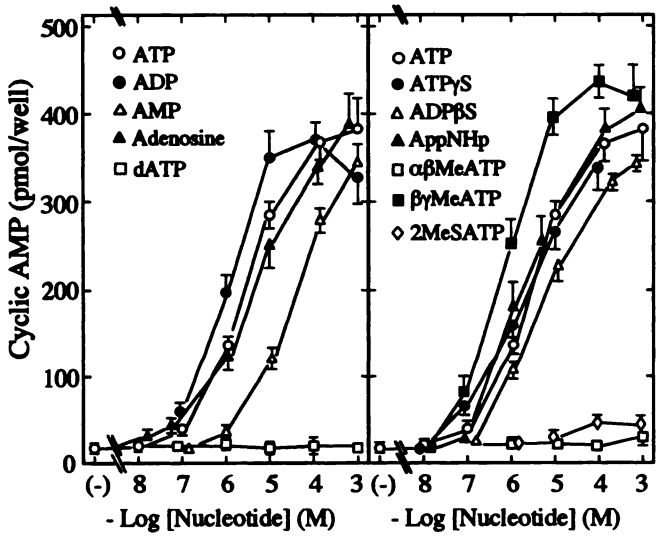

Fig. 6. Dose-dependent effects of adenine nucleotides and adenosine on cAMP levels in NG108–15 cells. Cells were incubated at 37° for 10 min in the presence of 100 μM Ro20–1724, with increasing concentrations of adenine nucleotides and adenosine as indicated. Data are mean ± standard error from triplicate determinations and are representative of results obtained in two experiments.

pharmacological characteristics do not correspond to those of any known subclass of P_1 or P_2 purinoceptors.

Effects of purinoceptor antagonists on ATP-induced cAMP accumulation and Ca^{2+} mobilization. We next investigated the effects of several purinoceptor antagonists on cAMP accumulation induced by ATP or adenosine (Fig. 7, A and B). Suramin, a P_2 purinoceptor antagonist, at concentrations up to 100 μM did not inhibit the cAMP accumulation induced by ATP (10 μM) or adenosine (10 μM). In contrast, 8SPT, IBMX, and XAC, which are known as P_1 purinoceptor antagonists, produced concentration-dependent inhibition of the cAMP accumulation induced by either ATP (10 μM) or adenosine (10 μM). These experiments were performed in the presence of Ro20–1724 (100 μM) to minimize the inhibitory effects of xanthine derivatives on the cAMP phosphodiesterase activity. The concentration-inhibition curves for the effects of the xanthine derivatives on the ATP-induced response were similar to those for the adenosine-induced response. The rank order of inhibitory potency was XAC > 8SPT ≥ IBMX. Although the basal cAMP level in NG108–15 cells was also significantly decreased in the presence of 8SPT, IBMX, and XAC, these compounds did not affect the cAMP accumulation induced by PGE_1 (data not shown).

The effects of suramin and xanthine derivatives on ATP-induced increases in $[Ca^{2+}]_i$ were also examined in fura-2-loaded cells. For this experiment, we used suramin (30 μM) and XAC (1 μM) as the test agents, because 8SPT itself has marked fluorescence, and a high concentration of suramin (above 50 μM) quenched the fura-2 fluorescence. As shown in Fig. 7C, suramin (30 μM) and XAC (1 μM) had little effect on ATP (10 μM)-induced increases in $[Ca^{2+}]_i$.

Influences of chronic exposure to NECA on the effects of ATP, β,γ-MeATP, adenosine, and PGE_1. Based on the sensitivity to xanthine derivatives, it is possible to assume that the effects of ATP and adenosine on cAMP accumulation are mediated by the same receptor population. If so, then chronic exposure of the cells to adenosine receptor agonists such as NECA should cause a parallel decrease in the ATP- and adenosine-induced cAMP responses, because such treatment has been demonstrated to produce desensitization of adenosine receptor-mediated adenylyl cyclase activation (26). As shown in Fig. 8, pretreatment of NG108–15 cells with NECA (10 μM) for 18 hr markedly reduced (by 80–90%) cAMP accumulation induced by NECA (10 μM) or adenosine (100 μM), indicating that the adenosine receptor had been desensitized. Under these conditions, subsequent responses to ATP (100 μM) and β,γ-MeATP (100 μM) were also attenuated by 70–80%, whereas PGE_1 (1 μM)-induced cAMP accumulation was not affected. In contrast, ATP (100 μM)-induced Ca^{2+} mobilization was not inhibited by the NECA pretreatment (data not shown).

Discussion

In NG108–15 cells, ATP was reported to induce a rapid increase in $[Ca^{2+}]_i$ as a consequence of phospholipase C-catalyzed polyphosphoinositide hydrolysis (22). In the present study, we demonstrate that extracellular ATP elicits marked cAMP accumulation in addition to phospholipase C activation and a rise in $[Ca^{2+}]_i$ in NG108–15 cells. ATP-induced cAMP accumulation in NG108–15 cells was greatly potentiated by Ro20–1724, a cAMP phosphodiesterase inhib-

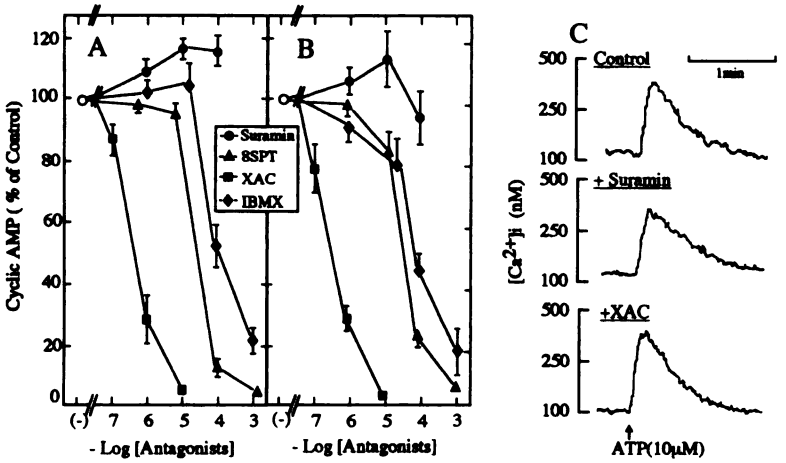

Fig. 7. Effects of suramin, XAC, 8SPT, and IBMX on cAMP accumulation induced by ATP (A) or adenosine (B) and on the ATP-induced increase in $[Ca^{2+}]_i$ (C) in NG108–15 cells. A and B, Cells were incubated at 37° for 10 min with ATP (10 μM) or adenosine (1 μM), in the absence (○) or presence of different concentrations of suramin (●), XAC (▲), 8SPT (■), or IBMX (♦). Experiments were performed in the presence of Ro20–1724 (100 μM). Data are mean ± standard error from triplicate determinations and are representative of results obtained in two experiments. The basal cAMP level was 18.4 ± 1.6 pmol/well, which was increased to 364 ± 14 pmol/well or 328 ± 19 pmol/well by 10 μM ATP or 10 μM adenosine, respectively. C, Fura-2-loaded cells were preincubated for 1 min without (control) or with suramin (30 μM; the highest concentration that did not affect the fura-2 fluorescence) or XAC (1 μM) and were stimulated with 10 μM ATP as indicated (*arrow*). Data shown are representative of results from three similar experiments.

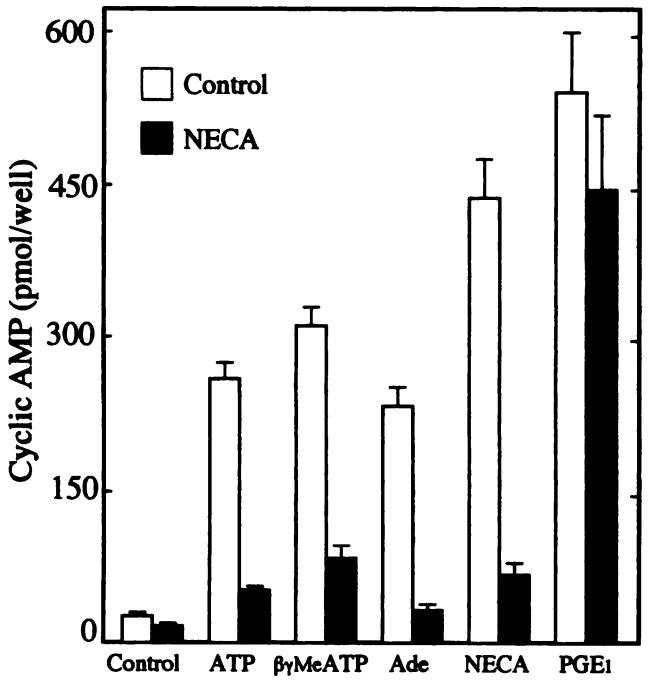

Fig. 8. Effect of pretreatment of NG108–15 cells with NECA on subsequent responses to ATP, β,γ-MeATP, adenosine, NECA, and PGE_1. NG108–15 cells were pretreated with (■) or without (□) 10 μM NECA for 18 hr. Cells were incubated in the presence of 100 μM Ro20–1724 at 37° for 10 min, with 100 μM ATP, 100 μM β,γ-MeATP, 100 μM adenosine (*Ade*), 10 μM NECA, or 1 μM PGE_1. Data are mean ± standard error from triplicate determinations and are representative of results obtained in two similar experiments.

itor, indicating that the effect is due to an increase in cAMP generation, most likely resulting from stimulation of adenylyl cyclase. Recently, ATP-induced cAMP accumulation has been reported in human T lymphocyte Jurkat cells (19), rat type II pneumocytes (12), bovine aortic smooth muscle cells (11), and mouse C2C12 myotubles (27). The present study appears to be the first indication that such cAMP responses to extracellular ATP are found in cells of neuronal origin.

Recent molecular cloning studies have revealed that several different subtypes of adenylyl cyclase exist in mammalian cells (28). Among them, type I (29), type III (30), and type VIII (31) adenylyl cyclases are known to be activated by increases in $[Ca^{2+}]_i$, in a manner dependent upon calmodulin. Because ATP-induced cAMP accumulation and increases in $[Ca^{2+}]_i$ showed very similar concentration-response curves in NG108–15 cells, such Ca^{2+}-sensitive adenylyl cyclases could possibly contribute to the ATP-induced cAMP accumulation. However, we have observed that NG108–15 cells minimally express these Ca^{2+}/calmodulin-sensitive adenylyl cyclases and predominantly possess the type VI adenylyl cyclase, an isoform that is inhibited by Ca^{2+}.[1] The following lines of evidence shown in the present study are also contradictory to the involvement of Ca^{2+}-sensitive adenylyl cyclases in the response to ATP: 1) the Ca^{2+} ionophore A23187 or BK, which evoked an increase in $[Ca^{2+}]_i$ similar in extent to that induced by ATP, did not increase, and instead slightly decreased, the cAMP level, 2) some purinoceptor agonists, such as 2Me-SATP and UTP, increased $[Ca^{2+}]_i$ without affecting cAMP levels, and 3) another purinoceptor agonist, β,γ-MeATP, selectively induced cAMP accumulation in the absence of increases in $[Ca^{2+}]_i$. Therefore, one can conclude that the effect of ATP on the cAMP level is independent of that on $[Ca^{2+}]_i$.

These results also lead to the idea that NG108–15 cells express at least two distinct ATP receptors that are coupled to different signal transduction mechanisms; one stimulates cAMP production by adenylyl cyclase activation, and the other increases $[Ca^{2+}]_i$ by phospholipase C activation. This idea is further supported by the findings that XAC inhibited ATP-induced cAMP accumulation without affecting Ca^{2+} mobilization and that chronic exposure to NECA resulted in a marked reduction in ATP-induced cAMP accumulation without changing the Ca^{2+} response. Therefore, the characteristics of a putative receptor that mediates ATP-induced cAMP accumulation are somewhat similar to those of P_1 purinoceptors. These unusual pharmacological characteristics of the ATP-mediated cAMP response are discussed below.

Recently, Lin *et al.* (22) reported that extracellular ATP increased $[Ca^{2+}]_i$, with a parallel formation of inositol phosphates, in NG108–15 cells. On the basis of the effects of various nucleotides and ATP analogues on the Ca^{2+} mobilization, those authors proposed that the effects of ATP are mediated by P_{2u} purinoceptors. Furthermore, Lustig *et al.* (32) recently cloned the P_{2u} purinoceptor cDNA from NG108–15 cells, indicating that P_{2u} purinoceptors play an

[1] I. Matsuoka, H. Nakanishi, and J. Hanoune, unpublished observations.

essential role in ATP-induced phospholipase C activation and increases in $[Ca^{2+}]_i$. The results obtained in the present study (Table 1) are consistent with this idea and further suggest that P_{2x} purinoceptors are not involved in the ATP-induced Ca^{2+} response, by demonstrating an inability of α,β-MeATP, a potent P_{2x} purinoceptor agonist, to increase $[Ca^{2+}]_i$.

Before the adenylyl cyclase-coupled ATP receptor is discussed, a possible indirect action of ATP through its hydrolyzed metabolites should be considered. It is well known that adenosine is a potent agonist to increase cAMP levels via A_2 adenosine receptors (1). NG108–15 cells have been shown to possess an adenosine receptor coupled to adenylyl cyclase activation (26). We also observed adenosine-induced cAMP production in NG108–15 cells. It is therefore possible that the ATP-induced cAMP accumulation reported here could potentially be explained by ATP hydrolysis to, or contamination by, adenosine. However, the following findings indicate that adenosine scarcely contributes to ATP-induced cAMP accumulation in NG108–15 cells. First, ATP was equipotent with, and in some case more effective than, adenosine in cAMP production in NG108–15 cells. Second, the addition of adenosine deaminase or ATP-regenerating agents did not impair the ATP-induced cAMP accumulation, whereas the effect of adenosine was abolished by addition of adenosine deaminase. Third, direct analysis of ATP hydrolysis by HPLC demonstrated no contamination by adenosine in the original ATP solution and little generation of adenosine by ATP hydrolysis during the course of the 10-min incubation period that we used for cAMP measurement. These results suggest that ATP itself can act as an agonist to stimulate cAMP production in NG108–15 cells. The existence of a specific receptor for ATP coupled to adenylyl cyclase activation is further supported by the evidence that several ATP analogues, including ATPγS, App(NH)p, and β,γ-MeATP, that are thought to be metabolically more stable than ATP were also able to stimulate cAMP production.

The effect of ATP on the cAMP level was first reported by Okajima *et al.* (10) in cultured hepatocytes, in which the specific receptor for ATP is negatively coupled to adenylyl cyclase, leading to inhibition of forskolin-induced cAMP accumulation. In NG108–15 cells, however, ATP had no inhibitory effect on forskolin-stimulated cAMP accumulation at concentrations ranging from 10 nM to 1 mM. The ATP receptor coupled to adenylyl cyclase in NG108–15 cells showed several unique characteristics. The first is that the receptor did not respond to either a P_{2x} agonist (α,β-MeATP), a P_{2y} agonist (2MeSATP), or a P_{2u} agonist (UTP). The order of potency of adenine nucleotides and analogues at this receptor [β,γ-MeATP > ADP ≥ ATP = App(NH)p = ATPγS > ADPβS] does not fit that expected for any known P_2 purinoceptors thus far reported (2, 6). These pharmacological characteristics are also quite different from recent observations that demonstrated ATP-induced cAMP accumulation in mouse C2C12 myotubles (27), because α,β-MeATP increased cAMP levels and suramin inhibited ATP-induced cAMP accumulation (27). ATP-induced cAMP accumulation in NG108–15 cells was not blocked by suramin but was inhibited by 8SPT, IBMX, and XAC. This is the second unusual feature as a ATP receptor, because these xanthine derivatives are characterized as P_1 purinoceptor antagonists (33, 34) and are generally found to be poor P_2 purinoceptor antagonists. It is of interest that ATP-induced cAMP accumulations demonstrated in bovine aortic smooth muscle cells (11) and rat type II pneumocytes (12) are also inhibited by P_1 purinoceptor antagonists, such as 8-cyclopentyl-1,3-dipropylxanthine and 8-phenyltheophylline, respectively.

In NG108–15 cells, adenosine elicited cAMP accumulation in a concentration range similar to that of ATP. In addition, AMP, which is thought to act at P_1 but not P_2 purinoceptors, also caused an increase in cAMP levels. A question arising from these results is whether the effects of ATP and adenosine on cAMP accumulation are mediated by two different receptors or by the same receptor population. It was shown that long term treatment of NG108–15 cells with NECA caused homologous desensitization of adenosine receptor-mediated adenylyl cyclase activation (26). The present study demonstrates that pretreatment of NG108–15 cells with NECA for 18 hr resulted in marked reductions in subsequent responses not only to P_1 receptor agonists (adenosine and NECA) but also to P_2 receptor agonists (ATP and β,γ-MeATP), without affecting the response to PGE_1. In addition, stimulation of NG108–15 cells with ATP in the presence of adenosine at the maximally effective concentration produced no further increase in response beyond that caused by each agonist alone.[2] These results support the hypothesis that ATP and adenosine share a common receptor. To date, pharmacological and biochemical studies suggest that P_1 adenosine receptors are heterogeneous and divisible into A_1, A_{2a}, A_{2b}, and A_3 subtypes (33). However, these adenosine receptors have been thought to be insensitive to ATP and its analogues. Indeed, recent molecular cloning and functional expression studies confirmed that ATP is a poor agonist at these adenosine receptors (35, 36). Keen *et al.* (26) reported that the characteristics of [^{3}H]NECA binding sites in NG108–15 cells were different from those of typical A_2 adenosine receptors. Taken together, these results suggest that the ATP receptor coupled to adenylyl cyclase activation in NG108–15 cells represents a novel purinoceptor subtype that cannot be explained by the P_1/P_2 purinoceptor classification based on the sensitivity to agonists and antagonists and the signal transduction mechanism.

With respect to the atypical purinoceptors, Shinozuka *et al.* (37) demonstrated that the prejunctional purinoceptors that mediate ATP-induced inhibition of noradrenaline release from the sympathetic nerves in rat caudal artery exhibited unusual characteristics that were different from those of known P_1 and P_2 receptors. Interestingly, the characteristics are quite similar to those of the adenylyl cyclase-coupled purinoceptors shown in the present study. For example, adenosine and ATP were equipotent in inhibiting noradrenaline release, and these effects of adenosine and ATP were inhibited by 8SPT (37). In addition, β,γ-MeATP was found to be a potent agonist, whereas α,β-MeATP had no effect (37). It is therefore possible that the adenylyl cyclase-coupled purinoceptor in NG108–15 cells and the prejunctional purinoceptor described by Shinozuka *et al.* could be classified into the same category. More recently, a prejunctional purinoceptor with a similar pharmacological profile has been found in rabbit ear artery, in which ATP and adenosine stimulated noradrenaline release from the sympathetic nerve terminal (38, 39).

[2] I. Matsuoka, Q. Zhou, and H. Nakanishi, unpublished observations.

In conclusion, extracellular ATP induces cAMP accumulation and Ca^{2+} mobilization by activating two distinct purinoceptors in NG108–15 cells. Although ATP-induced Ca^{2+} responses are mediated by the P_{2u} purinoceptor subtype, the cAMP response induced by ATP is mediated by a novel purinoceptor subtype that responds to both P_1 and P_2 purinergic agonists. In NG108–15 cells, cAMP is known to be involved in the regulation of cell functions, including promotion of differentiation (20, 21). The fact that ATP simultaneously activates two important signal transduction systems, i.e., the phospholipase C and adenylyl cyclase pathways, indicates that the physiological response of these cells to ATP might be regulated by cross-talk between these two signaling mechanisms. Although the existence of ATP receptors coupled to adenylyl cyclase stimulation in the nervous system remains to be investigated, the effect of ATP on cAMP accumulation in NG108–15 cells demonstrated in the present study could be important for understanding the physiological role of ATP, not only in neuronal cells but also in other organs.

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References

1. Stiles, G. L. Adenosine receptors: structure, function and regulation. *Trends Pharmacol. Sci.* **7**:486–490 (1986).
2. Gordon, J. L. Extracellular ATP: effects, sources and fate. *Biochem. J.* **233**:309–319 (1986).
3. Burnstock, G., and N. J. Buckley. The classification of receptors for adenosine and adenine nucleotides. *Methods Pharmacol.* **6**:193–212 (1985).
4. Williams, M. Purine receptors in mammalian tissues: pharmacology and functional significance. *Annu. Rev. Pharmacol. Toxicol.* **27**:315–345 (1987).
5. Van Calker, D., M. Muller, and B. Hamprecht. Adenosine regulates, via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* **33**:999–1005 (1979).
6. Harden, T. K., J. L. Boyer, H. A. Brown, C. L. Cooper, R. A. Jeffs, and M. W. Martin. Biochemical properties of a P_{2Y}-purinergic receptor. *Ann. N. Y. Acad. Sci.* **603**:256–266 (1990).
7. Cooper, C. L., A. J. Morris, and T. K. Harden. Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2y}-purinergic receptor. *J. Biol. Chem.* **264**:6202–6206 (1989).
8. Xing, M., F. Thevenod, and R. Mattera. Dual regulation of arachidonic acid release by P_{2u} purinergic receptors in dibutyryl cyclic AMP-differentiated HL-60 cells. *J. Biol. Chem.* **267**:6602–6610 (1992).
9. Dubyak, G. R., and C. El-Moatassim. Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* **265**:C577–C606 (1993).
10. Okajima, F., Y. Tokumitsu, Y. Kondo, and M. Ui. P_2-purinergic receptors are coupled to two signal transduction systems, leading to inhibition of cAMP generation and to production of inositol trisphosphate in rat hepatocytes. *J. Biol. Chem.* **262**:13483–13490 (1987).
11. Tada, S., F. Okajima, Y. Mitsui, Y. Kondo, and M. Ui. P_2 purinoceptor-mediated cyclic AMP accumulation in bovine vascular smooth muscle cells. *Eur. J. Pharmacol.* **227**:25–31 (1992).
12. Griese, M., L. I. Gobran, and S. A. Rooney. A_2 and P_2 purine receptor interactions and surfactant secretion in primary cultures of type II cells. *Am. J. Physiol.* **261**:L140–L147 (1991).
13. Illes, P., and W. Norenberg. Neuronal ATP receptors and their mechanism of action. *Trends Pharmacol. Sci.* **14**:50–54 (1993).
14. Steinberg, T. H., A. S. Newman, J. A. Swanson, and S. C. Silverstain. ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem.* **262**:8884–8888 (1987).
15. Su, C. Purinergic neurotransmission and neuromodulation. *Annu. Rev. Pharmacol. Toxicol.* **23**:397–411 (1983).
16. Nakanishi, H., and H. Takeda. The possibility that adenosine triphosphate is an excitatory transmitter in guinea-pig seminal vesicle. *Jpn. J. Pharmacol.* **22**:269–270 (1972).
17. Nakanishi, H., and H. Takeda. The possible role of adenosine triphosphate in chemical transmission between the hypogastric nerve terminal and seminal vesicle in the guinea-pig. *Jpn. J. Pharmacol.* **23**:479–490 (1973).
18. Okajima, F., K. Sato, M. Nazarea, K. Sho, and Y. Kondo. A permissive role of pertussis toxin substrate G-protein in P_2-purinergic stimulation of phosphoinositide turnover and arachidonate release in FRTL-5 thyroid cells: cooperative mechanism of signal transduction systems. *J. Biol. Chem.* **264**:13029–13037 (1989).
19. Tokumitsu, Y., Y. Yanagawa, and Y. Nomura. Stimulation of DNA synthesis in Jurkat cells by synergistic action between adenine and guanine nucleotides. *FEBS Lett.* **288**:81–85 (1991).
20. Higashida, H., T. Kato, T. K. Kano, M. Okuya, A. Miyake, and T. Tanaka. Proliferation and synapse formation of neuroblastoma-glioma hybrid cells: effects of glia maturation factor. *Brain Res.* **214**:287–299 (1981).
21. Nirenberg, M., S. Wilson, H. Higashida, A. Rotter, N. Krueger, N. Busis, R. Ray, J. G. Kenimer, and M. Adler. Modulation of synapse formation by cyclic adenosine monophosphate. *Science (Washington D. C.)* **222**:794–799 (1983).
22. Lin, T. A., K. D. Lustig, M. G. Sportiello, G. A. Weisman, and G. Y. Sun. Signal transduction pathways coupled to a P_{2u} receptor in neuroblastoma × glioma (NG108–15) cells. *J. Neurochem.* **60**:1115–1125 (1993).
23. Matsuoka, I., and T. Suzuki. Mepacrin-induced elevation of cyclic GMP and acceleration of reversal of ADP-induced aggregation in washed rabbit platelets. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9**:341–353 (1983).
24. Nakahata, N., T. M. Abe, I. Matsuoka, T. Ono, and H. Nakanishi. Adenosine inhibits histamine-induced phosphoinositide hydrolysis mediated via pertussis toxin-sensitive G protein in human astrocytoma cells. *J. Neurochem.* **57**:963–969 (1991).
25. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
26. Keen, M., E. Kelly, P. Nobbs, and J. MacDermot. A selective binding site for ^{3}H-NECA that is not an adenosine A_2 receptor. *Biochem. Pharmacol.* **38**:3827–3833 (1989).
27. Henning, R. H., M. Duin, H. A. den Hertog, and A. Nelemans. Characterization of P_2-purinoceptor mediated cyclic AMP formation in mouse C2C12 myotubes. *Br. J. Pharmacol.* **110**:133–138 (1993).
28. Tang, W.-J., and A. F. Gilman. Adenylyl cyclase. *Cell* **70**:869–872 (1992).
29. Krupinski, J., S. Coussen, H. A. Bakalyar, W.-J. Tang, P. G. Feinstein, K. Orth, C. Slaughter, R. R. Reed, and A. G. Gilman. Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. *Science (Washington D. C.)* **224**:1558–1564 (1989).
30. Choi, E. J., Z. Xia, and D. R. Storm. Stimulation of the type III olfactory adenylyl cyclase by calcium and calmodulin. *Biochemistry* **31**:6492–6498 (1992).
31. Cali, J. J., J. C. Zwaagstra, N. Mons, D. M. F. Cooper, and J. Krupinski. Type VIII adenylyl cyclase: a Ca^{2+}/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. *J. Biol. Chem.* **269**:12190–12195 (1994).
32. Lustig, K. D., A. K. Shiau, A. J. Brake, and D. Julius. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* **90**:5113–5117 (1993).
33. Collis, M. G., and S. M. Hourani. Adenosine receptor subtypes. *Trends Pharmacol. Sci.* **14**:360–366 (1993).
34. Bruns, R. F., G. H. Lu, and T. A. Pugsley. Characterization of A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.* **29**:331–346 (1986).
35. Yakel, J. L., R. A. Warren, S. M. Reppert, and R. A. North. Functional expression of adenosine A_{2b} receptor in *Xenopus* oocytes. *Mol. Pharmacol.* **43**:277–280 (1993).
36. Lindel, J., H. E. Taylor, A. S. Robeva, A. L. Tucker, J. H. Stehle, S. A. Rivkees, J. S. Fink, and S. M. Reppert. Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.* **44**:524–532 (1993).
37. Shinozuka, S., R. A. Bjur, and D. P. Westfall. Characterization of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. *Naunyn-Schmiedebergs Arch. Pharmacol.* **338**:221–227 (1988).
38. Ishii, R., K. Shinozuka, Y. Kobayashi, K. Hattori, T. Hashimoto, and K. Takeuchi. Methoxamine enhances the release of endogenous noradrenaline from rabbit ear artery: possible involvement of ATP. *Naunyn-Schmiedebergs Arch. Pharmacol.* **348**:46–52 (1993).
39. Ishii, R., K. Shinozuka, K. Hattori, K. Takeuchi, and T. Hashimoto. Characterization of prejunctional purinoceptor on adrenergic nerves of the rabbit ear artery. *Jpn. J. Pharmacol.* **64** (Suppl. I):187P (1994).

Send reprint requests to: Isao Matsuoka, Department of Pharmacology, Fukushima Medical College, Fukushima 960–12, Japan.