Extracellular ATP stimulates three different receptor–signal transduction systems in FRTL-5 thyroid cells

Activation of phospholipase C, and inhibition and activation of adenylate cyclase

Koichi SATO,* Fumikazu OKAJIMA and Yoichi KONDO

Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

In FRTL-5 thyroid cells, extracellular ATP, a P_2 -agonist, not only stimulates phospholipase C but also inhibits forskolin- or thyrotropin (TSH)-induced stimulation of adenylate cyclase in a pertussis toxin-sensitive manner [Okajima, Sato, Nazarea, Sho, & Kondo (1989) *J. Biol. Chem.* **264**, 13029–13037]. We have now found that, in pertussis toxin-treated cells, ATP can directly stimulate adenylate cyclase. Although adenylate cyclase modulation occurs through ATP metabolites such as AMP and adenosine, we show that extracellular ATP itself also regulates cyclic AMP production, based on the following: (1) the actions of ATP were imitated by hydrolysis-resistant ATP analogues, (2) the elimination of adenosine by adenosine deaminase decreased the effect of ATP only partially, at least at concentrations greater than 10 μ M-ATP, and (3) the amount of AMP produced from ATP was too low to account for the ATP effects. To identify the respective receptors for the three different actions of ATP, we established an antagonist profile. Suramin, which has been reported to be a P_2 -receptor antagonist, inhibited ATP-induced phospholipase C activation in a competitive fashion, but did not affect ATP-induced adenylate cyclase modulation. On the other hand, 8-cyclopentyl-1,3-diphenylxanthine competitively antagonized both the stimulatory and inhibitory ATP actions on cyclic AMP levels, but did not influence the activation of phospholipase C by ATP. The order of potency for various xanthine derivatives was clearly different with respect to their antagonistic effects on the stimulation and inhibition of adenylate cyclase induced by ATP. We conclude that ATP activates three receptors, each of which is coupled to a different signal transduction system in FRTL-5 cells, i.e. phospholipase C activation, and adenylate cyclase activation and inhibition.

INTRODUCTION

Extracellular ATP and its products of hydrolysis act via specific receptors on the cell surface to produce a variety of biological responses in many tissues and cells [1,2]. These purine nucleoside and nucleotide receptors have been classified into two subtypes, namely the P_1 -receptor, which has a high affinity for extracellular adenosine and AMP, and the P_2 -receptor, at which ATP and ADP bind more strongly [1]. The P_1 - and P_2 -receptors have been further classified into subclasses based on the differences in relative potencies of their respective agonists [1,2]. Two P_1 -receptor subclasses can also be distinguished by their function: one is responsible for negative (A_1) and the other for positive (A_2) modulation of adenylate cyclase (EC 4.6.1.1). These receptors have been purified as two different proteins from several tissues [3–7]. Four subclasses of P_2 -receptors have been defined, according to their tissue specificity as well as order of potency of agonists, and named as follows: P_{2x} and P_{2y} in smooth muscle, P_{2z} in immune cells and fibroblasts, and P_{2t} in platelets [2].

Extracellular ATP has been reported to stimulate phospholipase C (EC 3.1.4.3) in various cells, including hepatocytes [8–10], Ehrlich ascites tumour cells [11], thymocytes [12] and endothelial cells [13,14]. Boyer *et al.* have recently suggested that a 53 kDa membrane protein constitutes a P_2 -receptor in turkey erythrocytes [15]. We have found that, in FRTL-5 thyroid cells, extracellular ATP stimulates phospholipase(s), resulting in inositol phosphate production and arachidonate release [16–18].

However, the nucleotide inhibits thyroid-stimulating hormone (TSH)- and forskolin-activated adenylate cyclase [18,19]. In addition, a preliminary experiment showed that ATP stimulated cyclic AMP accumulation in pertussis toxin-treated cells. Therefore ATP may be involved in three signal transduction mechanisms in FRTL-5 cells, i.e. one leading to the stimulation of phospholipase C and the others to the inhibition and stimulation of cyclic AMP accumulation.

The aim of the present study is to establish whether the effects of ATP are caused by the interaction of ATP with different receptors, each coupled to a different signal transduction pathway, or with one receptor coupled to more than one pathway. Based on the effects of purinergic receptor antagonists on the actions of ATP, we found that ATP interacts with at least three different receptors coupled to different signal transduction systems.

EXPERIMENTAL

Materials

ATP, 8-bromo-ATP (Br-ATP), adenosine 5'-[α,β -methylene]triphosphate (pp[CH₂]pA), AMP, adenosine, N^6 -(L-2-phenylisopropyl)adenosine (PIA), forskolin, noradrenaline, adenosine deaminase (EC 3.5.4.4), phosphocreatine and creatine kinase (EC 2.7.3.2) were purchased from Sigma (St. Louis, MO, U.S.A.). Adenosine 5'-[γ -thio]triphosphate (ATP[γ S]), adenosine 5'-[β -thio]diphosphate (ADP[γ S]), adenosine 5'-[α -thio]triphosphate (ATP[α S]) and myokinase (EC 2.7.4.3) were from Boehringer–

Abbreviations used: Br-ATP, 8-bromo-ATP; pp[CH₂]pA, adenosine 5'-[α,β -methylene]triphosphate; ATP[γ S], adenosine 5'-[γ -thio]triphosphate; ADP[γ S], adenosine 5'-[β -thio]diphosphate; ATP[α S], adenosine 5'-[α -thio]triphosphate; PIA, N^6 -(L-2-phenylisopropyl)adenosine; DPX, 1,3-diethyl-8-phenylxanthine; CPX, 8-cyclopentyl-1,3-dimethylxanthine; XAC, xanthine amine congener {8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine}; TSH, thyroid-stimulating hormone (thyrotropin); RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

* To whom correspondence should be addressed.

Mannheim (Indianapolis, IN, U.S.A.). 8-Cyclopentyl-1,3-dimethylxanthine (CPX), 1,3-diethyl-8-phenylxanthine (DPX) and xanthine amine congener (XAC) were from Research Biochemicals Inc. (Wayland, MA, U.S.A.); *myo*-[2-³H]inositol (15.6 Ci/mmol) was from Du Pont-New England Nuclear (Boston, MA, U.S.A.); [2,8-³H]ATP (43.0 Ci/mmol) and [2-³H]AMP (12.0 Ci/mmol) were from Amersham International (Amersham, Bucks., U.K.). Bovine TSH was purified according to the method described in [20]. Pertussis toxin, suramin and RO 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] were generously provided by Dr Michio Ui (Tokyo University, Tokyo, Japan), Bayer (Wuppertal, Germany) and Roche Research Center (Kamakura, Japan) respectively. Reagents for the cyclic AMP radioimmunoassay were a gift from Yamasa Shoyu Co. (Choshi, Japan). Sources of reagents for cell culture and other purposes were as described in previous papers [16–19].

Cell culture

FRTL-5 cells, a cell line derived from normal rat thyroid [21], were provided by the Interthyr Research Foundation (Baltimore, MD, U.S.A.). The cells were grown in an atmosphere of CO₂/air (1:19) at 37 °C in Coon's modified F-12 medium supplemented with 5% (v/v) calf serum and six hormones, including 2 munits of TSH/ml, as described in [16,18]. When cells had become 60–70% confluent in 12-well plates, the culture medium was changed for a further 2 days to Ham's F-10 medium containing 5% (v/v) calf serum but without the six hormones. Where indicated, this medium was supplemented with pertussis toxin (20 ng/ml), [³H]inositol (4 µCi/ml) or both, as described previously [16,18]. In the case of experiments with noradrenaline, the cells were maintained in Ham's F-10 medium containing six hormones.

Measurement of [³H]inositol phosphate production

[³H]Inositol-labelled cells were washed once with Hepes-buffered medium [10 mM-Hepes (pH 7.4), 134 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH₂PO₄, 2.0 mM-CaCl₂, 2.5 mM-NaHCO₃, 1.2 mM-MgSO₄, 5 mM-glucose and 0.1% (w/v) BSA (fraction V)], preincubated for 10 min in the same medium containing 10 mM-LiCl, and incubated for further 5 min in medium containing 0.5 unit of adenosine deaminase/ml and the agent(s) to be tested. Reactions were terminated by removing the medium by aspiration, adding 1 ml of 0.1 M-HCl and freezing the cells. The supernatant (acid extract) of the thawed cells was used for the separation of [³H]InsP₂ and [³H]InsP₃ fractions, as described in [8,16]. Data were normalized to 10⁵ d.p.m. of radioactivity from unstimulated cells (before acid extraction), and are expressed as percentages of the basal values obtained in the absence of agonists.

Measurement of cyclic AMP

Cells were washed once with Ca²⁺-free Hepes-buffered medium containing 0.1 mM-EGTA [18], then incubated for 10 min at 37 °C in the same medium. The medium was replaced with the same medium (0.4 ml) containing 0.2 mM-RO 20-1724, 0.5 unit of adenosine deaminase/ml (except in the experiments shown in Fig. 1a) and various agents to be tested, including 2 nM-TSH and/or an ATP-regenerating system composed of 7.5 mM-phosphocreatine, 50 units of creatine kinase/ml and 10 units of myokinase/ml where indicated. For experiments with suramin (see Fig. 4), 50 µM-forskolin replaced TSH to avoid a possible interaction of TSH with suramin [22]. After a 5 min incubation the reaction was terminated by adding 100 µl of 1 M-HCl. Cyclic AMP in the acid extracts was measured by a sensitive and specific radioimmunoassay as described previously [23].

Measurement of the metabolism of ATP and AMP

To measure metabolic changes to and breakdown profiles of agonists, ³H-labelled ATP and AMP (1 µM/0.4 ml) were added to cells. Other experimental conditions were the same as those for the cyclic AMP experiments. After a 5 min incubation, 100 µl of the reaction medium was transferred to a tube containing 20 µl of 1 M-HCl and a mixture of ATP, AMP and adenosine (each at 5 mM). Samples of 10 µl were chromatographed on a silica gel-coated plastic sheet impregnated with a fluorescent indicator (Merck, article 5735). Metabolites produced during the incubation were assayed by counting the radioactivity in spots corresponding to metabolites [19].

Data presentation

All experiments were performed in duplicate or triplicate. The results presented represent results obtained with more than three different batches of intact cells, unless stated otherwise.

RESULTS

Extracellular ATP stimulates adenylate cyclase when G_i is inactivated by pertussis toxin treatment

As mentioned previously, we have shown that in FRTL-5 cells

Fig. 1. Effect of adenosine deaminase on ATP-, AMP- and adenosine-induced stimulation of cyclic AMP accumulation

Pertussis toxin-treated FRTL-5 cells were incubated with the indicated concentrations of ATP (○), AMP (△) and adenosine (□) in the presence of 0.2 mM-RO 20-1724 in a Ca²⁺-free Hepes-buffered medium as described in the Experimental section (a). The incubation medium was further supplemented with 0.5 unit of adenosine deaminase/ml (b). The 100% values in the presence and absence of adenosine deaminase were 0.28 ± 0.01 and 0.24 ± 0.01 nmol/10 mg of protein respectively. Results shown are means ± S.E.M. of three separate experiments. The response to AMP, corrected for its metabolic degradation (▲), was also plotted.

Fig. 2. Dose-dependent effects of ATP analogues on cellular cyclic AMP levels

FRTL-5 cells treated without (a) or with (b) pertussis toxin were incubated for 5 min with the indicated concentrations of ATP (○), ATP[γ S] (△), ATP[α S] (□), ADP[S] (●), Br-ATP (▲) or pp[CH₂]pA (■) in the presence (a) or the absence (b) of 2 nM-TSH in a Ca²⁺-free Hepes-buffered medium containing 0.2 mM-RO 20-1724 and 0.5 unit of adenosine deaminase/ml, as described in the Experimental section. Results are represented as means of two separate experiments and expressed as percentages of the value obtained in the absence of these nucleotides. The 100% values for (a) and (b) were 21.8 and 0.19 nmol/10 mg of protein respectively.

extracellular ATP and its analogues stimulate the production of inositol phosphates, but inhibit TSH-induced cyclic AMP accumulation in a pertussis toxin-sensitive manner [18]. We have now shown that in pertussis toxin-treated cells (where G_i, a necessary factor for the inhibitory action of ATP, is inactivated), higher doses of ATP, AMP or adenosine stimulate cyclic AMP production in a dose-dependent manner (Fig. 1a). However, since ectonucleotidases located at the cell surface may hydrolyse extracellular ATP [2,24], the observed increase in cyclic AMP accumulation might be caused by the presence of ATP metabolites such as AMP and adenosine, which are typical A₂ agonists and are known to stimulate adenylate cyclase. To estimate the contribution of these ATP metabolites to the observed action of ATP, the adenylate cyclase-stimulating activities of ATP, AMP and adenosine were assayed in the presence of adenosine deaminase, which inactivates adenosine. Under these conditions, adenosine completely lost its activity throughout the whole concentration range tested, whereas ATP and AMP remained active, although their dose-response curves were shifted to the right by about one order of magnitude. This indicates that the action of 10 μ M-ATP seems to be mostly attributable to its metabolite, adenosine, whereas at higher

Fig. 3. Effect of suramin on ATP- and noradrenaline-induced inositol phosphate production

Cells treated with pertussis toxin were prelabelled with [³H]inositol as described in the Experimental section. These cells were incubated for 5 min with the indicated concentrations of ATP (a) and noradrenaline (b) in the absence (○) or presence (●) of 300 μ M-suramin. Data were normalized to 10⁵ d.p.m. of the radioactivity in unstimulated cells, and are expressed as percentages of the value obtained in the absence of ATP or noradrenaline. The 100% values for (a) and (b) were $(5.3 \pm 0.2) \times 10^4$ and $(6.5 \pm 0.2) \times 10^4$ d.p.m. respectively. Results shown are means $\pm$ S.E.M. of three determinations in a single experiment. Two more experiments gave similar results.

concentrations the innate effect of ATP itself prevails (Fig. 1b). However, the possibility that the apparent effect of ATP is caused by the metabolic production of AMP is not excluded. To estimate metabolic changes during incubation, labelled ATP and AMP (10 nM–1 mM) were incubated with FRTL-5 cells for 5 min in the presence of adenosine deaminase, and the distribution of radioactivity among nucleotides separated on a t.l.c. plate was measured. A total of 55% of the radioactivity at 10 nM-ATP was metabolized, and about 5% was recovered in AMP. The breakdown of ATP tended to decrease with an increase in ATP concentration, with 40% degradation and 3% recovery in AMP at 1 mM-ATP. A similar type of metabolic change occurred with AMP, with 50% and 10% degradation at 10 nM and 1 mM respectively. The other ATP degradation products under these conditions were mostly inosine [19]. A small left shift occurred when the AMP dose-response curve was corrected for metabolic degradation (Fig. 1b). A similar shift is predictable for the ATP dose-response curve, but the overall low conversion rate of ATP to AMP ($\leq 5\%$) supports the idea that ATP itself stimulates cyclic AMP accumulation at concentrations greater than 10 μ M.

Order of potency of ATP analogues in stimulation and inhibition of cyclic AMP production

To characterize the stimulatory effect of ATP on adenylate cyclase activity, the order of potency of ATP analogues for this stimulatory action was compared with that for the inhibitory action on TSH-induced cyclic AMP accumulation. As shown in Figs. 2(a) and 2(b), the rank order of ATP and its analogues for the agonistic action was ATP = ATP[γ S] $\geq$ ATP[α S] > ADP[S] $\gg$ Br-ATP = pp[CH₂]pA, for both the stimulatory and inhibitory effects. This order, however, was different from that previously reported for the effects of ATP analogues on phospholipase

Fig. 4. Effect of suramin on ATP- and PIA-induced inhibition and stimulation of cyclic AMP production

Cells treated without (*a* and *c*) or with (*b* and *d*) pertussis toxin were incubated with the indicated concentrations of ATP (*a* and *b*) or PIA (*c* and *d*) in the absence (○) or the presence (●) of 300 μ M-suramin. Except for replacement of 2 nM-TSH by 50 μ M-forskolin in (*a*) and (*b*), all other experimental conditions were as described in the legend to Fig. 2. The 100% values for non-treated cells (activation by forskolin) and IAP-treated cells (basal) were 57.2 ± 1.5 and 0.18 ± 0.01 nmol/10 mg of protein respectively. Results shown are means $\pm$ S.E.M. of three separate experiments.

activation, expressed as arachidonate release, intracellular free Ca^{2+} increase and I^- efflux [16,18]. The effective doses for the inhibitory action were roughly 1–2 orders of magnitude lower than those for the stimulatory one. These results support the idea that ATP, similar to P_1 -agonists, can act as both a positive and a negative signal in the cyclic AMP pathway, although it is not clear that the pathways are activated through ATP-specific receptors.

A receptor coupled to the phospholipase C activation mechanism is distinguished from those coupled to adenylate cyclase modulation mechanisms by their different susceptibilities to antagonism by suramin

The multiple actions of ATP on three different signal transduction systems raised the question of whether these different effects are exerted via three different receptors or via a common receptor coupled to three different systems.

In previous studies we found that ATP both inhibits adenylate cyclase and stimulates phospholipase C. The former action is mediated by a receptor sensitive to DPX, a P_1 -antagonist, and pertussis toxin treatment. The effect of ATP on the phospholipase

Fig. 5. Effect of CPX on ATP-induced phospholipase C activation and on inhibition and stimulation of cyclic AMP production

In (*a*), after treatment of the cells with pertussis toxin and [^3H]inositol, these cells were incubated for 5 min with the indicated concentrations of ATP in the absence (○) or presence (●) of 10 μ M-CPX. Other experimental conditions and data presentation were as described in the legend to Fig. 3. The 100% value was $(6.3 \pm 0.3) \times 10^4$ d.p.m. In (*b*) and (*c*), cells treated without (*b*) or with (*c*) IAP were incubated with the indicated concentrations of ATP in the absence (○) or presence of 0.1 μ M- (▲), 1 μ M- (■) or 10 μ M- (●) CPX. Other experimental conditions and data presentation were as described in the legend to Fig. 2. The 100% values for (*b*) and (*c*) were 25.4 ± 0.1 and 0.19 ± 0.01 nmol/10 mg of protein, respectively. Results shown are means $\pm$ S.E.M. of three determinations in a single experiment. Similar results were observed in two more experiments.

C/Ca^{2+} system is mediated by two receptors, one sensitive and the other insensitive to DPX. The DPX-sensitive receptor-mediated action of ATP was also susceptible to pertussis toxin treatment, and hence this receptor, like the ATP receptor for adenylate cyclase inhibition, was characterized as a P_1 (A_1)-type ATP receptor [18,19]. As shown in [18], A_1 -receptor activation enhances phospholipase C stimulation through other agonists, and the participation of the A_1 -type ATP receptor in phospholipase C stimulation is explained by analogy with this A_1 receptor-mediated action. Thus at least two types of receptor, one sensitive and the other insensitive to DPX and pertussis toxin treatment, participate in the two actions of ATP.

We have now described a third effect of ATP, i.e. the stimulation of adenylate cyclase. To characterize the receptor(s) involved, we examined the effects of various receptor antagonists.

Fig. 6. Antagonistic effect of xanthine derivatives on ATP- and PIA-induced inhibition and stimulation of cyclic AMP

Cells treated without (a, c) or with (b, d) pertussis toxin were incubated with the indicated concentrations of CPX (○), DPX (△) or XAC (□) in the presence of 10 μ M-ATP (a, b) or 1 μ M-PIA (c, d). Other experimental conditions were as described in the legend to Fig. 2. (a) and (c) show dose-dependent effects of antagonists to the inhibitory actions of ATP (a) and PIA (c) on TSH-induced cyclic AMP accumulation. Data are expressed as percentages of the value obtained with TSH in the absence of other agonists and antagonists. The 100% value was 20.9 nmol of cyclic AMP/10 mg of protein (variation from the 100% value caused by the addition of purinergic antagonists was not statistically significant). (b) and (d) show dose-dependent effects of antagonists to stimulatory actions of ATP (b) and PIA (d) on cyclic AMP accumulation. Data are shown as percentages of the maximal cyclic AMP increase obtained with each agonist in the absence of the antagonist. The 100% values were 0.51 nmol/10 mg of protein for ATP and 0.46 nmol/mg of protein for PIA. The basal value was 0.14 nmol/10 mg of protein. Results are means of two separate experiments.

At first, to identify the DPX- and pertussis toxin-insensitive ATP receptor, we examined the influence of suramin on the action of ATP in pertussis toxin-treated cells. Suramin, an anti-trypansomiasis agent, has been reported to competitively inhibit P_2 -agonist-induced responses [25–27], suggesting that it is a putative antagonist of P_2 -receptors. As shown in Fig. 3, suramin competitively inhibited ATP-induced inositol phosphate production, whereas it hardly affected the inositol phosphate pro-

duction induced by noradrenaline via the α_1 -receptor, confirming that suramin does indeed act at the receptor level as a P_2 -receptor antagonist.

On the other hand, the effects of ATP and PIA (an adenosine derivative) on adenylate cyclase activity, including the inhibition of forskolin-induced cyclic AMP accumulation (Figs. 4a and 4c) and stimulation of adenylate cyclase in pertussis toxin-treated cells (Figs. 4b and 4d), were not influenced by suramin. Thus, suramin distinguished the ATP receptors responsible for modulation of adenylate cyclase activity from the typical P_2 -type purinergic receptor that mediates phospholipase C activation in pertussis toxin-treated cells.

Influence of xanthine derivatives on the ATP-induced inhibition and stimulation of cyclic AMP accumulation

In previous studies we have found that ATP-induced inhibition of cyclic AMP accumulation was competitively inhibited by DPX, a xanthine derivative and P_1 -receptor antagonist [18]. However, DPX did not influence the pertussis toxin-insensitive ATP action that resulted in arachidonate release [18]. We therefore examined the effects of various xanthine derivatives with P_1 -antagonist activity on ATP-induced responses. As shown in Fig. 5(a), 10 μ M-CPX did not affect ATP-induced inositol phosphate production in pertussis toxin-treated cells. Dose-response curves at ATP-induced stimulation and inhibition of cyclic AMP accumulation were shifted to the right in the presence of CPX, showing that CPX antagonizes both actions of ATP in a competitive fashion (Figs. 5b and 5c). These results are consistent with the suramin antagonism mentioned above, and confirm that ATP receptors in FRTL-5 thyroid cells can be classified into at least two categories, i.e. one sensitive to suramin and coupled to phospholipase C, and the other susceptible to CPX and linked to adenylate cyclase.

Given the P_1 -receptor-like nature of these ATP receptors, the effects of the same xanthine derivatives on the actions of PIA were examined under the same conditions as for ATP. Then we compared the rank order of xanthine derivatives and the two ATP receptors for adenylate cyclase modulation. As shown in Figs. 6(a) and 6(b), all ATP- and PIA-induced responses were antagonized by the xanthine derivatives in a dose-dependent manner. The order of antagonist potencies for the inhibitory action of either ATP or PIA was CPX > XAC > DPX (Figs. 6a and 6c), and that for the stimulatory actions of both agonists was XAC > CPX = DPX (Figs. 6b and 6d). These findings clearly show that the ATP-induced inhibition of TSH- or forskolin-activated adenylate cyclase is mediated by a receptor different from that involved in the ATP-induced stimulation of adenylate cyclase. The former is characterized as an A_1 -type receptor, while the latter is an A_2 -type.

DISCUSSION

The present study provides concrete evidence that extracellular ATP itself, as well as its products of hydrolysis, acts on receptors coupled to adenylate cyclase. This is based on three observations: (1) hydrolysis-resistant ATP derivatives had similar effects to those of ATP on cyclic AMP levels, (2) inactivation of adenosine by adenosine deaminase attenuated the actions of ATP only partially at ATP concentrations greater than 10 μ M, and (3) the amount of AMP produced from ATP under the present experimental conditions was too small to account for the extent of ATP-induced changes in cyclic AMP accumulation. When an ATP-regenerating system was added in the presence of adenosine deaminase, the response curve of ATP was shifted 0.5 order of magnitude to the right for both stimulation and inhibition of adenylate cyclase (results not shown), suggesting that preventing

ATP metabolism slightly attenuated its actions. However, the effect of ATP was still very strong, and we conclude that ATP itself as well as P_1 -agonists activate both stimulatory and inhibitory receptor systems which are coupled to adenylate cyclase. Thus, based on the present data and on previous studies, we now propose the presence of at least three receptors which can interact with extracellular ATP and which couple with three different signal transduction systems in FRTL-5 cells: (1) the activation of phospholipase C accompanied by the production of inositol phosphates (pertussis toxin-insensitive) [17,18], (2) the inhibition of TSH- or forskolin-induced cyclic AMP accumulation via a pertussis toxin-sensitive pathway [18,19], and (3) the stimulation of cyclic AMP accumulation, which is only observed in pertussis toxin-treated cells (Figs. 1 and 2).

Three lines of evidence support our proposal. Firstly, suramin, a P_2 -antagonist, inhibited competitively the production of inositol phosphates but had little effect on the inhibition or stimulation of cyclic AMP accumulation by ATP. Secondly, CPX, a xanthine derivative with P_1 -antagonist activity, inhibited the ATP effects on cyclic AMP levels in a competitive fashion, but did not influence ATP-induced inositol phosphate production. Thirdly, there is a notable difference in the order of potency of antagonists (CPX, DPX and XAC) between ATP-induced inhibition and stimulation of cyclic AMP accumulation. We have previously reported that part of the effect of ATP in stimulating phospholipase C is sensitive to pertussis toxin and DPX, and suggest that this is mediated by an A_1 -type receptor [18,19,25]. At this stage of investigation, we could not discriminate this receptor from the ATP receptor mediating adenylate cyclase inhibition.

It has recently been shown that ATP-induced contraction and relaxation in mouse vas deferens [26,27] or in smooth muscle cells of guinea-pig caecum [28] is antagonized by suramin. This agent has also been reported to interact with various enzymes, including Na^+ , K^+ -ATPase [29] and protein kinase C [30], and to interfere with the interaction of some growth factors with their receptors, probably because of binding of suramin to the growth factors themselves [31]. We found that TSH-induced cyclic AMP accumulation was markedly inhibited by suramin (results not shown). This result may be explained by binding of suramin to TSH. Finally, suramin has been shown to bind to BSA [22]; however, the presence of 0.1% (w/v) BSA in the reaction medium did not influence the effects of suramin. Thus the effects of suramin cannot always be explained by its P_2 -receptor-antagonistic nature. Nevertheless, under the present conditions, suramin does seem to act at the receptor level as a P_2 -antagonist, since it competitively inhibits ATP-induced phospholipase C activation but not that induced by noradrenaline. The only difference between the two systems seems to be the receptor species, i.e. one is a P_2 - and the other an α_1 -receptor [17]. Furthermore, suramin specifically recognized some of the ATP receptor subtypes, and it did not affect the ATP receptors which mediate modulation of adenylate cyclase activity. Therefore it seems reasonable to conclude that, under the present conditions, suramin interacts only with the P_2 -receptor subtype coupled to the phospholipase C/ Ca^{2+} system, but not with G-protein or phospholipase C molecules.

Boyer *et al.* [15] have recently shown that a P_2 -receptor in turkey erythrocytes is involved in the activation of phospholipase C via pertussis toxin-insensitive G-proteins, and the putative receptor was detected as a 53 kDa membrane protein by photo-affinity labelling with [α - ^{32}P]3'-O-(4-benzoyl)benzoyl ATP. We also have observed a 53 kDa protein labelled with the same [α - ^{32}P]3'-O-(4-benzoyl)benzoyl ATP in FRTL-5 cell membranes (F. Okajima & Y. Kondo, unpublished work). This labelled protein disappeared on SDS/PAGE in the presence of suramin, suggesting that the 53 kDa band in the gel contains at least one

P_2 -receptor coupled to phospholipase C via a pertussis toxin-insensitive G-protein.

The effects of ATP in rat hepatocytes are different from those in FRTL-5 cells. We found that ATP inhibits forskolin-induced cyclic AMP accumulation in a pertussis toxin-sensitive but a xanthine-derivative-insensitive manner, while adenosine and other P_1 -agonists had no inhibitory effect on cyclic AMP accumulation [8]. Based on these observations we proposed the presence of two different P_2 -receptor subtypes in hepatocytes, i.e. one coupled to the adenylate cyclase system and the other to the phospholipase C system. This is supported by antagonist experiments using suramin (H. Tomura, F. Okajima & Y. Kondo, unpublished work). In FRTL-5 cells, however, P_1 -agonists as well as ATP inhibit TSH- or forskolin-activated adenylate cyclase in a pertussis toxin-sensitive manner, but they stimulate cyclic AMP accumulation under conditions where G_i is inactivated by pertussis toxin. Regardless of the agonist species (either P_2 -agonists such as ATP or P_1 -agonists such as PIA), both the stimulatory and inhibitory responses of cyclic AMP in FRTL-5 cells were antagonized by xanthine derivatives but not by suramin. Rank orders of antagonistic potencies for P_2 -agonist-induced stimulatory and inhibitory actions were very similar to those for A_2 - and A_1 -type P_1 -receptor-mediated actions respectively. On this basis, one would not conclude that ATP receptors for modulating adenylate cyclase activity in FRTL-5 cells are different from P_1 -receptors. When we consider the potencies of agonists for both inhibitory and stimulatory actions on adenylate cyclase activity, however, the receptors do not appear to be P_1 -receptors, since the ATP affinities and those of P_1 -agonists, especially of AMP, are too close to fulfil the original definition of A_1 - and A_2 -receptors.

This work was supported in part by research grants from the Scientific Research Fund of the Ministry of Education, Science and Culture of Japan. K. S. is a Japan Society for the Promotion of Science Fellow. We thank Dr Martina Nazarea of Kyoto University for reading our manuscript.

REFERENCES

1. Burnstock, G. & Kennedy, C. (1985) *Gen. Pharmacol.* **16**, 433–440
2. Gordon, J. L. (1986) *Biochem. J.* **233**, 309–319
3. Nakata, H. (1989) *Mol. Pharmacol.* **35**, 780–786
4. Nakata, H. (1989) *J. Biol. Chem.* **264**, 16545–16551
5. Olah, M. E., Jacobson, K. A. & Stiles, G. L. (1989) *FEBS Lett.* **257**, 292–296
6. Munshi, R. & Linden, J. (1989) *J. Biol. Chem.* **264**, 14853–14859
7. Hutchison, K. A. & Fox, I. H. (1989) *J. Biol. Chem.* **264**, 19898–19903
8. Okajima, F., Tokumitsu, Y., Kondo, Y. & Ui, M. (1987) *J. Biol. Chem.* **262**, 13483–13490
9. Creba, J. A., Downes, P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747
10. Charest, R., Blackmore, P. F. & Exton, J. H. (1985) *J. Biol. Chem.* **260**, 15789–15794
11. Dubyak, G. R. (1986) *Arch. Biochem. Biophys.* **245**, 84–95
12. El-Moatassim, C., Dornand, J. & Mani, J.-C. (1987) *Biochim. Biophys. Acta* **927**, 437–444
13. Forsberg, E. J., Feuerstein, G., Shohami, E. & Pollard, H. B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5630–5634
14. Piroton, S., Raspe, E., Demolle, D., Erneux, C. & Boeynaens, J.-M. (1987) *J. Biol. Chem.* **262**, 17461–17466
15. Boyer, J. L., Cooper, C. L. & Harden, T. K. (1990) *J. Biol. Chem.* **265**, 13515–13520
16. Okajima, F., Sho, K. & Kondo, Y. (1988) *Endocrinology (Baltimore)* **123**, 1035–1043
17. Okajima, F., Sato, K. & Kondo, Y. (1989) *FEBS Lett.* **253**, 132–136
18. Okajima, F., Sato, K., Nazarea, M., Sho, K. & Kondo, Y. (1989) *J. Biol. Chem.* **264**, 13029–13037
19. Okajima, F. & Kondo, Y. (1990) *J. Biol. Chem.* **265**, 21741–21748
20. Yora, T. & Ui, N. (1978) *J. Biochem. (Tokyo)* **83**, 1173–1190

21. Ambesi-Impiombato, F. S., Parks, L. A. M. & Coon, H. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3455–3459
22. Marzouk, H. F. A. I., Hofland, L. J., den Holder, F. H., van Koetsveld, P. M., Steenbergen, J., Zuiderwijk, J., Abou-Hashim, E. M., El-Kannishy, M. H., de Jong, F. H. & Lamberts, S. W. J. (1990) *Mol. Cell. Endocrinol.* **72**, 95–102
23. Honma, M., Satoh, T., Takezawa, J. & Ui, M. (1977) *Biochem. M.* **18**, 257–273
24. Pearson, J. D., Carleton, J. S. & Gordon, J. L. (1980) *Biochem. J.* **190**, 421–429
25. Nazarea, M., Okajima, F. & Kondo, Y. (1991) *Eur. J. Pharmacol.* **206**, 47–52
26. Dunn, P. M. & Blakeley, A. G. H. (1988) *Br. J. Pharmacol.* **93**, 243–245
27. von Kugelgen, I., Bultmann, R. & Starke, K. (1989) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**, 760–763
28. Hertog, A. D., Nelemans, A. & den Akker, J. U. (1989) *Eur. J. Pharmacol.* **166**, 531–534
29. Fortes, P. A. G., Ellory, J. C. & Lew, U. L. (1973) *Biochim. Biophys. Acta* **318**, 262–272
30. Mahoney, C. W., Azzi, A. & Huang, K.-P. (1990) *J. Biol. Chem.* **265**, 5424–5428
31. Yayon, A. & Klagsbrun, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5346–5350

Received 16 April 1991/30 September 1991; accepted 26 November 1991