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The ATP^{4-} Receptor of Rat Mast Cells

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The concentration-dependence on exogenous ATP of activation and inhibition of mast-cell histamine secretion, phosphatidylinositol labelling and leakage of metabolites shows that all these functions are regulated by the free acid ATP^{4-}. Maximal histamine secretion and phosphatidylinositol labelling occur with ATP^{4-} at approx. 2μM, but higher concentrations, which cause inhibition of secretion and phosphatidylinositol labelling, are required to maximize leakage of ^{32}P-labelled metabolites. Both enhancement and inhibition of phosphatidylinositol labelling (due to low and high concentrations of ATP^{4-} respectively) are rapid in onset: histamine secretion is characterized by a delay, especially at low concentrations of ATP^{4-} (approx. 1μM). Phosphatidylinositol labelling and histamine secretion are dependent on extracellular Ca^{2+}. Metabolite leakage due to the presence of exogenous ATP^{4-} is slow and does not require Ca^{2+}. Of 18 analogues of ATP that were tested, only four were agonists for secretion, and only these four permitted leakage of ^{32}P-labelled metabolites. It is argued that activation and inhibition of histamine secretion, phosphatidylinositol labelling and metabolite leakage are all initiated by ATP^{4-} acting at the same receptor. For mast cells stimulated with ATP^{4-} enhancement of phosphatidylinositol metabolism is not sufficient by itself to cause Ca^{2+}-dependent secretion.

ATP is one of several agents that stimulate Ca^{2+}-dependent histamine secretion from mast cells (Keller, 1966; Sugiyama, 1971; Dahlquist & Diamant, 1974). The agonist form of ATP that interacts with receptors to permit Ca^{2+} movements and consequent secretion is the tetrabasic acid ATP^{4-} (Cockcroft & Gomperts, 1979*a*). This is a minor component of the total ATP, most of which is complexed to the bivalent cations Mg^{2+} and Ca^{2+} in physiological salt solutions (Taqui Khan & Martell, 1966). Unlike the other agonists for mast-cell secretion such as the immunoglobulin E-directed ligands (specific antigen and concanavalin A), chymotrypsin, compound 48/80 and ionophore A23187, the effect of increasing the concentration of ATP above its optimal value is to inhibit secretion due to itself (Cockcroft & Gomperts, 1979*a*) and also due to antigen or compound 48/80 (Grosman & Diamant, 1975) and ionophore A23187 (Diamant & Patkar, 1974).

The interaction of ATP^{4-} is unusual in a number of other ways. Although secretion commences promptly when ATP^{4-} is applied at a concentration optimal for secretion (about 2μM), below this concentration the commencement of secretion can be delayed up to at least 15 min (Cockcroft & Gomperts, 1979*a*). The reason for this is thought to stem from a delay in the entry of Ca^{2+}, which initiates secretion. The subsequent rate of secretion is slow at all concentrations of ATP^{4-}. We found that ATP^{4-} also promotes leakage of metabolites from mast cells in such a way that ATP^{4-} at a concentration optimal for secretion is permissive of phosphate movement, and that at inhibitory concentrations there is a loss of nucleotides and other phosphorylated metabolites without a loss of proteins (Cockcroft & Gomperts, 1979*b*).

We previously showed that stimulation of mast cells with the immunoglobulin E-directed ligands, and also chymotrypsin and compound 48/80, is accompanied by an increased rate of turnover of the anionic lipid phosphatidylinositol (Cockcroft & Gomperts, 1979*c*). Histamine secretion due to the presence of all of these ligands is a well-documented example of a secretory process in which the final stimulus to secretion certainly involves Ca^{2+} movements and probably requires an increase in the concentrations of Ca^{2+} in the cytosol (Gomperts, 1976). The finding that ligand-stimulated phosphatidylinositol turnover in many tissues as well as mast cells does not require the presence of Ca^{2+} has been used as evidence to support the hypothesis that

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phosphatidylinositol responses may be involved in the control of Ca^{2+} fluxes and consequent tissue activities (Michell, 1975; Michell *et al.*, 1977; Cockcroft & Gomperts, 1979*c*).

In the present paper we show that, in mast cells preincubated with [^{32}P]P_i, activation and inhibition of histamine secretion, phosphatidylinositol labelling and metabolite leakage are all mediated through receptors for ATP^{4-} having common specificity properties. The functions mediated by these ATP^{4-} receptors differ, so that whereas ATP^{4-}-induced metabolite leakage does not require Ca^{2+}, both ATP^{4-}-induced secretion and ATP^{4-}-induced phosphatidylinositol labelling are Ca^{2+}-dependent. This latter finding, taken together with the relative kinetics of phosphatidylinositol labelling and histamine secretion, sheds some doubt on the presumed precursor role of phosphatidylinositol-related events in the control of subsequent Ca^{2+}-mediated processes.

Materials

The following compounds were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) (note: the numbering refers to the listing of compounds in Table 2): ATP (1); 8-bromo-ATP (2); 1,N^6-etheno-ATP (3); GTP (4); CTP (5); 2′-deoxy-ATP (6); 9-β-D-arabinofuranosyl-ATP (8); [$\beta\gamma$-imido]ATP (p[NH]ppA) (10); [$\alpha\beta$-methylene]ATP (pp[CH_2]pA) (11); [$\beta\gamma$-methylene]ATP (p[CH_2]ppA) (12); adenosine tetraphosphate (ppppA) (13); adenosine 5′-monosulphate (17). ADP (14) and [$\beta\gamma$-thio]ATP (p[S]ppA) (9) were purchased from Boehringer (Mannheim, Germany) and 3′-*O*-methyl-ATP (7) was purchased from P–L Biochemicals (Milwaukee, WI, U.S.A.). γ-Methoxy-ATP (18) and γ-fluoro-ATP (19) were gifts from Dr. F. Eckstein (Max-Planck-Institut für Experimentelle Medizin, Göttingen, Germany). The concentrations of the adenine nucleotides were checked by measurements of the absorbance at 257.5nm at pH2.0, and the purity of the compounds was checked by Dr. E. G. Trams by high-pressure liquid chromatography. Ionophore A23187 was a gift from Dr. R. L. Hamill of Eli Lilly and Co. (Indianapolis, IN, U.S.A.).

Methods

Mast cells of male Sprague–Dawley rats were obtained by peritoneal lavage and purified to better than 90% homogeneity by centrifugation through Ficoll (30%) as previously described (Cockcroft & Gomperts, 1979*c*). The cells were suspended at approx. 5×10^5 cells/ml in a phosphate-free buffered salt solution (pH7.5) containing 137mM-NaCl, 2.7mM-KCl, 10μM-EGTA and 20mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], and 1.0mM-$MgCl_2$ and 1.8mM-$CaCl_2$ unless otherwise specified.

To measure phosphatidylinositol labelling, cells were incubated in the presence of [^{32}P]P_i (100μCi/ml) for 30min so as to label intracellular precursor pools. For the experiments described in Figs. 1 and 2 and in Table 1 about 10^5 cells in 200μl were then transferred to tubes containing ATP and $MgCl_2$ as specified (final volume 400μl) and the incubations were continued for further periods as indicated. To terminate the reaction the cells were quenched by addition of 1ml of ice-cold 0.15M-NaCl (buffered at pH7 with 10mM-potassium phosphate). For the time-course experiment illustrated in Fig. 3, labelled cells (4.5×10^6 cells in 3ml) were added to ATP contained in small volumes to give appropriate final concentrations. To terminate these incubations, duplicate samples (100μl each) were withdrawn at various times and quenched by addition to 1ml of buffered NaCl as above. The cells were sedimented by centrifugation (at 400*g* for 5min) at 4°C. The supernatants were retained for the determination of secreted histamine. Extraction of lipids from the cell pellets and separation of phosphatidylinositol by descending chromatography on formaldehyde-treated papers were as previously described (Cockcroft & Gomperts, 1979*c*).

To measure the leakage of ^{32}P-labelled mast-cell metabolites, mixed peritoneal mast cells in 2ml were first incubated with [^{32}P]P_i (100μCi/ml) for 2h and the mast cells were then isolated by sedimentation through Ficoll as described previously (Cockcroft & Gomperts, 1979*b*). About 2×10^4 ^{32}P-labelled mast cells were transferred to tubes and incubated in 400μl containing ATP or other compounds as specified. At the times indicated, the incubations were terminated as described above. Samples of the supernatant were taken for determination of secreted histamine and for counting of radioactivity as previously described (Cockcroft & Gomperts, 1979*b*).

The amount of histamine secreted into the extracellular fluid as a consequence of applying a stimulus was measured fluorimetrically as previously described (Cockcroft & Gomperts, 1979*a*). Histamine secretion is expressed as a percentage of the total cell histamine.

The concentrations of ATP^{4-} in solutions of ATP containing Mg^{2+} and Ca^{2+} were calculated by the use of a simplified equilibrium equation as described previously (Cockcroft & Gomperts, 1979*a*), with $\log K_{(Mg^{2+})} = 4.28$ and $\log K_{(Ca^{2+})} = 3.97$ (Taqui Khan & Martell, 1966).

All results presented are the averages of duplicate determinations from fully representative experiments that were repeated on at least three occasions.

Results

ATP-induced histamine secretion and phosphatidylinositol labelling

ATP applied to rat mast cells that have been preincubated for 30min with [^{32}P]P_i stimulates the incorporation of radioactivity into phosphatidylinositol. The concentration-dependence of the ATP-induced phosphatidylinositol response and also of histamine secretion during the subsequent 20min is shown in Fig. 1. Both these responses rise to a maximum as the concentration of ATP is increased; above this they become inhibited so that with the $ATP_{(total)}$ concentration above 180μM the rate of phosphatidylinositol labelling falls below the control value, and with the $ATP_{(total)}$ concentration above 240μM the histamine secretion falls to the value observed with unstimulated cells. Similar results were obtained when [^{3}H]inositol was used as the precursor of phosphatidylinositol labelling (results not shown).

As we have previously shown, the agonist form of ATP for histamine secretion is the free acid ATP^{4-} (Cockcroft & Gomperts, 1979*a*). The experiments shown in Fig. 2 and Table 1 were designed to demonstrate that it is also this form of ATP that stimulates phosphatidylinositol labelling. In the experiment of Fig. 2, the concentration of $ATP_{(total)}$ was fixed at 150μM throughout (except for the zero point of ATP concentration) and the concentrations of ATP^{4-} were regulated by varying the concentrations of Mg^{2+} in accordance with equilibrium considerations as previously described (Cockcroft & Gomperts, 1979*a*). It is clear that the two measured responses show a similar dependence on ATP^{4-} and that the two curves are displaced from each other to a similar degree as in Fig. 1, in which the concentration of ATP^{4-} was regulated by changing

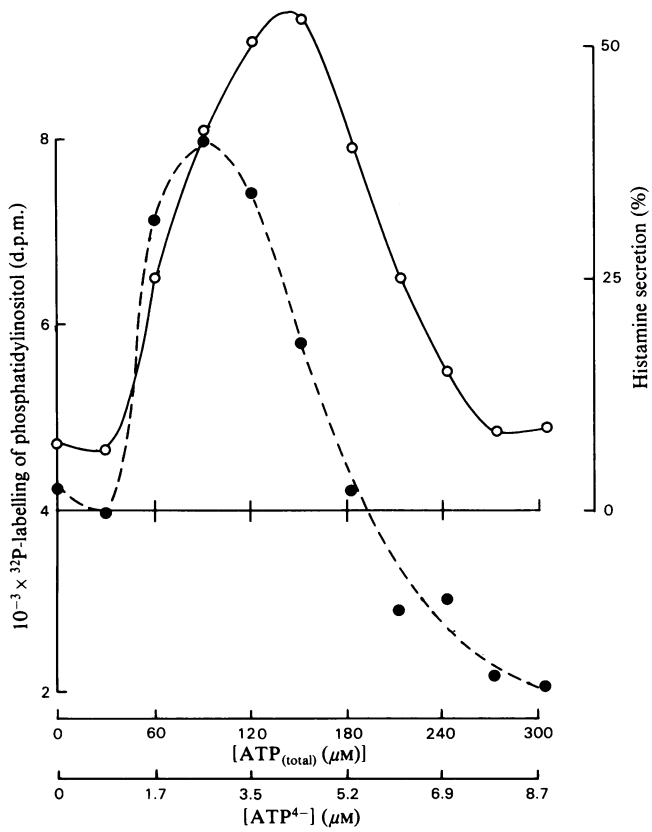

Fig. 1. *Concentration–effect relationships for ATP-induced histamine secretion and ATP-induced phosphatidylinositol labelling by mast cells*

Purified mast cells were labelled with ^{32}P[P_i] for 30min and then treated with ATP for a further 20min. The scales of the abscissa indicate both the total ATP applied to the mast cells and the calculated concentrations of ATP^{4-} in the presence of 1mM-Mg^{2+} and 1.8mM-Ca^{2+}. Experimental details are given in the text. ○, Histamine secretion (right-hand scale); ●, phosphatidylinositol labelling (left-hand scale).

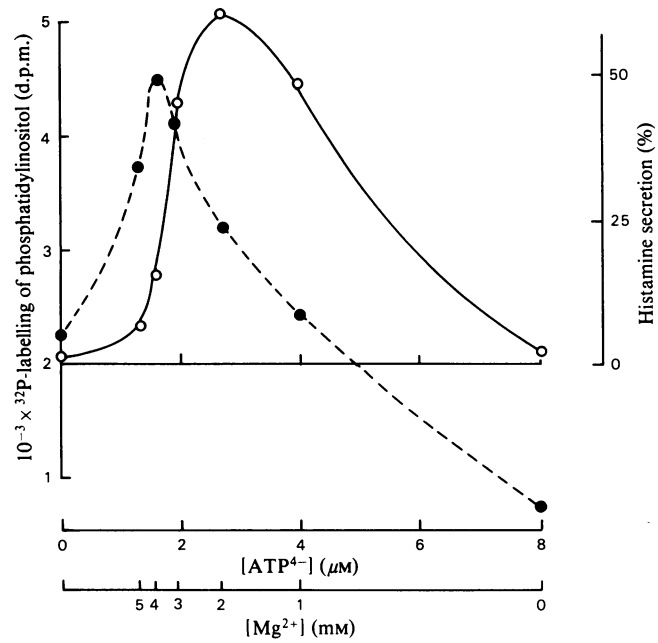

Fig. 2. *Concentration-effect relationships for ATP^{4-}-induced histamine secretion and ATP^{4-}-induced phosphatidylinositol labelling by mast cells*

In this experiment the incubations with ATP were for 30 min. For all (non-zero) concentrations of ATP, the $ATP_{(total)}$ concentration was set at 150 μM and the concentrations of ATP^{4-} were regulated by varying the concentration of Mg^{2+} as indicated in the scales of the abscissa. The Ca^{2+} concentration was 2.18 mM throughout. Varying the Mg^{2+} concentration in the range 1–5 mM in the absence of ATP had no effect on either histamine secretion or phosphatidylinositol labelling (see Table 1, which refers to further results from this experiment). Experimental details are given in the text. O, Histamine secretion (right-hand scale); ●, phosphatidylinositol labelling (left-hand scale).

Table 1. *Effects of Ca^{2+} and Mg^{2+} on ATP-induced histamine secretion and phosphatidylinositol labelling by mast cells*

Experimental details are given in the text.

[ATP (total)] (μM)	[Ca^{2+}] (mM)	[Mg^{2+}] (mM)	[ATP^{4-}] (calc.) (μM)	Histamine secretion (%)	$10^{-3} \times {}^{32}$P in phosphatidylinositol (d.p.m.)
0	0 and 2.18	0–5	0	1.5 ± 0.3*	2.3 ± 0.1*
50	0	1	2.63	2.5	1.9 ± 0.05†
50	2.18	0	2.63	55	4.0 ± 0.3‡
100	0	2	2.63	3.5	2.0 ± 0.1†
100	2.18	1	2.63	57	5.0 ± 0.45‡
150	2.18	2	2.63	64	4.0 ± 0.6‡
200	2.18	3	2.63	61	4.5 ± 0.05‡
250	2.18	4	2.63	58	4.4 ± 0.3‡
300	2.18	5	2.63	58	5.1 ± 0.5‡

* In the absence of ATP there was no systematic variation in either histamine secretion or phosphatidylinositol labelling due to variation of the concentration of Ca^{2+} (0 and 2.18 mM) or Mg^{2+} (0–5 mM); therefore the control experiments are presented as averages of all the experiments with zero ATP (± S.E.M., $n = 16$). In the presence of ATP results presented are means of duplicate determinations (± range).

† Significant difference as a group from equivalent incubations in the presence of Ca^{2+} ($P < 0.005$).

‡ Significant difference as a group from equivalent incubations in the absence of ATP ($P < 0.001$).

the $ATP_{(total)}$ concentration with that of Mg^{2+} maintained constant. An alternative approach was to fix the concentration of ATP^{4-} at a constant value and to vary the concentration of $ATP_{(total)}$. This was also done by varying the concentration of Mg^{2+} in accordance with equilibrium considerations. In these circumstances (see Table 1) the extent of histamine secretion and the labelling of phosphatidylinositol

remained invariant as the $ATP_{(total)}$ concentration was varied between 50 and 300μM; that of ATP^{4-} was 2.6μM throughout. Mg^{2+} had no effect on phosphatidylinositol labelling in the absence of ATP.

Results shown in Table 1 confirm that secretion due to ATP^{4-} is dependent on extracellular Ca^{2+}, as shown previously (Cockcroft & Gomperts, 1979*a*), and also show that the ATP^{4-}-induced phosphatidylinositol labelling is Ca^{2+}-dependent. This latter finding was unexpected, as we previously found that increased phosphatidylinositol turnover due to other agonists for mast-cell secretion is independent of the presence of Ca^{2+} (Cockcroft & Gomperts, 1979*c*). The sensitivity of the ATP^{4-}-induced phosphatidylinositol labelling to extracellular Ca^{2+} does not arise from an inhibitory effect of Mg^{2+} (which could dominate in the absence of Ca^{2+}), because when we applied ATP at micromolar concentrations in the absence of bivalent cations (i.e. $ATP_{(total)}$ concentration approximately equal to that of ATP^{4-}) we were unable to elicit a phosphatidylinositol response.

Fig. 3 illustrates the time course of histamine secretion and phosphatidylinositol labelling at different concentrations of ATP. At concentrations of ATP^{4-} up to 4.3μM, the labelling of phosphatidylinositol commences immediately and continues to increase for at least 40min. The commencement of histamine secretion (Fig. 3*b*) is subject to a form of delay, which, as we have previously shown (Cockcroft & Gomperts, 1979*a*), varies with the concentration of ATP^{4-}. Addition of ATP^{4-} at 8.7μM, which is inhibitory to secretion, causes immediate and maintained inhibition of phosphatidylinositol labelling.

To test whether the self-inhibition by ATP of ATP-induced secretion (illustrated in Figs. 1 and 2) involves limitation of Ca^{2+} movements, we added ATP to mast cells together with ionophore A23187. The results are shown in Fig. 4. In the absence of Mg^{2+}, ATP inhibits ionophore-A23187-mediated secretion over the range 30–120μM, but the presence of Mg^{2+} negates this effect, so that, with Mg^{2+} at 3.0mM, ATP exerts no inhibition over this range of concentrations (Ca^{2+} was 1.8mM throughout).

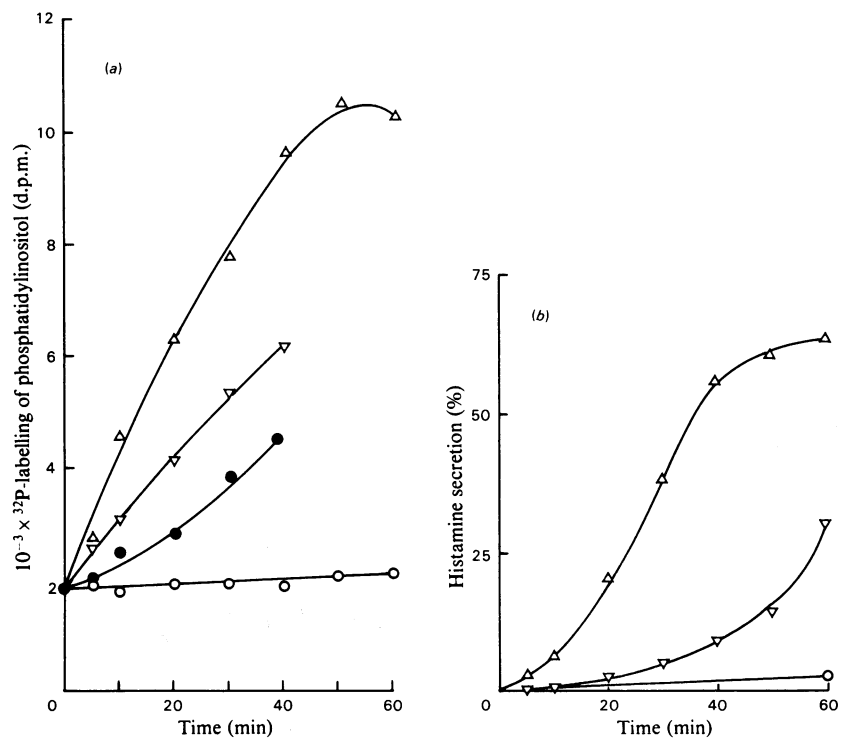

Fig. 3. *Time courses of* (*a*) *ATP-induced phosphatidylinositol labelling and of* (*b*) *ATP-induced histamine secretion by mast cells*

ATP (0, 50, 150 and 300μM) was applied to purified mast cells in the presence of 1mM-Mg^{2+} and 1.8mM-Ca^{2+} to give ATP^{4-} concentrations of 0μM (●), 1.4μM (▽), 4.3μM (△) and 8.7μM (○). In the absence of applied ATP, histamine secretion during 60min was less than 5%. Experimental details are given in the text.

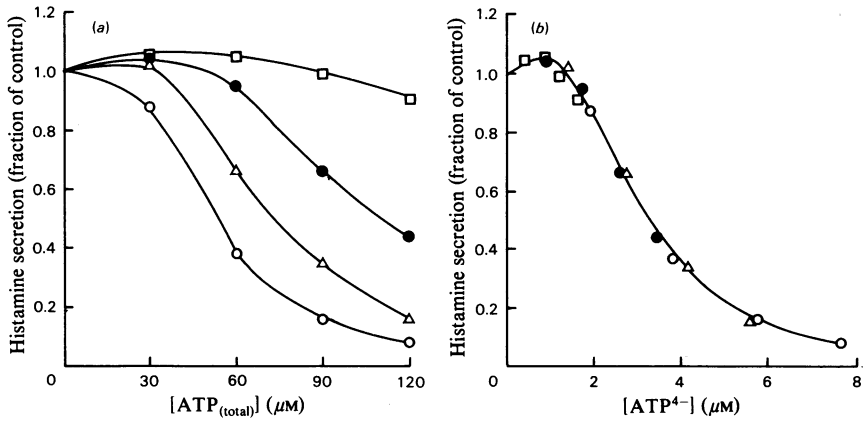

Fig. 4. *Inhibition by ATP of ionophore-A23187-mediated histamine secretion by mast cells in the presence of different concentrations of Mg^{2+}*

(*a*) Incubations were for 10 min. The concentration of ionophore A23187 was 0.5 μM, and that of Ca^{2+} was 1.8 mM throughout. ○, 0 mM-Mg^{2+}; △, 0.3 mM-Mg^{2+}; ●, 1.0 mM-Mg^{2+}; □, 3.0 mM-Mg^{2+}. The results are expressed as a fraction of the secretion induced by ionophore A23187 in the absence of ATP. This was 75.8 ± 1.0% (±S.E.M., $n = 8$) and was unaffected by Mg^{2+} in the range 0–3 mM. Experimental details are given in the text. (*b*) Data of (*a*) expressed as a function of ATP^{4-} concentration.

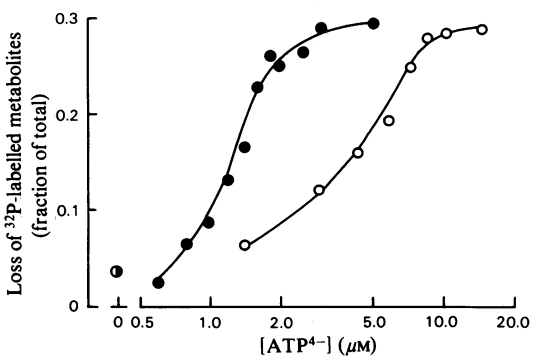

Fig. 5. *ATP^{4-}-induced leakage of ^{32}P-labelled metabolites by mast cells*

Peritoneal cells were incubated for 2 h with carrier-free [^{32}P]P_i, and the mast cells were then isolated by centrifugation through Ficoll as described in the Methods section. Incubations with ATP were for 10 min both in the absence of bivalent cations (●) and in the presence of 1 mM-Mg^{2+} plus 1.8 mM-Ca^{2+} (○). Experimental details are given in the text. Histamine secretion during 10 min with 150 μM-$ATP_{(total)}$ in presence of Mg^{2+} and Ca^{2+} was 10%.

The results have been replotted in Fig. 4(*b*) in terms of the calculated concentrations of ATP^{4-}, and it becomes clear that it is the free acid ATP^{4-} that is the effective inhibitor of ionophore-A23187-mediated secretion. We have found that ATP has no effect on the fluorescence-emission spectrum of ionophore A23187 and that it has no effect on the rate of ionophore-A23187-mediated efflux of Ca^{2+} from Ca^{2+}-loaded liposomes (results not shown), so that it would appear that the inhibition by ATP^{4-} of ionophore-A23187-induced histamine secretion is mediated through an effect at the cellular level. Since under these circumstances the ionophore can be expected to be carrying Ca^{2+} into the cells by diffusion through the lipid bilayer of the plasma membrane in the normal way (Gomperts, 1977), the inhibition due to ATP^{4-} must be exerted at a later stage in the secretory process.

ATP-stimulated leakage of ^{32}P-labelled metabolites

We have previously shown that ATP at concentrations inhibitory to secretion also causes substantial losses of ^{32}P-labelled metabolites from pre-labelled cells. The experiment shown in Fig. 5 shows that this will also occur if ATP is applied to mast cells in the micromolar range in the absence of bivalent cations. In this case, however, metabolite leakage commences with ATP^{4-} concentration (approximately equal to that of $ATP_{(total)}$) less than 1 μM and becomes maximal with ATP^{4-} at 3 μM, whereas in the presence of bivalent cations loss of ^{32}P-labelled metabolites is first detectable with ATP^{4-} at about 1.4 μM and becomes maximal at 8.6 μM. It would thus appear that the bivalent cations are able to confer some form of protection on the system against this effect of ATP.

Specificity of the ATP^{4-} receptor

We tested a number of other nucleotides and

Table 2. *Effects of ATP analogues on activation and inhibition of histamine secretion and leakage of ^{32}P-labelled metabolites from mast cells*

Experimental details are given in the text and in the legend to Fig. 6.

	Concn. of nucleotide (μM)			
	50% initiation of secretion	Optimal secretion	50% inhibition of secretion	50% metabolite leakage
(A) No modification				
(1) ATP	70	100	200	160
(B) Modification to adenine moiety				
(2) 8-Bromo-ATP		Inactive		Inactive
(3) 1,N^6-Etheno-ATP		Inactive		Inactive
(4) GTP		Inactive		Inactive
(5) CTP		Inactive		Inactive
(C) Modification to ribose moiety				
(6) 2′-Deoxy-ATP	530	600	1200	1000
(7) 3′-*O*-Methyl-ATP	*	Active*	*	Active*
(8) 9β-D-Arabinofuranosyl-ATP	50	75	140	120
(D) Modification to triphosphate moiety				
(9) [$\beta\gamma$-Thio]ATP	140	225	420	340
(10) [$\beta\gamma$-Imido]ATP		Inactive		Inactive
(11) [$\alpha\beta$-Methylene]ATP		Inactive		Inactive
(12) [$\beta\gamma$-Methylene]ATP		Inactive		Inactive
(13) Adenosine tetraphosphate		Inactive		Inactive
(14) ADP		Inactive		Inactive
(15) AMP		Inactive		Inactive
(16) Adenosine		Inactive		Inactive
(17) Adenosine 5′-monosulphate		Inactive		Inactive
(18) γ-Methoxy-ATP		Not tested†		Inactive†
(19) γ-Fluoro-ATP		Not tested†		Inactive†

* Because of limited availability we were unable to test compound (7) at concentrations above 350μM in the presence of Mg^{2+} and Ca^{2+}; at this concentration histamine secretion occurred but was sub-maximal. Measurements of metabolite leakage with this compound were made only in the absence of bivalent cations; under these conditions leakage was maximal with 3′-*O*-methyl-ATP^{4-} at 15μM.

† Because of limited availability these compounds could only be tested in the absence of Mg^{2+} and Ca^{2+}.

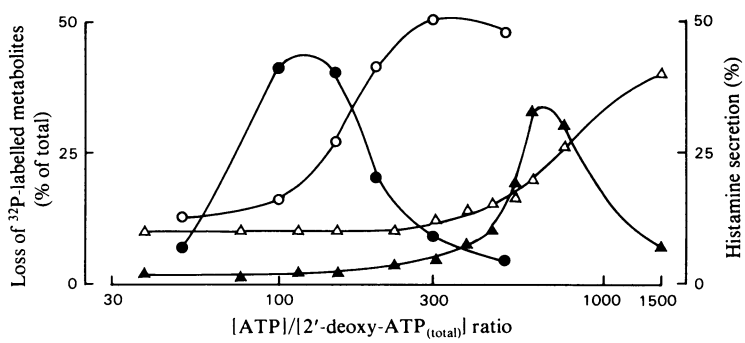

Fig. 6. *Concentration–effect relationships for histamine secretion and leakage of ^{32}P-labelled metabolites from mast cells treated with ATP and 2′-deoxy-ATP*

● and ○, ATP; ▲ and △, 2′-deoxy-ATP. ● and ▲, Histamine secretion; ○ and △, leakage of ^{32}P-labelled metabolites. The mast cells were preincubated for 2h with [^{32}P]P_i and washed before incubation with nucleotides and Mg^{2+} (1mM) and Ca^{2+} (1.8mM) for 30min. Radioactivity and histamine concentration in the supernatant were measured. Experimental details are given in the text.

synthetic analogues of ATP as agonists for histamine secretion and leakage of ^{32}P-labelled metabolites. The results are presented in Table 2, and these refer to incubations of 30 min duration. In addition to ATP, only [$\beta\gamma$-thio]ATP, 2′-deoxy-ATP, 3′-*O*-methyl-ATP and 9-β-D-arabinofuranosyl-ATP were capable of stimulating mast cells to secrete histamine. Furthermore, only these compounds were permissive of leakage of ^{32}P-labelled metabolites. None of the other compounds tested was effective in stimulating either function when applied at concentrations up to 1 mM. Since we could not tell whether the analogues differ significantly from ATP in their affinity for the bivalent cations Mg^{2+} and Ca^{2+}, we also tested them up to 50 μM in a bivalent-cation-free medium for their effect on leakage of ^{32}P-labelled metabolites: none of the inactive compounds listed in Table 2 showed any activity in these experiments. Also, none of the non-agonist compounds was antagonistic to leakage of ^{32}P-labelled metabolites due to ATP applied in the presence (ATP at 300 μM, non-agonists at 1 mM) or absence (ATP at 5 μM, non-agonists at 50 μM) of bivalent cations.

Although the (total) concentrations of the four active analogues required to stimulate the mast cells are different from those found for ATP, the relative concentrations of any single compound needed to elicit histamine secretion and leakage of ^{32}P-labelled metabolites are rather similar. This relationship is illustrated for ATP and 2′-deoxy-ATP in Fig. 6. (Although equilibrium data for complex-formation between Mg^{2+}, Ca^{2+} and the active ATP analogues have not been reported, these are unlikely to differ materially from those for ATP, and so it is probable that the affinity of the receptor for 2′-deoxy-ATP^{4-} is indeed less than for ATP^{4-}, as Fig. 6 suggests.)

Discussion

The biological function of ATP^{4-} receptors on mast cells is not known. One must consider the possibility that extracellular ATP can become available as a consequence of the normal functions of some tissues in which it is a secretory product. The adrenal medulla (Hillarp & Thieme, 1959) and platelets (Day & Holmsen, 1971) are good examples of this. ATP could also become available as a result of burns and other forms of traumatic shock (Green & Stoner, 1950). The half-life of ATP in the circulation is about 3–5 min (Holmsen *et al.*, 1969). It has been suggested that ATP released from nerves during axon reflex processes produces vasodilation of skin vessels by releasing histamine from mast cells in the vicinity (Kiernan, 1974), but our work indicates that, depending on concentration, ATP released *in vivo* could also have an inhibitory effect on mast-cell secretion.

The results show that histamine secretion and phosphatidylinositol labelling are both stimulated by the same form of ATP, and, in agreement with our previous results, the experiments illustrated in Fig. 2 and Table 1 provide confirmation that this is the uncomplexed ATP^{4-}. As presented here, the concentration–effect relationships for the two responses do appear to be slightly different, with the optimum concentration of ATP for histamine secretion being higher than that needed to achieve maximal phosphatidylinositol labelling. It is probable that this difference is largely trivial in origin, because the experiments as carried out necessarily represent a compromise in time. The separate measurements of ATP-mediated histamine secretion and phosphatidylinositol labelling present a conflict of requirements such that a prolonged incubation (75 min) is ideal to ensure termination of secretion, but short incubations are needed to prevent distortion of the pattern of phosphatidylinositol labelling by ATP-induced leakage from the cells of the labelled precursors [^{32}P]P_i and [^{32}P]ATP. Figs. 1 and 2 describe the ATP-induced responses during 20 min and 30 min of incubation. At these times the secretion due to the lower concentrations of ATP would have been far from complete, so the curve describing histamine secretion appears to be shifted to higher ATP concentrations. Conversely, the curve describing phosphatidylinositol labelling appears shifted to lower ATP concentrations because of the progressive loss with time of labelled intracellular precursors at the higher concentrations of ATP^{4-}. Obviously, measurements of phosphatidylinositol breakdown [which is understood to be more immediately coupled to receptor function (Michell, 1975)] would be preferable to the labelling experiments reported in the present paper, as it would not be subject to these errors.

The results shown in Fig. 5 demonstrate that the ATP-induced leakage of phosphorylated metabolites is also due to the ligand in the free acid form ATP^{4-}. The requirement for higher concentrations of ATP^{4-} when bivalent cations are present is not due to depletion of ATP^{4-} by complex-formation, though it could possibly arise from a competitive effect of these complexes at the ATP^{4-}-receptor sites. Alternatively, the bivalent cations could confer some form of protection on the cell against this detrimental effect of ATP^{4-}.

The finding that it is the free acid form ATP^{4-} that stimulates and inhibits histamine secretion, stimulates phosphatidylinositol labelling and permits metabolite leakage suggests that all these functions may be initiated by interactions at the same receptor.

Of 18 other nucleotides and analogues tested, only four were active in stimulating histamine secretion, and only these four were permissive of metabolite leakage. The specificity requirements for ligands

active at the ATP^{4-} receptor are fairly precise. Our present data [and those of others with respect to histamine secretion only (Diamant, 1969; Sugiyama, 1971)] suggest that there is a defined requirement for an unmodified adenine ring. In addition, we find that, with the single exception of the substitution of S for O in the $\beta\gamma$-linkage of the triphosphate moiety, the specificity requirements in this section of the ATP molecule are also fairly well defined. The finding that γ-fluoro-ATP and γ-methoxy-ATP both failed to induce metabolite leakage provides some further support for the proposition that it is the tetrabasic ATP^{4-} that is recognized by the receptor; neither of these compounds, which are otherwise minimally modified, are able to dissociate beyond the tribasic form even at high pH. In contrast, three ATP analogues having modifications to the ribose moiety were effective as agonists for both histamine secretion and induced metabolite leakage. Mainly because of limitations of the purse we did not test a larger range of ATP analogues, nor did we test them independently as agonists for phosphatidylinositol labelling. In spite of this, the evidence for the idea of a common receptor mediating activation and inhibition of histamine secretion, phosphatidylinositol labelling and metabolite leakage is strong.

For each of the agonists, the effect of raising the concentration to approximately 3-fold above that required to maximize secretion permits the loss of 40–50% of the intracellular pool of phosphorylated metabolites during 30min. However, we consider that metabolite loss from the cells is an insufficient explanation for the inhibition of the secretory process that occurs at supraoptimal concentrations of ATP^{4-}. As shown in Fig. 4, when the Ca^{2+}-ionophore A23187 is applied to mast cells together with inhibitory concentrations of ATP^{4-}, it is unable to induce secretion. Within the resolution of our time scale the onset of inhibition due to ATP^{4-} is immediate, yet ATP^{4-}-induced leakage follows a slow and prolonged time course (Cockcroft & Gomperts, 1979*b*). However, as shown in Table 2, for all three of the agonists that were tested in both systems, the concentrations required to cause metabolite leakage and to inhibit histamine secretion appear to coincide. It is likely that these are both manifestations of a common cellular phenomenon initiated by ligands binding to the ATP^{4-} receptor.

When mast cells are stimulated with low concentrations of ATP^{4-} (approx. 1μM) there is a delay in the initiation of histamine secretion. However, as we showed previously (Cockcroft & Gomperts, 1979*a*), histamine secretion commences promptly on addition of Ca^{2+} to cells pretreated for 15min with ATP in the absence of Ca^{2+}. This implies that the delay that normally occurs represents the time taken for Ca^{2+} sensitivity to develop (i.e. probably the time taken for Ca^{2+} channels to open). By contrast, phosphatidylinositol labelling due to ATP^{4-} only occurs when Ca^{2+} is present and then commences immediately.

Previous experience with mast cells supported Michell's (1975) hypothesis concerning a precursor role of phosphatidylinositol metabolism in the control of Ca^{2+} movements and hence secretion. This role for phosphatidylinositol metabolism does not hold in the case of ATP stimulation of mast cells. We have now shown that the enhanced secretion due to addition of Ca^{2+} after an initial preincubation in its absence (with ATP^{4-} concentration maintained constant at 1–2μM) (Cockcroft & Gomperts, 1979*a*) is not due to stimulation of phosphatidylinositol metabolism, since under these circumstances it does not occur. On the other hand, addition of ATP^{4-} (1μM) to mast cells in the presence of Ca^{2+} causes an immediate enhancement in the rate of phosphatidylinositol labelling, whereas secretion is delayed for 15min or more. Because phosphatidylinositol labelling due to other ligands is not Ca^{2+}-dependent, the dependence on Ca^{2+} must come at an ATP^{4-}-dependent stage in the generation of metabolic precursors for phosphatidylinositol synthesis. The question arises whether this requirement for Ca^{2+} is expressed externally or within the cell. Exocytotic degranulation follows rapidly on the internalization of Ca^{2+} (Bennett *et al.*, 1979), and so the contrasting kinetic patterns of phosphatidylinositol labelling and secretion seem to suggest that the Ca^{2+} requirement for the former process is expressed at a separate location. This is probably on the cell exterior, but it cannot be at the ATP^{4-}-binding site.

Our work with rabbit neutrophils showed that, in that system, activation resulting in mobilization of Ca^{2+} and secretion of lysosomal enzymes does not require enhanced phosphatidylinositol turnover (Cockcroft *et al.*, 1980). In the present work we have demonstrated that enhanced phosphatidylinositol turnover due to activation of the ATP^{4-} receptor is not sufficient by itself to cause Ca^{2+}-dependent secretion. Quite clearly, a reassessment of the role of phosphatidylinositol metabolism in cellular activation processes is required.

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