Characterization of Calcium-Ion-Activated Adenosine Triphosphatase in the Plasma Membrane of Rat Mast Cells

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(Received 8 December 1975)

The properties of a Ca^{2+} -activated adenosine triphosphatase shown to be present in homogenates of purified rat peritoneal mast cells were investigated. The enzyme was activated by Ca^{2+} , Mg^{2+} , and to a lesser extent by Mn^{2+} and Co^{2+} . Ca^{2+} alone was necessary for full activity and the further addition of Mg^{2+} did not have any effect. The chelating agents EGTA [ethanedioxybis(ethylamine)tetra-acetate] and EDTA completely inhibited the reaction. The pH optimum was 7.8. Reduced glutathione, cysteine, dithiothreitol, *N*-ethylmaleimide, urea, ADP, NaF, increasing ionic strength and Triton X-100 all inhibited the reaction. On subcellular fractionation of mast-cell homogenates by density-gradient centrifugation, the distribution of Ca^{2+} -activated adenosine triphosphatase resembled that of 5'-nucleotidase, but differed from that of the other markers used, suggesting localization in the plasma membrane. Further experiments indicated that the enzyme is present on the external surface of the plasma membrane.

Histamine is stored in mast cells in membrane-bound cellular organelles or granules, and all the available evidence favours the hypothesis that its release occurs by discharge of the granular materials through the cell membrane involving the process of membrane fusion (Bach, 1974; Schachter, 1973; Lagunoff, 1973). This process has been termed degranulation, and appears to correspond to exocytosis in endocrine secretory systems (Röhlich *et al.*, 1971).

The physiological stimulus for the release of histamine is the anaphylactic antibody-antigen reaction, although other substances are able to effect histamine release artificially (Goth, 1967; Stanworth, 1973). Histamine release induced by many such agents is a highly selective process, other soluble constituents of the mast cell not being released concomitantly with histamine (Schachter, 1973).

In common with other systems that store secretory products within granules, there appear to be the same common denominators in the release process, namely the requirement for energy and Ca^{2+} (Rubin, 1974). Our present knowledge of enzymes coupled to such energy-requiring systems would suggest the involvement of some kind of ATPase* (Rubin, 1970, 1974; Poste & Allison, 1973; Bach, 1974). Two types of ATPase could be implicated: first, a Ca^{2+} -transporting ATPase similar to that described in the erythrocyte, which ordinarily keeps Ca^{2+} ions out of the cell by the expenditure of ATP; other Ca^{2+} -transporting ATPases have been described in mitochondria and the sarcoplasmic reticulum (Schatzmann, 1975).

Secondly, ATPase activities have been related to contractile proteins, and in this connexion it is interesting that there are striking similarities between the processes regulating secretion and those regulating contraction in their requirement for both Ca^{2+} and ATP (Rubin, 1970; Harris *et al.*, 1974). Thus a similar mechanism may exist in the two triggering processes, which involves the interaction of Ca^{2+} , ATP and ATPase. The action of Ca^{2+} may be to alter the viscosity of the cytoplasm to facilitate the interaction of granular membrane with the plasma membrane, or Ca^{2+} may conjugate with adenine nucleotide to form the connecting link between the two membranes (Poisner & Trifarò, 1967).

Evidence for the existence of such an ATPase in mast cells is at present lacking. For the present study mast cells from the peritoneal cavity of the rat provided a suitable model. With these points in mind the presence of a cation-stimulated ATPase in the mast cell was confirmed and its properties were investigated. Later experiments showed that the enzyme is localized at the external surface of the plasma membrane.

Materials and Methods

Materials

AMP (disodium salt), ATP (Tris salt), bovine serum albumin (fraction V and type F), 2-deoxy-D-glucose, 2-glycerophosphate, DL-lactic acid (sodium salt), 2,4-dinitrophenylhydrazine, Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid],

* Abbreviation: ATPase, adenosine triphosphatase.

histamine, *o*-phthalaldehyde, ouabain, NAD⁺ and NADH were from Sigma (London) Chemical Co. Kingston-upon-Thames, Surrey KT2 7BH, U.K. Plastic apparatus (test tubes and pipettes) used throughout were from Sterilin, Richmond, Surrey, U.K. Cellulose nitrate centrifuge tubes were from Beckman, Croydon, Surrey CR9 3LG, U.K.

Purification of mast cells

The method of Cooper & Stanworth (1974) was used except that all solutions were prepared in 20mM-Hepes/0.9% NaCl/0.5mM-glucose, pH 7.4 (Hepes medium), instead of 0.1M-phosphate-buffered saline, pH 7.3. Preparations contained at least 95% pure mast cells, and were checked for viability by light microscopy and Toluidine Blue staining.

Preparation of mast-cell homogenates

Peritoneal mast cells were purified as above and suspended in a small volume (1–2 ml) of Hepes medium (+0.5% bovine serum albumin type F).

Since mast-cell granules lose histamine when in contact with Na⁺ or K⁺ ions (Green, 1967), all sucrose media used for preparation and subfractionation of homogenates were buffered at pH 7.4 with solid Tris. The cells were washed twice with 0.25M-sucrose, re-suspended in 1–2 ml of the same medium and pipetted into a 20 ml ice-chilled cup of an MSE 100W Ultrasonicator. The microprobe used (titanium exponential microprobe; end diameter 3 mm) was pre-cooled to –20°C. The cells were then sonicated at 20 kHz (4 μ m peak-to-peak) for 10 s. The preparations were examined microscopically and it was shown that more than 95% of the mast cells were disrupted. Under these conditions a high proportion of the histamine-containing granules keep their perigranular membrane intact (Üvnas, 1974).

Subfractionation of mast-cell homogenates

Mast cells from eight to ten rats (6–8 $\times$ 10⁶ cells) were isolated and homogenized as described above. All operations were performed at 4°C. The homogenates were then subfractionated by one of the following methods.

(1) *Discontinuous sucrose-gradient centrifugation.* A two-step gradient of 2 ml of 1.0M-sucrose below 2 ml of 0.5M-sucrose was prepared in a 5 ml centrifuge tube, and 1 ml of mast-cell homogenate in 0.25M-sucrose was layered on top of the gradient and then centrifuged at 12000 rev./min (7160 $g_{av.}$) for 90 min in a SW65 rotor in a Beckman L2-65 preparative ultracentrifuge. The tubes were then fractionated in a tube slicer (Randolph & Ryan, 1950); the fractions were stored at –20°C until ready for characterization.

(2) *Flotation sucrose-gradient centrifugation.* Mast-cell homogenates prepared as described above were centrifuged at 40000 rev./min (102000 $g_{av.}$) for 1 h in a Ti65 rotor to sediment all particulate material. The pellet was resuspended in 2.5 ml of 1.0M-sucrose, and pipetted into a 5 ml centrifuge tube. An overlay of 2.5 ml of 0.5M-sucrose was applied and the tube centrifuged at 20000 rev./min (28700 $g_{av.}$) for 15 h. The tubes were then fractionated as described above.

(3) *Continuous sucrose-gradient centrifugation.* Mast-cell homogenate was prepared as above and centrifuged at 2000 rev./min (750 $g_{av.}$) in an MSE Major centrifuge for 5 min. The pellet was washed three times and the combined supernatants were centrifuged at 40000 rev./min (102000 $g_{av.}$) in a Ti65 rotor for 1 h. The pellet was rehomogenized in 0.5M-sucrose in a Potter-Elvehjem Teflon-glass homogenizer and made up to a final volume of 2.5 ml. This was placed in the right-hand chamber of a gradient maker and 2.5 ml of 2M-sucrose placed in the left-hand chamber. A continuous gradient was thus formed in which the sample was uniformly distributed throughout. The tube was then spun at 40000 rev./min (102000 $g_{av.}$) in a SW65 rotor and subfractionated in a tube slicer, as described above.

Chemical methods

Protein was determined by the method of Lowry *et al.* (1951). E_{750} was measured. Standard curves using bovine serum albumin fraction F were run concurrently. P_i was determined by the method of Baginski *et al.* (1967).

Histamine was assayed by a manual (Shore *et al.*, 1959) or automated (Evans *et al.*, 1973) spectrofluorimetric procedure.

Enzyme assays

Ca²⁺-activated ATPase (EC 3.6.1.3). The final assay method was developed as described in the Results and Discussion section. The following components were preincubated at 37°C for 2 min in a total mixture volume of 0.25 ml: 30 mM-Tris/HCl buffer (pH 7.8), 4 mM-CaCl₂ and 10–50 μ g of mast-cell homogenate protein. The reaction was started by addition of ATP (Tris salt), pH 7.8, to give a final concentration of 4 mM. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 0.25 ml of ice-cold 20% (w/v) trichloroacetic acid. Centrifugation was generally unnecessary. After 5 min at 4°C in an ice bath, suitable samples were taken and P_i release was determined by the method of Baginski *et al.* (1967), the volumes of the reagents being scaled down by a factor of 4 to increase the sensitivity. In each experiment appropriate controls were incubated, one without ATP, one without mast-cell homogenates and one without Ca²⁺. ATPase activity was

calculated as the P_i release in the complete assay minus the release in the controls.

($Na^+ + K^+$)-stimulated ATPase (EC 3.6.1.3). This enzyme was assayed by the method of Cooper & Hawthorne (1975), except that the final volume of the assay mixture was 0.25 ml and the incubation time was 60 min; 10–50 μ g of mast-cell homogenate protein was used.

Acid phosphatase (EC 3.1.3.2). The method of Hübscher & West (1965) was used. The final assay volume was 0.25 ml and the incubation time was 2 h; 10–50 μ g of mast-cell homogenate protein was used.

5'-Nucleotidase (EC 3.1.3.5). The composition of the assay mixture was as follows. The total volume of the assay mixture was 0.25 ml, comprising 20 mM-Tris/HCl buffer (pH 7.4), 10 mM-sodium potassium tartrate, 10 mM-MgCl₂, 100 mM-KCl, 5 mM-AMP and 10–50 μ g of mast-cell homogenate protein. No-substrate and no-enzyme controls were also performed. The reaction was started by addition of substrate, and incubation was for 90 min at 37°C. The reaction was terminated, and the released P_i measured as described above.

Lactate dehydrogenase (EC 1.1.1.27). This was assayed by the colorimetric method of King as described by Varley (1967) except that the final assay volumes were scaled down by a factor of 4 to give the necessary sensitivity, and incubation was allowed to proceed for 30 or 60 min; 10–50 μ g of mast-cell homogenate protein was used. Pyruvate standards were run concurrently with each assay.

Results and Discussion

Enzyme characterization (Fig. 1)

Metal-ion requirements. To determine which types of ATPase are present in the mast cell it was first necessary to establish their metal-ion dependency. As the results in Table 1 show, ATP breakdown was stimulated by Ca^{2+} and Mg^{2+} equally and the other ions activated in the order $Mn^{2+} > Co^{2+} > Zn^{2+} > Sr^{2+}$. The metal-ion-requirement curves for Ca^{2+} , Mg^{2+} and $Ca^{2+} + Mg^{2+}$ are shown in Fig. 1. Optima were reached at 4 mM. Sr^{2+} , which can replace Ca^{2+} in histamine release (Foreman & Mongar, 1972), did not activate the ATPase significantly. The presence of Mg^{2+} did not appear to increase Ca^{2+} -stimulated ATP breakdown. This appears to be different from the ($Ca^{2+} + Mg^{2+}$)-activated ATPase of the erythrocyte, which requires the presence of Mg^{2+} for full activity (Schatzmann, 1975).

ATP requirement. An optimum was reached at 4 mM-ATP in all cases, which is equimolar with the metal-ion concentration. Substrate-requirement studies carried out in which the Ca^{2+} and ATP concentrations were maintained in equimolar ratios revealed a similar curve.

Time-course. The rate of ATP breakdown was constant for 60 min, activity declining after this time.

Enzyme concentration. ATPase activity was proportional to enzyme concentration over the range used (0–100 μ g of homogenate protein).

Effect of pH. ATP breakdown was most active at slightly alkaline pH, optima being at pH 7.8–8.6.

Effect of inhibitors on Ca^{2+} -activated ATPase

As Table 2 shows, the presence of the end product of the reaction, ADP, completely inhibited Ca^{2+} -ATPase activity at a concentration of 10 mM. Increasing ionic strength inhibited ATPase activity, which is the reverse of that expected if the enzyme has actomyosin-like properties (Harris *et al.*, 1974). Triton X-100 inhibited the enzyme at low concentration (0.01–1%). Very small amounts of detergent (0.0005–0.001%) had no effect. Therefore 'super-activation' of Ca^{2+} -activated ATPase by low concentrations of detergents was not observed, as has been shown for ($Na^+ + K^+$)-activated ATPase (Roelofsens *et al.*, 1971). EDTA and EGTA [ethanedioxybis(ethylamine)tetra-acetate] both completely inhibited at concentrations at which it would be expected that all available metal ion is chelated; NaF and various thiol-containing compounds were also inhibitory (Fig. 2). Histamine release has been shown to depend on the presence of thiol groups (Edman *et al.*, 1964). Ouabain, the inhibitor of ($Na^+ + K^+$)-stimulated ATPase, and of iodoacetamide had no effect on the enzyme. Caffeine and theophylline, both inhibitors of the actomyosin ATPase (Martonosi, 1971), also lowered the activity of mast-cell Ca^{2+} -activated ATPase.

Subcellular localization of Ca^{2+} -activated ATPase

Mast-cell homogenates were subfractionated by various sucrose-gradient techniques as described above. To determine the fate of the different cell components various markers were used. These were 5'-nucleotidase (plasma membrane), lactate dehydrogenase (cytoplasm), acid phosphatase (lysosomes and granule membranes) and histamine (granules). The last three markers have been shown to be present in the mast cell (Bloom, 1974), and histamine has been proved to be present in the mast-cell granule (Schachter, 1973). 5'-Nucleotidase has been shown to be present in the plasma membrane of all tissues so far examined, but not as yet in the mast cell (Goldfischer *et al.*, 1964; de Duve, 1971; Birnie, 1972).

Discontinuous-gradient experiments. The distribution of Ca^{2+} -activated ATPase resembled that of 5'-nucleotidase, suggesting localization in the plasma membrane, and was very different from that of the other markers used (Fig. 3a, Table 3a).

Flotation experiment. Since plasma membranes contain a high proportion of lipid, they are lighter than other cell components. Flotation has been used for preparing plasma membranes in which the cell

Fig. 1. Characteristics of mast-cell ATPase

Assay conditions were as described in the Materials and Methods section, except where indicated. Metal requirement: (a) ●, Ca^{2+} ; ■, Sr^{2+} ; (b) Mg^{2+} ; (c) $Ca^{2+} + 4 \text{ mM-Mg}^{2+}$. ATP requirement: (d) ●, Ca^{2+} concentration constant at 4 mM; ■, Ca^{2+} and ATP always in equimolar concentrations; (e) Mg^{2+} ; (f) $Ca^{2+} + 4 \text{ mM-Mg}^{2+}$. Time-course: (g) Ca^{2+} ; (h) Mg^{2+} ; (i) $Ca^{2+} + 4 \text{ mM-Mg}^{2+}$. Effect of protein concentration: (j) Ca^{2+} ; (k) Mg^{2+} ; (l) $Ca^{2+} + 4 \text{ mM-Mg}^{2+}$. Effect of pH: (m) Ca^{2+} ; (n) Mg^{2+} ; (o) $Ca^{2+} + 4 \text{ mM-Mg}^{2+}$.

Table 1. Effect of bivalent cations on ATP hydrolysis in mast-cell homogenates

Assay conditions were as described in the Materials and Methods section except that various bivalent cations (as chloride salts) were included at a concentration of 4 mM; 27 μg of mast-cell homogenate protein was present.

Cation	Specific activity (nmol of P_i /min per mg of protein)
Ca^{2+}	10.5
Mg^{2+}	10.9
Mn^{2+}	7.3
Co^{2+}	6.0
Zn^{2+}	2.2
Sr^{2+}	1.0

fraction is layered below a gradient and spun to equilibrium. The membranes float upwards and are separated from other components (Birnie, 1972). This also appeared to be the case with the mast-cell membranes. There was a high concentration of both Ca^{2+} -activated ATPase and 5'-nucleotidase at the top of the gradient (Fig. 3b and Table 3b), again suggesting a plasma-membrane localization.

Continuous gradient experiment. Roodyn (1965) has discussed the difficulties of interpreting subcellular-fractionation data, and has suggested that a clear association between the subcellular component in question and the enzyme should be demonstrated in a variety of preparations of varying purity. Hence a third method of cell fractionation was tried, that is the use of a continuous gradient. de Duve *et al.* (1959) suggested that artifacts involving bulk sedimentation

Table 2. Effect of various inhibitors on Ca^{2+} -activated ATPase

Assay conditions were as described in the Materials and Methods section apart from the inclusion of the various inhibitors; 22 μg of mast-cell homogenate protein was present.

Inhibitor	Concentration	Inhibition of Ca^{2+} -activated ATPase (%)
ADP	10 mM	100.0
	1 mM	58.8
	0.1 mM	11.1
Sucrose	0.3 M	33.3
	10 mM	0.0
Ouabain	1 mM	0.0
KCl	0.5 M	42.4
	0.1 M	29.4
Urea	1 M	27.2
Triton X-100	1%	73.4
	0.1%	70.0
	0.01%	46.8
Caffeine	10 mM	35.5
Theophylline	10 mM	35.5

of material can be minimized when the material is homogeneously distributed within the whole tube. This type of experiment was performed and the results (Fig. 3c and Table 3c) show that again distributions of Ca^{2+} -activated ATPase and 5'-nucleotidase were similar. This time there was a much greater percentage of histamine in the particle-free supernatant and this presumably represents removal

Fig. 2. Effect of EDTA, EGTA, NaF and thiol agents on Ca^{2+} -activated ATPase

Assay conditions are described in the Materials and Methods section except for inclusion of inhibitor at various concentrations; $25 \mu\text{g}$ of mast cell homogenate was present. $\circ$, Dithiothreitol; $\bullet$, *N*-ethylmaleimide; $\blacktriangledown$, cysteine; $\blacksquare$, glutathione (reduced); $\blacktriangle$, NaF; $\diamond$, EGTA; $\blacklozenge$, EDTA.

of histamine from the granules by the washing procedures.

Is Ca^{2+} -activated ATPase an ecto-enzyme?

Any activity shown by isolated cells suspended in an iso-osmotic medium containing substrate can presumably be attributed to ecto-enzyme, on the assumption that only the outer aspect of the plasma membrane of intact cells would be capable of interacting with substrate presented in such a manner. On the other hand when disrupted cells are used, both sides of the plasma membrane will be exposed to the substrate. If, therefore, an enzyme is to qualify as an ecto-enzyme it should demonstrate no difference in activity when measured in intact cells as compared to cell homogenates, whereas endo-enzymes, in contrast, would fail to show any activity in intact cells as they are restricted to the inner surface of the plasma membrane.

As ecto-ATPases have already been described in other cells (Wallach & Ullrey, 1962; Trams & Lauter, 1974) it seemed of interest to ascertain whether the Ca^{2+} -activated ATPase identified in mast cells in the present study was located on the outside or inside of the plasma membrane. Since it is possible to isolate pure whole mast cells as well as mast-cell homogenates, it seemed a relatively simple

Fig. 3. Subcellular distribution of Ca^{2+} -activated ATPase and markers in mast-cell fractions

Relative specific activity represents the percentage of total activity recovered in a fraction/percentage of total protein recovered in that fraction. (a) Discontinuous sucrose gradients. Fractions are labelled as follows: S, 0.25M-sucrose layer; M1, 0.25M-/0.5M-sucrose interface; M2, 0.5M-/1.0M-sucrose interface; G, pellet at bottom of tube. The results of three separate experiments are shown. (Vertical bars represent s.d.) (b) Flotation experiment. Fractions are: S, high-speed (100000g) supernatant; 1, 0.5M-sucrose layer; 2, 0.5M-/1.0M-sucrose interface; 3, pellet at bottom of tube. (c) Continuous gradient experiment. Fractions are: S, high-speed (100000g) supernatant; P, low-speed (750g) pellet. Fractions 1-8 (0.6ml) taken from the continuous gradient are numbered from the top (1) to the bottom (8) of the tube. Fraction 8 represents the pellet at the bottom of the tube.

Table 3. *Subcellular distribution of Ca²⁺-activated ATPase and markers in subfractions of mast-cell homogenates*

Specific activities are given as nmol/min per mg of protein for the enzymes and ng of histamine/mg of protein for histamine. Fractionation procedures are described in the Materials and Methods section. The values for the percentage distribution of the activities in the fractions are based on the total recovered activity. Results in (a) represent the mean $\pm$ s.d. of three separate experiments. Values for the amount of protein in the original homogenates are (a) 0.89 $\pm$ 0.07 mg; (b) 1.55 mg; (c) 1.48 mg.

(a) Discontinuous sucrose gradients

	Distribution in fractions $\pm$ s.d. (%)				Homogenate (Specific activity)	Recovery (%)
	Supernatant	Interface 1	Interface 2	Granules		
Protein	23.1 $\pm$ 5.2	4.2 $\pm$ 1.2	8.6 $\pm$ 2.6	64.1 $\pm$ 8.8	—	96.7 $\pm$ 4.1
Ca ²⁺ -activated ATPase	14.4 $\pm$ 8.2	28.4 $\pm$ 4.1	25.5 $\pm$ 7.0	30.7 $\pm$ 4.9	12.7 $\pm$ 2.4	102.1 $\pm$ 6.1
5'-Nucleotidase	19.6 $\pm$ 14.8	30.8 $\pm$ 8.8	23.4 $\pm$ 5.6	26.2 $\pm$ 2.7	12.0 $\pm$ 3.5	103.3 $\pm$ 4.3
Acid phosphatase	36.7 $\pm$ 0.7	6.2 $\pm$ 1.5	4.4 $\pm$ 3.1	52.3 $\pm$ 3.6	5.1 $\pm$ 0.9	96.4 $\pm$ 4.4
Histamine	9.8 $\pm$ 3.1	2.5 $\pm$ 0.3	13.7 $\pm$ 6.9	74.0 $\pm$ 4.0	18.5 $\pm$ 4.6	100.8 $\pm$ 6.4
Lactate dehydrogenase	70.3 $\pm$ 9.5	11.8 $\pm$ 1.6	4.3 $\pm$ 1.7	13.6 $\pm$ 11.4	41.2 $\pm$ 3.9	99.3 $\pm$ 7.2

(b) Flotation experiment

	Distribution in fractions (%)				Homogenate (Specific activity)	Recovery (%)
	Supernatant	Upper layer	Interface	Pellet		
Protein	24.4	2.2	9.6	63.9	—	91.2
Ca ²⁺ -activated ATPase	12.2	10.1	14.3	63.4	11.8	109.7
5'-Nucleotidase	12.8	11.3	20.0	56.0	10.8	93.9
Acid phosphatase	41.1	0.0	0.0	58.9	2.2	94.3
Histamine	17.4	0.0	6.7	75.9	14.6	99.6
Lactate dehydrogenase	73.0	0.9	8.9	17.8	51.0	93.6

(c) Continuous gradient experiment

	Distributions in fractions (%)										Homo- genate (Specific activity)	Recovery (%)
	Super- natant	Low- speed pellet	1	2	3	4	5	6	7	8		
Protein	30.4	16.2	2.4	2.4	2.8	2.8	3.8	4.0	4.5	30.2	—	100.0
Ca ²⁺ -activated ATPase	10.8	7.2	3.8	6.1	13.5	16.3	9.3	7.9	5.0	20.1	11.9	94.6
5'-Nucleotidase	10.7	7.1	3.6	5.6	13.5	15.6	12.0	9.5	3.5	19.0	9.9	92.3
Acid phosphatase	41.9	19.8	0.0	0.0	0.0	0.0	0.0	1.8	2.6	33.9	4.1	102.0
Histamine	70.8	9.9	0.7	0.7	0.7	0.7	0.8	1.0	1.0	13.6	11.9	88.4
Lactate dehydrogenase	82.4	2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.8	43.3	101.5

Table 4. *Effect of deoxyglucose on the release of histamine and lactate dehydrogenase from mast cells incubated with ATP*

Whole mast cells (4×10^5 cells) were incubated with or without 5 mM-deoxyglucose for 10 min at 37°C under the conditions for the Ca²⁺-activated ATPase assay (30 mM-Tris/HCl, pH 7.8, 4 mM-CaCl₂, 4 mM-ATP and 0.9% NaCl in a final volume of 0.25 ml) and also under conditions optimal for ATP-induced histamine release (Hepes medium, pH 7.4, 0.5 mM-CaCl₂ and 0.5 mM-ATP in a final volume of 0.25 ml). No-substrate and no-enzyme controls were also performed. The cells were then spun at 750g for 5 min and the supernatants decanted. After 0.25 ml of 0.9% NaCl had been added to the pellets, they were sonicated (20 kHz; 8 μ m peak-to-peak) for 10 s. Both the supernatants and pellets were then spun at 750g for 10 min. Samples (50 μ l) were then taken for histamine and lactate dehydrogenase assays. The results represent the mean $\pm$ s.d. of three separate experiments.

Agent	% Lactate dehydrogenase release	% Histamine release
4 mM-ATP + 5 mM-deoxyglucose + 4 mM-Ca ²⁺	7.1 $\pm$ 1.7	3.2 $\pm$ 0.5
4 mM-ATP + 4 mM-Ca ²⁺	5.9 $\pm$ 0.9	8.1 $\pm$ 2.0
0.5 mM-ATP + 5 mM-deoxyglucose + 0.5 mM-Ca ²⁺	7.4 $\pm$ 1.3	4.3 $\pm$ 1.4
0.5 mM-ATP + 0.5 mM-Ca ²⁺	7.3 $\pm$ 0.9	35.4 $\pm$ 1.1
Control	4.7 $\pm$ 2.4	2.1 $\pm$ 0.5

Table 5. Activities of Ca^{2+} -activated ATPase and plasma-membrane enzymes in whole mast cells and homogenates

Whole mast cells or homogenates were incubated in assay media optimal for the enzymes being tested, except that 0.9% NaCl was included to maintain iso-osmoticity and 5 mM-deoxyglucose to prevent ATP-induced histamine release. No-enzyme and no-substrate controls were performed. Approx. 80 μg of mast-cell protein was used in each assay. The reaction volumes were 0.25 ml in all cases. Incubation was for 10 min at 37°C and the reactions were terminated by the addition of 0.25 ml of cold 20% (v/v) trichloroacetic acid. After centrifugation the supernatants were analysed for liberated P_i as described in the Materials and Methods section. The results represent the mean $\pm$ S.D. of four separate experiments.

Enzyme	Enzyme specific activity (nmol of P_i /min per mg of protein)	
	Whole cells	Homogenates
Ca^{2+} -activated ATPase	8.4 ± 1.8	8.7 ± 1.0
Mg^{2+} -activated ATPase	10.6 ± 0.3	13.6 ± 0.6
$(\text{Na}^+ + \text{K}^+)$ -activated ATPase	0.0 ± 0.0	7.8 ± 3.1
5'-Nucleotidase	0.4 ± 0.5	11.8 ± 2.0

task to measure and compare the ATPase activities of these two types of preparation.

The complication of the histamine release induced by ATP in the presence of Ca^{2+} (Diamant & Krüger, 1967) was overcome by the use of deoxyglucose, a known inhibitor of histamine release (Goth, 1967). This was shown to inhibit ATP-induced histamine release from rat mast cells (Table 4), accompanying release of lactate dehydrogenase (an indicator of cell damage) proving to be low. When Ca^{2+} -activated ATPase was assayed (incubation time 10 min) in the presence of 5 mM-deoxyglucose, equal enzyme activity was observed in both whole-cell and homogenate preparations. Mg^{2+} -activated ATPase activity was also found to be similar in both types of preparation; whereas $(\text{Na}^+ + \text{K}^+)$ -activated ATPase and 5'-nucleotidase activity were not detected in the whole-cell preparation (Table 5).

It is interesting to note that $(\text{Na}^+ + \text{K}^+)$ -activated ATPase has been shown to be present on the inner surface of the erythrocyte plasma membrane (Coleman, 1973), but 5'-nucleotidase is generally considered to be an ecto-enzyme (Trams & Lauter, 1974). The apparent activity of the latter enzyme observed in mast-cell homogenates did not appear to be attributable to non-specific acid phosphatase since it did not exhibit detergent activation, and at pH 5.4 only 20% of the activity at pH 7.4 was recorded. Moreover, 1 mM-NaF (which completely inhibits acid phosphatase) caused only 20% inhibition of enzyme activity. Further, the subcellular distribution of

mast-cell 5'-nucleotidase observed (Fig. 3 and Table 3) was completely different to that of acid phosphatase. Alkaline phosphatase was undetectable in the mast-cell homogenates and therefore need not be considered. Hence we concluded that the activity observed was indeed that of 5'-nucleotidase.

Conclusion

In summary, it would appear that there is present in the rat mast cell a Ca^{2+} -activated ATPase located on the outer surface of the plasma membrane. This would tend to rule out the possibility that the function of Ca^{2+} -activated ATPase in the mast cell is that of an ion pump, which ordinarily excludes Ca^{2+} ions from the cell by expenditure of ATP. In contrast, the Ca^{2+} -activated ATPase of the erythrocyte has been shown to be present on the inner surface of the plasma membrane (Schatzmann, 1975). It is, however, possible that the true orientation of the ATPase is normally towards the interior of the cell, but that the enzyme molecules are not fixed rigidly in this configuration, so that presentation of the substrate ATP to the external surface causes a reversal of their positions. Indeed, extracellular ATP has been shown to produce a reversible increase in the cation (Na^+ and K^+) permeability of dog erythrocytes and to affect the surface properties of chick-embryo fibroblasts and ascites-tumour cells (Elford, 1975), besides causing movement of Ca^{2+} ions across the mast-cell membrane leading to histamine release (Dahlquist, 1974).

Therefore it has been suggested that ATP induces an increased membrane permeability to cations. Our identification of a cation-activated ATPase at the surface of the mast cell indicates, however, that this is an energy-requiring process (at least as far as this cell type is considered). Moreover, thrombosthenin, a contractile protein with the characteristics of smooth muscle actomyosin Ca^{2+} -activated ATPase found in blood platelets, has also been shown to be localized on the outside of the plasma membrane, and, like the mast-cell enzyme, is inhibited by ADP (Moake *et al.*, 1970). The mast cell may possess a similar contractile protein system. In this connexion, therefore, it is interesting to note that membrane-associated actin filaments have recently been detected in the rat mast cell (Röhlich, 1975). Although it seems unlikely that the cell is going to encounter extracellular concentrations of ATP in the mM range *in vivo*, various mammalian cells have been shown to be capable of synthesizing ATP on their outer surface (Agren *et al.*, 1971). Further, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase have also been shown to be ecto-enzymes capable of synthesizing ATP extracellularly (Fenselau & Long, 1974), and it has been shown that adenylate kinase is present on the external surface of the platelet plasma membrane (Packham *et al.*, 1975). It is possible therefore that

such enzymes could provide the necessary substrate for the ATPase identified in the present study.

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