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PLASMA MEMBRANE ADENOSINE TRIPHOSPHATASES IN RAT PERITONEAL MAST CELLS AND MACROPHAGES—THE RELATION OF THE MAST CELL ENZYME TO HISTAMINE RELEASE

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Abstract—Adenosine triphosphatases activated by calcium or magnesium have been demonstrated on the outer surface of rat peritoneal mast cells and macrophages. The plasma membrane ATPases in the two types of cells have similar but not identical properties. Mg^{2+} is somewhat more effective than Ca^{2+} in stimulating both the enzymes. They are not influenced by sodium and potassium and not inhibited by ouabain and oligomycin. Ethacrynic acid inhibits both, but the mast cell enzyme is more sensitive to it. The enzyme on the macrophage has five to thirty-seven times higher activity (average seventeen times) than that on the mast cell. The apparent K_m of the enzymes in intact cells, incubated with adenosine triphosphate for 5 min, is estimated to be 36 μM for mast cells and 30 μM for macrophages. The optimal pH for the mast cell and the macrophage enzymes is 6.7 and 7.1 respectively. The activities of the two enzymes rise similarly with temperature up to 37° but differ at 47°, the macrophage enzyme being less active at this temperature than at 37°. Phosphatidyl serine, which stimulates anaphylactic and dextran-induced histamine release, causes about 40 per cent stimulation of the plasma membrane ATPase of mast cells in the absence of Ca^{2+} and Mg^{2+} but has no appreciable effect in their presence. No change in the mast cell enzyme could, however, be observed in relation to histamine release induced by dextran, compound 48/80 and ATP. But ethacrynic acid, which in 1 mM concentration inhibits 50 per cent of the mast cell enzyme activity, also causes pronounced inhibition of histamine release induced by all the three agents in the same concentration. The inhibition is not influenced by the presence of glucose, suggesting that ethacrynic acid does not inhibit histamine release by blocking energy metabolism. Ethacrynic acid apparently acts at another site. The site of action could very well be plasma membrane ATPase. There is also a correlation between the inhibition of the mast cell enzyme by sodium fluoride and lack of calcium and their inhibitory effect on histamine release. The possible involvement of the plasma membrane ATPase of mast cells in the process of exocytosis leading to histamine release is discussed.

Calcium or magnesium activated ATPases have been demonstrated in the plasma membrane or the secretory granule of various types of secretory cells [1–5]. In the adrenal medulla a correlation has been shown between Mg^{2+} ATPase of chromaffin granules and catechol amine release [2]. For platelets it has been suggested that the Ca^{2+}–Mg^{2+} ATPase in the contractile element, actomyosin, of the platelet membrane by bringing about a change in the form of the membrane may promote the release reaction [5]. Ca^{2+}–Mg^{2+} ATPases have also been demonstrated in the granules of polymorphonuclear leukocytes [6] and in the plasma membranes of hematopoietic cell lines secreting immunoglobin [7], and in each case it has been proposed that the enzyme is involved in the secretory process. Furthermore, it has been suggested that plasma membrane ATPases of macrophages and leukocytes may participate in the phagocytic process [8–10]. However, the steps leading to secretion or phagocytosis remain to be explored.

In the present study we have demonstrated Ca^{2+}–Mg^{2+} activated ATPases on the outer surface of the plasma membrane of rat peritoneal mast cells and macrophages, and attempted to explore the relation of the mast cell enzyme to the secretion of histamine.

MATERIALS AND METHODS

Male Wistar rats, 250–550 g, were used for all experiments except for one group in which mast cells from male Sprague–Dawley rats were incubated with compound 48/80 for 20 sec. Mast cells were separated from the other peritoneal cells (mostly macrophages) by differential centrifugation in concentrated human serum albumin as described previously [11, 12]. Plasma membrane ATPase activities were determined in both the mast cell and the macrophage fractions. The cells, pooled from 6 to 24 rats, were suspended in Krebs–Ringer solution (NaCl 139.8 mM, KCl 4.7 mM, $MgSo_4$ 1.2 mM, $CaCl_2$ 2.5 mM) with Tris–HCl buffer 3.1 mM and 1 mg/ml human serum albumin, final pH 7.5. $CaCl_2$ was however omitted in experiments with fluoride. The purity of the cells was determined by fixing and staining samples of mast cells and macrophages in a solution containing 10 ml formalin, 2 ml acetic acid, 10 mg toluidine blue and 88 ml 0.9% NaCl [13].

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The mast cells were differentiated by the staining of their granules from the macrophages. Five hundred cells were counted for each preparation. The purities of the mast cells and macrophages were 97.2 ± 0.23 per cent and 97.8 ± 0.19 per cent (mean ± S.E.M.) respectively.

For the determination of ATPase activities of the plasma membrane, the cells were incubated with adenosine 5′ (γ^{32}P) triphosphate and the terminal ^{32}P-orthophosphate released in the reaction was measured. Usually four samples of mast cells were used, two of them being exposed to histamine releasers or other agents and the other two serving as controls. In some experiments six samples were used to study the effect of two different agents. 0.5×10^6–1.8×10^6 mast cells were used for each sample in most of the experiments for the determination of ATPase activity, but in a few experiments with very short incubation period a larger number of mast cells up to 7×10^6 per sample was used. Samples of macrophages were incubated simultaneously to determine the ATPase activity of these cells. The number of macrophages used was about equal to that of mast cells in the earlier experiments. Because of the high enzyme activity in the macrophages their number was later reduced to 1/3 to 1/10 of that of the mast cells. The cells were prewarmed at 37° for 5–20 min in 15 ml plastic centrifuge tubes and the incubation was thereafter continued usually for 15 min with γ^{32}P-ATP and cold ATP giving a total ATP concentration of 5–18 μM. The initial specific activity of γ^{32}P-ATP, 1160–3750 μCi/μmole, was reduced by the addition of cold ATP to 67–411 μCi/μmole. The volume of the cell suspensions after the addition of ATP and other reagents was usually 1.5 ml. The reaction was terminated by adding 0.5 ml trichloroacetic acid giving a final concentration of 0.3 N. The TCA-extract was neutralized with NaOH solution and the orthophosphate was extracted after conversion to phosphomolybdic acid in a mixture of isobutyl alcohol and benzene [14–16]. The samples and all solutions were kept at 0–4° except during the incubation at 37°. An aliquot of the isobutyl alcohol–benzene layer was counted for ^{32}P in instagel in a Nuclear Chicago Mark II liquid scintillation counter and from this value the amount of inorganic phosphate (P_i) released was calculated. Control samples without cells were run through identical procedures to determine the nonenzymatic hydrolysis of ATP. This value (average 2.5 per cent) was deducted to obtain the ATPase activity on the plasma membrane of the cells. Separate control experiments were also run to check the recovery of P_i by adding ^{32}P-orthophosphate to mast cells, macrophages and to the solution free from cells. In all cases complete recovery was obtained.

In each experiment, the mast cell samples gave ATPase values for predominantly mast cells but the activity of a small number of contaminating macrophages was included in the mast cell values. As the plasma membrane ATPase activity was found to be several times higher in the macrophages (see Results) as compared to the mast cells, the enzyme activity of the mast cell samples had to be corrected for the part contributed by the few macrophages in spite of the high degree of the purity of the cells. The macrophage samples likewise gave ATPase values for mostly macrophages but with a few contaminating mast cells. Since the purities of the mast cell and macrophage samples were known in each experiment the values for 100 per cent mast cells and 100 per cent macrophages could be calculated. These calculated ATPase values for pure mast cells and pure macrophages have been presented in the tables and figures. It has been a routine procedure to determine the enzyme activities in both the types of cells, even when the macrophage values are not presented, and from these results to calculate the corrected values for 100 per cent pure mast cells and macrophages. Some workers have used reduced glutathione in the medium for determining the plasma membrane ATPase activity of intact cells [17]. In a few of our preliminary experiments, reduced glutathione 0.67 mM was added to the samples for ATPase determination. Its presence however made no difference in the enzyme activity and it was therefore omitted.

When the effect of histamine liberators on ATPase activity was studied, histamine release was determined in mast cell samples of the same suspension used for the enzyme assay. The concentration of the releasers, the density of the cell population and the experimental conditions were the same as for the enzyme assay. The incubation procedure and the general methodology for determining histamine release from mast cells have been described earlier [18]. Histamine was determined fluorometrically [19]. The spontaneous histamine release, 3.3 ± 0.29 per cent (mean ± S.E.M.), has been deducted from the values presented. The concentration of ATP used for the determination of the enzyme activity was kept low (5–18 μM) in most of the experiments to eliminate the risk of histamine release by ATP itself [20]. With these concentrations there was no histamine release from mast cells in the usual medium containing 2.5 mM Ca^{2+} and 1.2 mM Mg^{2+}. However, in experiments in which we wished to study the relation of ATPase to ATP-induced histamine release, the concentration of ATP was raised to 25–80 μM with reduction of Ca^{2+} concentration to 0.25 mM and omission of Mg^{2+}.

γ^{32}P-ATP was purchased from the Radiochemical Centre, Amersham. Cold ATP, ouabain and oligomycin were purchased from Sigma Chemical Co. Dextran (mol. wt 70,000) was obtained from Pharmacia, human serum albumin from Kabi, instagel from Packard Instrument Company Inc. and phosphatidyl-*l*-serine from Schwarz/Mann. Compound 48/80, a condensation product of *p*-methoxyphenethylmethylamine with formaldehyde was kindly supplied by AB Leo, Hälsingborg, Sweden. Ethacrynic acid was obtained through the courtesy of Sharp Dohme & Co.

RESULTS

General characterization of the enzymes. The apparent K_m of plasma membranes ATPases, using intact cells incubated with ATP for 5 min, were estimated to be 36 μM for mast cells and 30 μM for

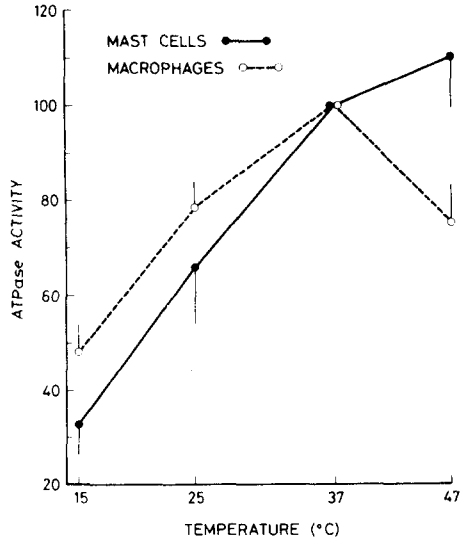

Fig. 1. Effect of temperature on plasma membrane ATPase activity of mast cells and macrophages. The results are shown as percentages of the respective values at 37°. Mean ± S.E.M. from four experiments. Concentration of ATP 8 μM. ATPase activities at 37° expressed as nmoles P_i released/10^6 cells in 15 min were 1.62 ± 0.32 for mast cells and 18.52 ± 6.46 for macrophages (mean ± S.E.M.).

macrophages. The enzyme activity both in mast cells and macrophages showed relatively large individual variations but the activity in the macrophages was always several times higher than in the mast cells. A comparison of the enzyme activity of the two types of cells was made in 18 experiments in which the number of macrophages per sample was 1/3 to 1/10 of the number of mast cells. The enzyme activity expressed as P_i released per 10^6 cells was on the average 17 times higher (range, 5 to 37 times) in the macrophages as compared to the mast cells. The temperature curves for mast cells and macrophages are shown in Fig. 1. They are similar up to 37°, the enzyme activity for both rising with increasing temperature. But there was a difference at 47°. While the enzyme activity in mast cells still continued to rise, although at a slower rate, that in the macrophages dropped below the level at 37°. The effect of pH is shown in Fig. 2. This was studied between pH 5.6 and 8.7. Imidazole–HCl buffer was used for pH 5.6–7.1 and Tris–HCl buffer for pH 7.1–8.7. The time of incubation with ATP was reduced to 5 min and the concentration of the buffers increased to 25 mM in these experiments. With this concentration of buffer there was no change in the pH of the medium after incubation of the cells for 5 min. The mast cell and macrophage enzymes showed distinct peaks at pH 6.7 and 7.1 respectively, cf. Fig. The Krebs–Ringer solution used generally for other ATPase experiments was buffered with 3.1 mM Tris–HCl buffer to give a pH 7.5. Because of the lower buffer concentration the pH of the medium changed in these experiments after incubation for 15 min to 7.1–7.2. The optimal pH for macrophages lies within this range, and that for mast cells, although close to it, is slightly lower. The pH, determinated at room temperature (22°), is given here, cf. Fig. The change with temperature was small both for imidazole and Tris buffer. The pH of the buffered solutions decreased at 37° on the average 0.12 unit for Tris buffer and 0.10 unit for imidazole buffer.

In order to determine if the ATPase on the cell surface was solubilized in the medium during the incubation, the cell suspensions were incubated as usual for 15 min at 37° but without ATP. The suspension was then cooled to 0–4° and the cells separated from the supernatant by centrifugation. ATPase in the supernatant was determined thereafter as usual by incubation with ATP and the enzyme activity expressed as percentage of the total activity in control samples, which were run through the same process except that the centrifugation was omitted. Only a small amount of enzyme was found to be solubilized into the medium. Expressed as percentage of control values 7.5 ± 1.32 per cent of the mast cell enzyme and 6.3 ± 0.94 per cent of the macrophage enzyme (mean ± S.E.M.) were found in the medium in a series of four experiments. P_i released from ATP was determined as a routine in the trichloracetic acid extract of the cell suspension, but in three control experiments the cells were centrifuged at 0–4° after incubation with ATP and the released P_i was determined thereafter in the TCA-extract of the supernatant. The amount of P_i recovered from the supernatant obtained from mast cells and macrophages accounted for 94 ± 1.2 per

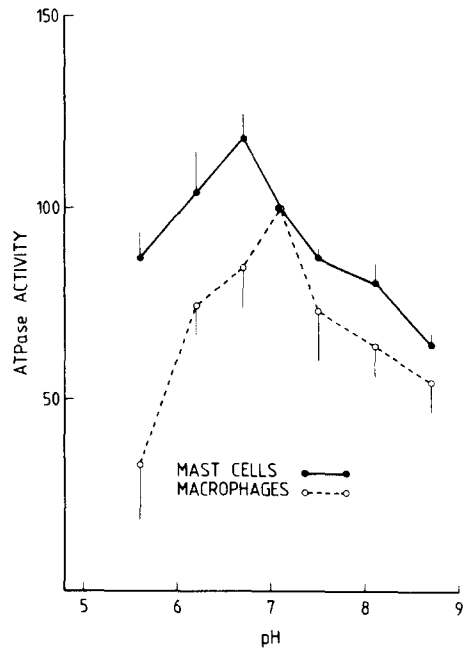

Fig. 2. Effect of pH of the medium on plasma membrane ATPase activity of mast cells and macrophages. The results are shown as percentages of the respective values at pH 7.1. Mean ± S.E.M. from three experiments for pH 5.6–7.1 with 25 mM imidazole buffer and three experiments for pH 7.1–8.7 with 25 mM Tris buffer. Conc. of ATP 17 μM. Incubation time with ATP 5 min. ATPase activities at pH 7.1 expressed as nmoles P_i released/10^6 cells in 5 min were for mast cells: 2.14 ± 0.53 with imidazole buffer, 2.86 ± 1.23 with Tris buffer, and for macrophages: 27.58 ± 5.62 with imidazole buffer, 25.58 ± 2.58 with Tris buffer (mean ± S.E.M.).

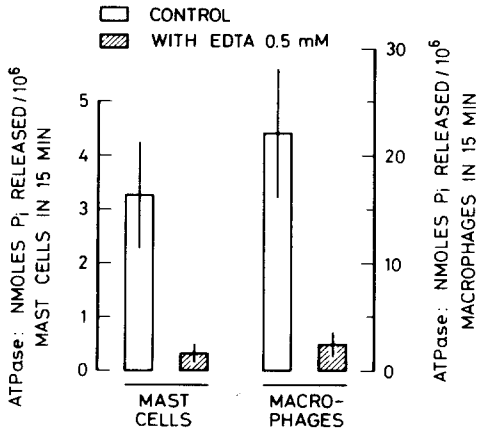

Fig. 3. Effect of EDTA on plasma membrane ATPase activity of mast cells and macrophages. The cells were incubated in Ca^{2+}, Mg^{2+}-free solution both for controls and samples with EDTA. Concentration of ATP 14 μM. Mean ± S.E.M. from three experiments.

cent and 97 ± 1.5 per cent (mean ± S.E.M.) of the enzyme activities of the respective control samples, indicating the location of the ATPase on the outer surface of the cell membrane.

Ion requirement. The usual incubation medium contained Na^+, K^+, Ca^{2+} and Mg^{2+}. When samples of the same cell suspension were incubated in the presence and absence of Ca^{2+} and Mg^{2+} the enzyme activities were reduced in a Ca^{2+}, Mg^{2+}-free solution both in mast cells and macrophages and could be restored by supplying Ca^{2+}, Mg^{2+} or both. The residual activity in a Ca^{2+}, Mg^{2+}-free solution appears to be supported by Ca^{2+} and/or Mg^{2+} from the plasma membrane, as this is further reduced by ethylenediaminetetraacetic acid (EDTA). In the experiments shown in Fig. 3 the incubation medium was free from Ca^{2+} and Mg^{2+}. Addition of EDTA, 0.5 mM reduced the average enzyme activities to 10 and 11 per cent of the control values for the mast cells and the macrophages respectively. Although in presence of EDTA the enzyme activity in both the types of cells was greatly reduced it was not completely abolished (cf. Tables 1–3). A small residual activity remained even when, in some of the experiments, the cells were continuously exposed to EDTA from the time of isolation of the cells for a total period of 2–3 hr prior to incubation with ATP for the determination of ATPase activity.

It is apparent from Tables 1 and 2 that Ca^{2+} or Mg^{2+} caused pronounced stimulation of the enzyme activity in mast cells and macrophages. Compared to the values in presence of EDTA the activity in the mast cells was increased on the average six and ten times in presence of Ca^{2+} and Mg^{2+} respectively. The stimulation of ATPase in the macrophages by Ca^{2+} or Mg^{2+} was even more pronounced, the average increase being 23 times with Ca^{2+} and 33 times with Mg^{2+}. The enhancement of the enzyme activity when both Ca^{2+} and Mg^{2+} were added together was of the same order of magnitude (Table 3). When the effects of Ca^{2+} and Mg^{2+} were compared in samples of the same cell suspension (Table 4), Mg^{2+} was

Table 1. Effect of Ca^{2+} on the plasma membrane ATPase activity of mast cells and macrophages

	ATPase activity (nmoles P_i released per 10^6 cells in 15 min)			
	Mast cells		Macrophages	
Expt.	Ca^{2+}, Mg^{2+}-free sol.	With 2.5 mM Ca^{2+}	Ca^{2+}, Mg^{2+}-free sol.	With 2.5 mM Ca^{2+}
1	0.25	1.52	0.14	5.67
2	0.23	1.15	0.28	16.40
3	0.04	0.63	0.47	11.04
4	0.18	0.95	0.95	9.92
	$P < 0.01$		$P < 0.025$	
Mean	0.18	1.06	0.46	10.76

P for paired data. Concentration of ATP 8 μM. EDTA 0.5 mM added to all samples.

Table 2. Effect of Mg^{2+} on the plasma membrane ATPase activity of mast cells and macrophages

	ATPase activity (nmoles P_i released per 10^6 cells in 15 min)			
	Mast cells		Macrophages	
Expt.	Ca^{2+}, Mg^{2+}-free sol.	With 2.5 mM Mg^{2+}	Ca^{2+}, Mg^{2+}-free sol.	With 2.6 mM Mg^{2+}
1	0.16	1.61	0.30	8.40
2	0.12	3.64	0.33	7.07
3	0.25	2.31	0.08	8.62
4	0.39	1.47	0.30	8.80
	$P < 0.05$		$P < 0.001$	
Mean	0.23	2.26	0.25	8.22

P for paired data. Concentration of ATP 6 μM. EDTA 0.5 mM added to all samples.

Table 3. Effect of Ca^{2+} + Mg^{2+} on the plasma membrane ATPase activity of mast cells and macrophages

	ATPase activity (nmoles P_i released per 10^6 cells in 15 min)			
	Mast cells		Macrophages	
Expt.	Ca^{2+}, Mg^{2+}-free sol.	With 2.5 mM Ca^{2+} + 2.5 mM Mg^{2+}	Ca^{2+}, Mg^{2+}-free sol.	With 2.5 mM Ca^{2+} + 2.5 mM Mg^{2+}
1	0.14	1.25	0.38	10.83
2	0.13	0.96	0.19	10.60
3	0.08	1.21	0.11	3.42
4	0.09	0.58	1.72	19.54
	$P < 0.01$		$P < 0.05$	
Mean	0.11	1.00	0.60	11.09

P for paired data. Concentration of ATP 6 μM. EDTA 0.5 mM added to all samples.

found to be on the average 31 and 37 per cent more effective in stimulating the membrane ATPase activities of the mast cells and the macrophages respectively.

The effect of Na^+ and K^+ on the plasma membrane ATPases of mast cells and macrophages was studied by replacing NaCl and KCl in the usual medium by lithium chloride. An attempt to exclude monovalent cations by using sucrose had to be given up because of large spontaneous histamine release in a sucrose medium, as also observed by other workers [21, 22]. The enzyme activity in the Na^+, K^+-free solution containing Li^+ was compared with the activities in solutions in which equimolar parts of LiCl were replaced by 90 mM NaCl or 10 mM KCl. The enzyme activities expressed as nmoles P_i released per 10^6 cells in 15 min in (a) Na^+, K^+-free solution, (b) with 90 mM Na^+ and (c) with 10 mM K^+ were as follows: for mast cells (a) 3.96 ± 0.53, (b) 4.03 ± 0.37, (c) 3.49 ± 0.32 and for macrophages (a) 19.45 ± 0.93, (b) 20.05 ± 0.75, (c) 19.40 ± 1.15 (mean $\pm$ S.E.M. from three experiments). There was thus no appreciable influence of Na^+ and K^+ ions on the mast cell and macrophage membrane ATPases.

Effect of phosphatidyl serine. Phosphatidyl serine has been shown to enhance histamine release from rat mast cells, and it has been speculated that an ATPase may be involved in the enhancing process [23, 24]. The effect of phosphatidyl serine on the plasma membrane ATPases of mast cells and macrophages was therefore studied. A stimulation of the mast cell enzyme by phosphatidyl serine (50 μg/ml) was seen in the absence of Ca^{2+} and Mg^{2+} but the results varied widely. Expressing the ATPase activity with phosphatidyl serine as a percentage of the control in each experiment, the values with Ca^{2+} and Mg^{2+} in the medium were 112 ± 7.7 for mast cells and 101 ± 2.9 for macrophages (mean $\pm$ S.E.M., nine experiments). In the absence of Ca^{2+} and Mg^{2+} the values were 141 ± 11.7 for mast cells and 102 ± 6.3 for macrophages (mean $\pm$ S.E.M., nine experiments). Lack of Ca^{2+} has been shown to lead to loss of phospholipids in the Ca^{2+} transporting ATPase of sarcoplasmic reticulum [25]. It was therefore thought that the effect of phosphatidyl serine on mast cell ATPase might be shown better in the absence of calcium. There was indeed on the average 41 per cent stimulation of the mast cell enzyme in the absence of Ca^{2+} and Mg^{2+} (P for paired data < 0.025) but no significant effect was seen in the presence of these ions. The stimulation is mild and it is difficult to evaluate if it may be an indirect effect of phosphatidyl serine or a direct stimulant effect on the enzyme.

Effect of inhibitors. The inhibitory effect of EDTA (0.5 mM) on plasma membrane ATPases of mast cells and macrophages has been shown above to be due to chelation of Ca^{2+} and Mg^{2+}. The effect of four other inhibitors of different types of ATPases has

Table 4. Comparison between the effects of Ca^{2+} and Mg^{2+} on the plasma membrane ATPase activity of mast cells and macrophages

	ATPase activity (nmoles P_i released per 10^6 cells in 15 min)			
	Mast cells		Macrophages	
Expt.	With 2.5 mM Ca^{2+}	With 2.5 mM Mg^{2+}	With 2.5 mM Ca^{2+}	With 2.5 mM Mg^{2+}
1	5.19	6.64	38.86	60.70
2	4.01	4.88	57.99	69.32
3	2.66	4.25	60.93	88.29
4	3.01	3.79	45.61	61.27
	$P < 0.025$		$P < 0.025$	
Mean	3.72	4.89	50.85	69.90

P for paired data. Concentration of ATP 17 μM. EDTA 0.5 mM added to all samples.

Table 5. Effect of inhibitors on the plasma membrane ATPase activity of mast cells and macrophages

Expt.	Inhibitor	ATPase activity (nmoles P_i released per 10^6 cells in 15 min)			
		Mast cells		Macrophages	
		Control	With inhibitor	Control	With inhibitor
1	Ethacrynic acid, 1 mM	1.12	0.63	11.92	10.52
2	,,	1.26	0.66	19.12	15.48
3	,,	1.46	0.61	11.05	9.72
			$P < 0.05$		$P > 0.1$
Mean (expts 1–3)		1.28	0.63	14.03	11.98
4	Ethacrynic acid, 5 mM	1.12	0	11.92	3.55
5	,,	1.26	0.13	19.12	5.68
6	,,	1.46	0	11.05	3.59
			$P < 0.01$		$P < 0.05$
Mean (expts 4–6)		1.28	0.04	14.03	4.27
7	Sodium fluoride, 10 mM	7.07	4.97	17.17	16.76
8	,,	7.98	4.79	23.75	22.35
9	,,	7.36	4.98	23.39	22.09
10	,,	5.19	3.71	25.76	23.11
			$P < 0.01$		$P > 0.050$
Mean (expts 7–10)		6.90	4.61	22.52	21.08

P for paired data. Concentration of ATP 8 μM for expts 1–6 and 17 μM for expts 7–10.

been studied. Ouabain, a well-known inhibitor of N^+, K^+-ATPase and oligomycin, an inhibitor of mitochondrial ATPase had no effect on the plasma membrane ATPases of mast cells and macrophages even in high concentrations (1–9 mM for ouabain and 1–10 μg/ml for oligomycin). The effect of two other inhibitors of ATPase is shown in Table 5. Ethacrynic acid caused 50 per cent inhibition of mast cell membrane ATPase in 1 mM concentration and practically complete inhibition in 5 mM concentration. Ethacrynic acid did not produce any significant inhibition of the macrophage enzyme at 1 mM concentration but 5 mM ethacrynic acid caused 70 per cent inhibition. Sodium fluoride (10 mM) caused 33 per cent inhibition of the mast cell enzyme. It had no appreciable effect on the macrophage enzyme.

Mast cell membrane ATPase activity in relation to histamine release. An attempt was made to determine if any change in the mast cell membrane ATPase activity occurs in relation to histamine release. The cells were incubated at 37°. At this temperature histamine release induced by dextran occurs in 40 sec [26] and that by compound 48/80 in 10 sec [27]. For compound 48/80 the period of observation to determine the immediate effect on ATPase was increased to 20 sec in order to obtain more reliable results, while for dextran the observation period was 40 sec, i.e. the same as the time required for histamine release. It may be seen from Fig. 4 that there was no change in mast cell membrane ATPase activity either during 20–40 sec, i.e. in relation to histamine release or for 15 min after the exposure of the cells to the releasers.

Any change in mast cell membrane ATPase in relation to histamine release induced by ATP was also studied. The enzyme activity was determined for 15 min after exposure of the cells to ATP. ATP-induced histamine release is a slower process taking 5–15 min at 37° [28], at different ATP and Ca^{2+} concentrations. The time of observation of ATPase activity (15 min) thus covers the period of histamine release quite well. As Ca^{2+} in higher concentrations and Mg^{2+} ions have been shown to inhibit ATP-induced histamine release [28], Mg^{2+} was omitted from the medium and two concentrations of Ca^{2+} were used, viz. 0.25 mM for histamine release and 2.5 mM for the inhibition of the release. As shown in Fig. 5, when ATP was used in a concentration of 25–35 μM it caused 11–19 per cent histamine release in presence of 0.25 mM Ca^{2+} but the release was negligible when Ca^{2+} concentration was increased to

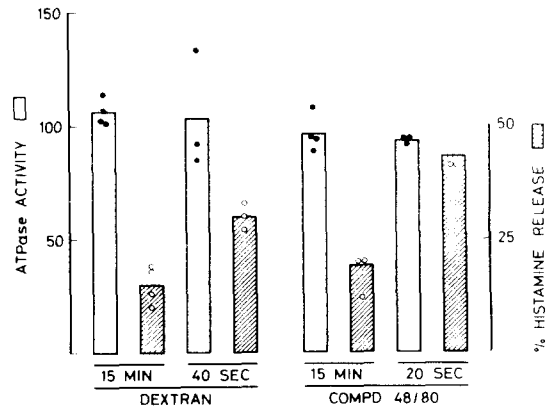

Fig. 4. Plasma membrane ATPase activity of mast cells in relation to histamine release induced by dextran (10 mg/ml) and compound 48/80 (2–4 μg/ml) at 37°. The ATPase values for mast cells exposed to the releasers are shown as percentages of the respective control values. The control values for ATPase in the absence of the releasers were 1.75 ± 0.22, 0.14 ± 0.03 and 0.20 ± 0.06 (mean ± S.E.M.) nmoles P_i released/10^6 cells in 15 min, 40 and 20 sec respectively. Concentration of ATP 8 μM for 15 min and 40 sec and 17 μM for 20 sec. Each point represents one experiment.

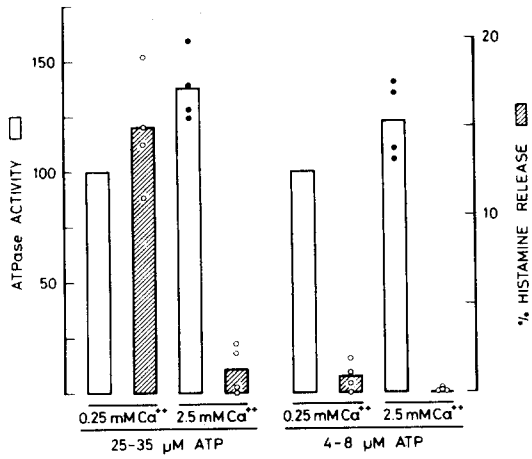

Fig. 5. Plasma membrane ATPase activity of mast cells in relation to histamine release induced by 25–35 μM ATP and corresponding control experiments with 4–8 μM ATP. The ATPase values for mast cells exposed to ATP with 2.5 mM Ca^{2+} are shown as percentages of the corresponding ATPase values with 0.25 mM Ca^{2+}. Mg^{2+} was omitted from the medium. Expressed as nmoles P_i released/10^6 cells in 15 min, the ATPase activities in presence of 0.25 mM Ca^{2+} with 25–35 μM and 4–8 μM ATP were 4.79 ± 0.54 and 0.91 ± 0.32 (mean ± S.E.M.) respectively. Each point represents one experiment.

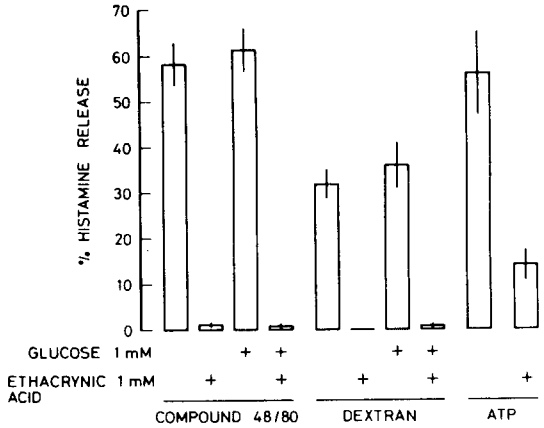

Fig. 6. Inhibition of histamine release from mast cells induced by compound 48/80, dextran and ATP with ethacrynic acid. Concentrations of the releasers: 1 μg/ml compound 48/80, 10 mg/ml dextran and 80 μM ATP. Mean ± S.E.M. from three experiments for each releaser.

2.5 mM. The histamine release with ATP in presence of the lower calcium concentration was not associated with any stimulation of ATPase activity. When the calcium concentration was increased to 2.5 mM the ATPase activity was on the average 38 per cent higher probably due to the higher concentration of calcium. When the concentration of ATP was reduced to 4–8 μM, histamine release was negligible both at 0.25 mM and 2.5 mM Ca^{2+} concentrations. The change in the ATPase activity with the higher Ca^{2+} concentration was however in the same direction as with 25–35 μM ATP. The likely conclusion would be that histamine release induced by ATP is not associated with an appreciable change in the mast cell membrane ATPase activity. This conclusion appears also to be borne out by experiments with oligomycin. We have reported above that oligomycin in concentrations 1–10 μg/ml had no effect on mast cell membrane ATPase. In three other experiments oligomycin (2 μg/ml) caused 60 per cent inhibition of histamine release induced by ATP (50–60 μM) but the ATPase activity of the mast cells was unchanged in the absence and presence of oligomycin: 1.91 ± 0.34 and 1.94 ± 0.26 (mean ± S.E.M.) nmoles P_i released/10^6 cells in 15 min respectively.

Since ethacrynic acid and fluoride inhibited the plasma membrane ATPase of mast cells it was of interest to explore their effect on histamine release. Using mixed rat peritoneal cells we have previously shown that ethacrynic acid inhibits dextran-induced histamine release and its potentiation by phosphatidyl serine [24]. More recently, ethacrynic acid has been shown to inhibit histamine release from human basophils induced by antigen challenge or ionophore A23187 [29, 30]. The experiments illustrated in Fig. 6 with pure mast cells show a pronounced inhibitory effect of ethacrynic acid on histamine release induced by dextran, compound 48/80 and ATP. Using 1 mM ethacrynic acid histamine release induced by compound 48/80 and dextran was found to be practically completely blocked. The same concentration of ethacrynic acid caused 75 per cent inhibition of ATP-induced histamine release ($P < 0.025$). It has been shown that the inhibition of compound 48/80-induced histamine release by blocking the oxidative pathway is largely ineffective in presence of glucose [31, 32], but the same type of inhibition of histamine release induced by ATP is unaffected by glucose [33]. It may be seen in Fig. 6 that the presence of glucose did not make any significant difference in the inhibition of histamine release caused by ethacrynic acid. Histamine release was, on the other hand, largely restored by glucose in control samples of the same cell suspensions, used for the experiments with compound

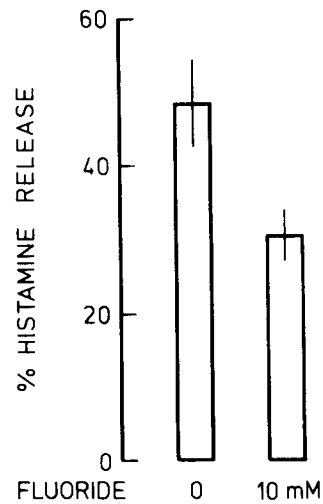

Fig. 7. Inhibition of histamine release from mast cells induced by compound 48/80 (3 μg/ml) with sodium fluoride (10 mM). Mean ± S.E.M. from four experiments. $P < 0.05$.

48/80 and dextran, when the release was inhibited by blocking the respiratory chain with antimycin A. Ethacrynic acid thus does not appear to have any additional inhibitory effect on the energy metabolism to account for the inhibition of histamine release. Fluoride (10 mM) caused 37 per cent inhibition of histamine release induced by compound 48/80, as shown in Fig. 7. In two separate experiments the inhibition was stronger (58–59 per cent), and unaffected by the presence of glucose. Other inhibitors of glycolysis have been previously shown to have no effect on compound 48/80-induced histamine release in presence of oxygen [18]. It is therefore unlikely that the fluoride effect on histamine release is due to inhibition of glycolysis.

DISCUSSION

The Ca^{2+}–Mg^{2+} activated ATPases demonstrated in intact rat peritoneal mast cells and macrophages may be considered as ecto-ATPases, located on the outer surface of the plasma membrane. Sugiyama [34] studied ATPase in intact mast cells and found that the enzyme was stimulated by Ca^{2+} and Mg^{2+}. As pointed out by the author, his results with mast cells cannot be considered specific for this type of cells due to the relatively heavy contamination with macrophages, which had several times higher activity. The discrepancy between his and our results with mast cells seems to stem from contamination of Sugiyama's mast cell preparations with macrophages. His assertion that the contamination with macrophages was largely responsible for the ATP-splitting activity of the mast cells does not apply to our preparations in which the purity has been high and the enzyme activity contributed by the few contaminating macrophages has been taken into account in reporting the results for pure (100 per cent) mast cells.

When the present article was in preparation we came across the work of Cooper and Stanworth [35] on the plasma membrane ATPase of rat mast cells. Most of their work is with homogenates of mast cells. The observations on the effects of Ca^{2+} and Mg^{2+} agree with ours. So does their characterization of the enzyme as an ecto-ATPase. However, the pH optimal for mast cell ATPase is lower in our experiments with intact cells (6.7) than in theirs with cell homogenate (7.8–8.6). Chelation of metal ions by EDTA in the homogenate caused complete inhibition of mast cell ATPase [35]. In our preparation of intact cells EDTA usually caused 90 per cent or more inhibition of the mast cell ATPase, but complete inhibition of the enzyme was not observed even after prolonged exposure to EDTA. Probably some Ca^{2+} and/or Mg^{2+} ions, which remain available to the enzyme in the intact cell even after incubation with EDTA, would account for this difference. Cooper and Stanworth have used mast cells which were "at least 95 per cent pure". Considering the remarkably higher activity of ATPase in the macrophages, a substantial part of the reported enzyme activity would be contributed by the macrophages even at this level of purity. However, many of the observations agree with ours because of the general similarity between the plasma membrane ATPases of mast cells and macrophages. But in addition to the high activity of the macrophage membrane ATPase, we have pointed out some of its other differences from the mast cell enzyme, viz. temperature and pH dependence (Figs 1 and 2) and sensitivity to some inhibitors (Table 5).

We could not demonstrate any change in the mast cell membrane ATPase during or after histamine release induced by dextran, compound 48/80 or adenosine triphosphate. However, this does not rule out very transient stimulation of the enzyme during or immediately before exocytosis of the mast cell granules. We have therefore sought evidence for the involvement of the plasma membrane ATPase in histamine release by using inhibitors of the enzyme. Ethacrynic acid (1 mM), which inhibited the enzyme, also inhibited histamine release induced by dextran, compound 48/80 and ATP in the same concentration. Histamine release may be inhibited by two types of inhibitors: (a) those blocking the generation of ATP and (b) those acting on the triggering mechanism in the mast cell membrane. The effect of inhibitors of oxidative energy metabolism is counteracted by glucose through glycolytic ATP production. Since the inhibitory effect of ethacrynic acid could not be counteracted by glucose it may be regarded as an inhibitor of type (b) acting on the cell membrane. Conceivably, the two effects of ethacrynic acid, viz. the inhibition of histamine release and the inhibition of plasma membrane ATPase are related to each other, the enzyme inhibition being the cause of the inhibition of histamine release. Since Ca^{2+} is required for histamine release [36] and the chelation of Ca^{2+} by EDTA causes pronounced inactivation of the enzyme—the Ca^{2+} effect also points to the involvement of the enzyme in histamine release. Furthermore, sodium fluoride has been shown to inhibit the plasma membrane ATPase in mast cell homogenate [35] and intact mast cells (Table 5). This inhibition appears to correlate well with the fluoride inhibition of compound 48/80-induced histamine release (Fig. 7). Recently a correlation has been shown between the inhibitory effects of flavones on histamine release from rat mast cells and on Ca^{2+}-ATPase of sarcoplasmic reticulum [37]. More work is however required to explore the relation of plasma membrane ATPase in mast cells to histamine release. ATP-induced histamine release seems to be a special case. Mg^{2+}, which activates the enzyme, has an inhibitory effect on histamine release induced by ATP [28]. This may be either due to another site of action of Mg^{2+} at a later stage in ATP-induced histamine release or because of a fundamental difference between the release induced by ATP and other selective releasers like antigen, dextran and compound 48/80. ATP-induced histamine release differs from histamine release induced by other selective releasers in having a slower time course [28] taking minutes instead of seconds and in being associated with pronounced changes in the plasma membrane permeability [38, 39].

Na^{+}–K^{+} independent, divalent cation dependent ecto-ATPases have been demonstrated in several other types of cells, like Ehrlich ascites carcinoma cells, HeLa cells, and leukocytes [40–43]. DePierre

and Karnovsky, who have studied the ecto-ATPase in polymorphonuclear leukocytes, could not assign any functional role to the enzyme [43]. Woodin and Wienecke [6] demonstrated a Ca^{2+}–Mg^{2+} dependent ATPase in the granules of polymorphonuclear leukocytes and proposed that the granule ATPase by hydrolyzing ATP in the plasma membrane would promote the fusion of granule membrane with the cell membrane. A similar model for membrane fusion reaction involving Ca^{2+}, ATP and ATPase has also been advanced by Poste and Allison [44]. Ågren *et al.* [7] have recently studied Mg^{2+}–Ca^{2+} ATPases on the outer surface of human peripheral lymphocytes and hematopoietic cell lines and found a correlation between high cell surface ATPase activity and cytokinesis probably involving a mechano-chemical coupling process. It is possible that a similar mechanism is involved in the secretory reaction from mast cells. Actin filaments attached to the plasma membrane have lately been demonstrated in mast cells [45]. The possible sequence of events in secretion from the mast cells may conceivably be initiated through a coupling between the Ca^{2+}–Mg^{2+} ATPase and the contractile protein in the plasma membrane. This could bring about the initial reaction of exocytosis by promoting membrane movement and invagination causing the approximation of the plasma membrane to the perigranular membrane.

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REFERENCES

1. P. Banks, *Biochem. J.* **95,** 490 (1965).
2. A. M. Poisner and J. M. Trifaró, *Molec. Pharmac.* **3,** 561 (1967).
3. S. P. Wilson and N. Kirshner, *J. Neurochem.* **27,** 1289 (1976).
4. G. L. Harris, D. H. Cove and N. Crawford, *Biochem. Med.* **11,** 10 (1974).
5. H. Holmsen, in *Biochemistry and Pharmacology of Platelets, Ciba Foundation Symposium 35* (new series), p. 175. Elsevier, Amsterdam (1975).
6. A. M. Woodin and A. A. Wieneke, *Biochem. J.* **90,** 498 (1964).
7. G. Agren, K. Nilsson and G. Ronquist, *Acta physiol. scand.* **98,** 67 (1976).
8. R. J. North, *Ultrastruct. Res.* **16,** 83 (1966).
9. F. Medzihradsky, H. Lin and M. J. Marks, *Life Sci.* **16,** 1417 (1975).
10. F. Medzihradsky, G. G. Bole, L. M. Nork and H. Lin, *Res. Commun. Chem. Path. Pharmac.* **14,** 277 (1976).
11. N. Chakravarty and E. Zeuthen, *J. Cell Biol.* **25,** 113 (1965).
12. N. Chakravarty, *J. Cell Biol.* **25,** 123 (1965).
13. D. Lagunoff and E. P. Benditt, *Am. J. Physiol.* **196,** 993 (1959).
14. I. Berenblum and E. Chain, *Biochem. J.* **32,** 295 (1938).
15. J. B. Martin and D. M. Doty, *Analyt Chem.* **21,** 965 (1949).
16. O. Lindberg and L. Ernster, in *Methods of Biochemical Analysis* (Ed. D. Glick), Vol. 3, p. 1. Interscience Publishers, Inc. (1956).
17. G. Ågren, J. Pontén, G. Ronquist and B. Westermark, *J. Cell Physiol.* **78,** 171 (1971).
18. N. Chakravarty, *Acta physiol. scand.* **72,** 425 (1968).
19. P. A. Shore, A. Burkhalter and V. H. Cohn, *J. Pharmac. exp. Ther.* **127,** 182 (1959).
20. B. Diamant and P. G. Krüger, *Acta physiol. scand.* **71,** 291 (1967).
21. H. Yamasaki and K. Sugiyama, Japan *J. Pharmac.* **22,** 325 (1972).
22. D. E. Cochrane and W. W. Douglas, *J. Physiol., Lond.* **257,** 433 (1976).
23. A. Goth, H. Ray Adams and M. Knoohuizen, *Science N.Y.* **173,** 1034 (1971).
24. N. Chakravarty, A. Goth and P. Sen, *Acta physiol. scand.* **88,** 469 (1973).
25. N. M. Green, in *Calcium Transport in Contraction and Secretion, Proc. Int. Symp.* (Eds. E. Carafoli *et al.*), p. 339. Holland Publishing Co., Bressanone, Italy (1975).
26. F. Svendstrup and N. Chakravarty, *Expl Cell Res.* **106,** 223 (1977).
27. G. D. Bloom and N. Chakravarty, *Acta physiol. scand.* **78,** 410 (1970).
28. R. Dahlquist and B. Diamant, *Acta Pharmac. Tox.* **34,** 368 (1974).
29. A. M. Magro and E. J. Cragoe, *Fedn Proc.* **36,** 1216 (1977).
30. A. M. Magro, Personal Communication. *Clin. Exp. Immun.* (in press) (1978).
31. B. Diamant and B. Uvnäs, *Acta physiol. scand.* **53,** 315 (1961).
32. A. M. Rothschild, I. Vugman and M. Rocha e Silva, *Biochem. Pharmac.* **7,** 248 (1961).
33. C. Peterson, *Acta physiol. scand. (suppl.)* **413,** 5 (1974).
34. K. Sygiyama, Japan *J. Pharmac.* **21,** 531 (1971).
35. P. H. Cooper and D. R. Stanworth, *Biochem. J.* **156,** 691 (1976).
36. J. L. Mongar and H. O. Schild, *Physiol. Rev.* **42,** 226 (1962).
37. C. M. S. Fewtrell and B. D. Gomperts, *Nature, Lond.* **265,** 635 (1977).
38. K. Sugiyama, Japan *J. Pharmac.* **21,** 209 (1971).
39. P. G. Krüger, B. Diamant and R. Dahlquist, *Int. Arch. Allergy, Appl. Immun.* **46,** 676 (1974).
40. D. F. H. Wallach and D. Ullery, *Cancer Res.* **22,** 228 (1962).
41. G. Ronquist and G. Ågren, *Cancer Res.* **35,** 1402 (1975).
42. H. B. Bosmann, A. Hagopian and E. H. Eylar, *Archs Biochem. Biophys.* **128,** 51 (1968).
43. J. W. DePierre and M. L. Karnovsky, *J. biol. Chem.* **249,** 7121 (1974).
44. G. Poste and A. C. Allison, *J. Theor. Biol.* **32,** 165 (1971).
45. P. Röhlich, *Expl Cell Res.* **93,** 293 (1975).