Presence of a high-affinity Ca^{2+} - and Mg^{2+} -dependent ATPase in rat peritoneal mast-cell membranes

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Purified perigranular and plasma membranes isolated from rat peritoneal mast cells were examined for Ca^{2+} - and Mg^{2+} -dependent ATPase activity. Isolated perigranular membranes contained only a low-affinity Ca^{2+} - or Mg^{2+} -dependent ATPase ($K_m > 0.5 \text{ mM}$). The plasma membranes contained both a low-affinity Ca^{2+} - or Mg^{2+} -dependent ATPase ($K_m = 0.4 \text{ mM}$, $V_{\max} = 20 \text{ nmol of } P_i/\text{min per mg}$), as well as a high-affinity Ca^{2+} - and Mg^{2+} -dependent ATPase ($K_m = 0.2 \mu\text{M}$, $V_{\max} = 6 \text{ nmol of } P_i/\text{min per mg}$).

Although the release of histamine from mast cells may be elicited by a variety of secretagogues, an absolute requirement for Ca^{2+} exists. A rapid increase in intracellular Ca^{2+} concentration is required to trigger exocytosis of the histamine-containing granules from mast cells. Although a plasma-membrane Ca^{2+} , Mg^{2+} -ATPase (or Ca^{2+} pump) has been demonstrated in a variety of cell types (reviewed by Penniston, 1983), a role for such a Ca^{2+} pump in the histamine release mechanism has only been postulated (Fewtrell & Gomperts, 1977a,b; Magro *et al.*, 1983). Pharmacological agents shown to inhibit the Ca^{2+} , Mg^{2+} -ATPase found in muscle sarcoplasmic reticulum also inhibit mast-cell degranulation (Fewtrell & Gomperts, 1977a,b). Unlike the Na^+ + K^+ -dependent ATPase, which is specifically inhibited by ouabain, no inhibitor specific for the high-affinity Ca^{2+} , Mg^{2+} -ATPase has been identified. Therefore studies measuring the direct effect of potential inhibitors on the mast-cell Ca^{2+} , Mg^{2+} -ATPase could be useful for elucidating the role of various transport ATPases in the secretory process. To initiate these studies, rat peritoneal mast-cell membranes were examined for the high-affinity Ca^{2+} , Mg^{2+} -ATPase. This paper reports evidence for such a high-affinity Ca^{2+} , Mg^{2+} -ATPase in mast-cell plasma membranes.

Abbreviations used: Ca^{2+} , Mg^{2+} -ATPase, Ca^{2+} - and Mg^{2+} -dependent ATPase; HEDTA, *N*-hydroxyethyl-ethylenediaminetriacetic acid.

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Ca^{2+} has been detected by ion-probe analysis in the membrane-bound histamine-containing granules (Chock *et al.*, 1982; Hein & Caulfield, 1982, 1983). The granules may form an intracellular Ca^{2+} -storage site, thereby serving as an integral component of the mechanism for the maintenance of low intracellular Ca^{2+} concentrations. Therefore the perigranular membrane may contain components of a Ca^{2+} -transport mechanism necessary for Ca^{2+} sequestration within the granule matrix. Accordingly, the perigranular membranes were also examined for high-affinity Ca^{2+} , Mg^{2+} -ATPase activity.

Materials and methods

Membrane isolation and purification

Male Sprague-Dawley rats (300–400 g) were killed by CO_2 inhalation and mast cells were obtained by peritoneal lavage. The mast cells were purified (>95% pure) by centrifugation through 38% (w/v) albumin (Sullivan *et al.*, 1975) and disrupted by repeating a cycle of single 0.5 s sonication pulses, followed by centrifugation to separate the released granules (Amende & Donlon, 1985). Plasma membranes were separated from intact granules by centrifuging the sonicated material through a self-generating Percoll gradient (Kruger *et al.*, 1980). The plasma membranes remained at the Percoll/buffer interface and were washed three times with 50 mM-Hepes/tetramethylammonium hydroxide, pH 7.5. Perigranular membranes were prepared by the osmotic lysis of intact granules (Percoll pellet) (Uvnas *et al.*, 1970). These membrane fractions

were examined by using membrane marker enzymes. The plasma-membrane fraction was enriched 4–6-fold in 5'-nucleotidase (a plasma-membrane marker) specific activity, and mitochondrial contamination as measured by succinate dehydrogenase total activity was 15% of the initial homogenate activity. The perigranular membranes contained 10% of the initial homogenate 5'-nucleotidase specific activity and 1% of the succinate dehydrogenase activity.

ATPase activity determination

ATPase assays were conducted as described previously (Amende *et al.*, 1983). The high-affinity Ca^{2+} , Mg^{2+} -ATPase was assayed in the presence of 0.2 mM-Tris/ATP, 80 mM-KCl, 20 mM-Hepes/tetramethylammonium hydroxide (pH 7.5), 1 mM- NaN_3 and 0.8 mM-ouabain, with 1 mM-HEDTA as the Ca^{2+} -chelating agent, with various amounts of CaCl_2 added as needed for each Ca^{2+} concentration, determined as described by Amende *et al.* (1983). The Ca^{2+} concentration was varied from 0.11 to 57 μM in the presence of 0 and 200 μM - Mg^{2+} . Phosphate was determined by an automated assay (Raess & Vincenzi, 1980). Symbols and bars in Figures represent means $\pm$ S.E.M. for triplicate assays. Values for K_m and $V_{\max}$ were calculated from Lineweaver–Burk double-reciprocal plots of the data.

The assay media for the low-affinity Ca^{2+} - and Mg^{2+} -dependent ATPases contained 3 mM-ATP, 20 mM-Hepes/tetramethylammonium hydroxide, 80 mM-KCl, 1 mM- NaN_3 , 3 mM-ouabain, and CaCl_2 (Ca^{2+} -dependent ATPase), MgCl_2 (Mg^{2+} -dependent ATPase plus high-affinity Ca^{2+} , Mg^{2+} -ATPase activated by the micromolar amounts of Ca^{2+} present in the assay system) or MgCl_2 and 1 mM-EGTA (Mg^{2+} -dependent ATPase only), as indicated in the Figure legends.

Protein content was determined by a modification of the Lowry method (Peterson, 1977), with bovine serum albumin as standard.

Materials

Hepes, HEDTA, ouabain and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results and discussion

Fig. 1(a) shows the high-affinity Ca^{2+} , Mg^{2+} -ATPase activity found in the enriched rat peritoneal mast-cell plasma-membrane fraction. In the absence of Mg^{2+} , only a Ca^{2+} -dependent ATPase is present, with a K_m for Ca^{2+} of approx. 3 μM and $V_{\max}$ of around 12.3 nmol of P_i /min per mg. When assayed in the presence of 200 μM - Mg^{2+} , both high- and low-affinity components of the ATPase are

Fig. 1. Effect of Ca^{2+} and Mg^{2+} on the ATPase activity of mast-cell (a) plasma and (b) perigranular membranes. The isolated mast-cell membranes were assayed for ATPase activity as described in the Materials and methods section. The free Ca^{2+} concentration was varied from 0 to 60 μM in the presence of (▲) 0 and (●) 200 μM - Mg^{2+} .

apparent. The high-affinity component has a K_m for Ca^{2+} of approx. 0.2 μM , with a $V_{\max}$ of 6–7 nmol of P_i /min per mg, whereas the low-affinity ATPase shows an approx. K_m of 0.9 μM and an estimated $V_{\max}$ of 11.5 nmol of P_i /min per mg. The high-affinity component is activated by Mg^{2+} . This is the first report of a high-affinity Ca^{2+} , Mg^{2+} -ATPase in mast-cell plasma membranes. The low-affinity component was previously identified (Cooper & Stanworth, 1976; Magro, 1977; Chakravarty & Echetebeu, 1978). The addition of 200 μM - Mg^{2+} to the assay medium for perigranular-membrane ATPase activity caused a small increase in activity (Fig. 1b). This low Ca^{2+} , Mg^{2+} -ATPase activity may be due to the presence of plasma membranes in the final perigranular-membrane preparation. The 5'-nucleotidase activity (a plasma-membrane enzyme marker) of the perigranular membranes was 10% of that of the whole mast-cell sonicated preparation, indicating that some residual plasma-membrane contamination of the perigranular-membrane preparation had occurred (Amende & Donlon, 1985).

Another approach to detecting the Ca^{2+} , Mg^{2+} -ATPase is shown in Fig. 2. Various low-affinity ATPases were assayed in the plasma-membrane fraction (Fig. 2a), with millimolar amounts of ATP, Mg^{2+} , Ca^{2+} and Mg^{2+} +1mM-EGTA (Amende *et al.*, 1983). The activity of the Mg^{2+} -dependent ATPase is greater than that of the Ca^{2+} -dependent ATPase. The addition of EGTA chelates the small amount of contaminating Ca^{2+} present in the assay buffer (1–2 μM) and thereby eliminates the high-affinity Ca^{2+} , Mg^{2+} -ATPase component. Thus, in the plasma membrane (Fig. 2a), EGTA addition decreases the activity of the Mg^{2+} -dependent ATPase. However, the Mg^{2+} -dependent ATPase of the perigranular membrane does not show a similar EGTA inhibition (Fig. 2b). The addition of EGTA to the perigranular Mg^{2+} -dependent ATPase has no effect. These results are consistent with those shown in Fig. 1(b) and indicate the absence of the high-affinity Ca^{2+} , Mg^{2+} -ATPase from perigranular membranes. The perigranular membranes contain only the low-affinity ATPase. These data support the histochemical observations by Chakravarty &

Nielson (1980), who showed the presence of a low-affinity ATPase in perigranular membranes as well as the plasma membranes. Our results show that the high-affinity ATPase is present in the plasma membrane, but not in the perigranular membranes. The mechanism by which Ca^{2+} accumulates in the mast-cell granule (Chock *et al.*, 1982; Hein & Caulfield, 1982, 1983) is still unknown, since no high-affinity ATPase could be demonstrated in the perigranular membranes.

This demonstration of the high-affinity Ca^{2+} , Mg^{2+} -ATPase in mast-cell plasma membranes provides a new experimental approach to the study of the mast-cell degranulation process. The high-affinity Ca^{2+} , Mg^{2+} -ATPases so far described function to maintain the low intracellular Ca^{2+} concentrations that are found in resting mast cells. It is reasonable to hypothesize that inhibition of the mast-cell Ca^{2+} , Mg^{2+} -ATPase would result in the following sequence: Ca^{2+} efflux would be blocked, thereby causing an increase in intracellular Ca^{2+} , which would lead to histamine release. Although several pharmacological inhibitors of similar ATPases have been applied to the mast cell and result in decreased histamine release (Fewtrell & Gomperts, 1977a,b; Chakravarty, 1980), none were specific inhibitors of the ATPase. Their effects on the mast-cell Ca^{2+} , Mg^{2+} -ATPase are unknown.

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Fig. 2. Variation in (a) plasma-membrane and (b) perigranular-membrane ATPase activity with concentration of bivalent cation at 3mM-Tris/ATP

Enzyme activity was determined for various concentrations of CaCl_2 (Ca^{2+} -dependent ATPase), MgCl_2 (both Mg^{2+} -dependent ATPase and high-affinity Ca^{2+} , Mg^{2+} -ATPase) or MgCl_2 with 1mM-EGTA (Mg^{2+} -dependent ATPase only).

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