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Ethacrynic acid inhibitable Ca^{2+} and Mg^{2+}-activated membrane adenosine triphosphatase in rat mast cells

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SUMMARY

A crude plasma membrane fraction from the homogenate of purified rat mast cells demonstrates a high degree of Ca^{2+}-dependent and Mg^{2+}-dependent adenosine triphosphatase (ATPase) activity. The microsomal and mitochondrial fractions show negligible amounts of the Ca^{2+} and Mg^{2+}-activated ATPases. The broad ATPase inhibitor, ethacrynic acid, effectively blocks the mast cell ATPase activity while ouabain demonstrates little inhibitory effect. Correspondingly, ethacrynic acid inhibits histamine release from antigen-challenged mast cells while ouabain does not. Both ATPase inhibition and histamine release inhibition by ethacrynic acid require the presence of the olefinic bond in the ethacrynic acid molecule.

INTRODUCTION

Studies demonstrating a drop in the intracellular levels of adenosine triphosphate (ATP) during the release of histamine from rat mast cells (Diamant *et al.*, 1974; Johansen & Chakravarty, 1975) have promoted the hypothesis that energy requirements for anaphylaxis are supplied by ATP utilization. As a substrate, ATP is acted upon by a variety of enzymes including adenosine triphosphatase (ATPase). Although the importance of ATPases in making the energy of ATP available has long been established, the ATPase system of histamine-releasing cells has only recently been extensively investigated (Cooper & Stanworth, 1976).

In addition to requiring cations as cofactors, most ATPases demonstrate cationic specificity enabling a categorization of them based on their activating cation. The presence of Ca^{2+} is a prerequisite to the secretory stage of antigen-induced histamine release (Mongar & Schild, 1957) and there is a growing interest in how Ca^{2+} links stimulus and secretion. Various roles have been postulated for Ca^{2+} (Mongar, Svec & Foreman, 1973; Orr, Hall & Allison, 1972), including suggestions that the Ca^{2+} coupling of stimulus and secretion is effected through Ca^{2+}-activated ATPases. The postulate that ATPases play a central role in Ca^{2+} coupling has been made more feasible by demonstrating a high level of Ca^{2+}-activated ATPases in histamine-releasing cells (Cooper & Stanworth, 1976).

Reports illustrating the fact that agents which are known to inhibit ATPases similarly inhibit histamine release could be considered supportive of the hypothesis that Ca^{2+} coupling is, in part, dependent upon Ca^{2+}-activated ATPases. Recently, Fewtrell & Gomperts (1977) were able to show that a flavone's ability to inhibit IgE-mediated histamine release from rat mast cells correlates with the flavone's capacity to restrict Ca^{2+}-dependent ATPase activity associated with sarcoplasmic reticulum membranes of rabbit skeletal muscle. It has also been shown that ethacrynic acid,† an established ATPase inhibitor,

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† Ethacrynic acid: [2,3 dichloro-4-(2-methylenebutyryl)-phenoxyacetic] acid.

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effectively blocks the *in vitro* release of histamine from human basophils (Magro, 1977). Whether these ATPase inhibitors can affect the ATPases of histamine-releasing cells has not been established.

The cationic requirements of mast cell ATPases parallel the cationic requirements for histamine release in that both are dependent upon the presence of Ca^{2+}–Mg^{2+} while being relatively insensitive to Na^+–K^+. As well as by cationic specificity, ATPases can be categorized according to their susceptibility to ATPase inhibitors. Ouabain-inhibitable ATPases are specifically Na^+–K^+-dependent whereas broader ATPase inhibitors, such as ethacrynic acid, are required to inhibit the Na^+–K^+-independent ATPases which are activated by divalent cations (Duggan & Noll, 1965; Proverbio, Robinson & Whittembury, 1970). In addition to determining the effect of ouabain and ethacrynic acid upon characterized and localized mast cell ATPases, an objective of this present report is to determine if these ATPase inhibitors affect the mast cell ATPases in a manner similar to their effect upon the histamine release process.

MATERIALS AND METHODS

Reagents and solutions. Solutions were prepared in soap-free vessels with twice distilled and deionized water utilizing reagent-grade chemicals. All reagents and enzymatic substrates were obtained from Sigma except ethacrynic acid which was supplied by Merck, Sharpe and Dohme.

Animals. Wistar rats of the Albany Strain, inbred at Griffin Laboratory Farms, Guilderland, New York, were used.

Preparation of ATPase. Mast cells were purified by gradient centrifugation on bovine serum albumin according to the method of Sullivan *et al.* (1975). Peritoneal mast cells from sixteen rats (200–250 g) yielded approximately 10^7 mast cells of 90–95% purity. The plasma membrane mitochondrial and microsomal fractions of the purified mast cell homogenate were obtained by the methods of Boegman, Manery & Pinteric (1970) and Robinson (1970). Essentially intact cells were homogenized manually in a hypnotic (0·15 M) sucrose solution. The supernatant of the homogenate was checked for histamine and the homogenated cells were checked microscopically under phased light to insure lysis. The homogenate was then centrifuged for 7 min at 900 *g* in a Sorvall RC-3 centrifuge. This pellet contains a crude plasma membrane fraction and it is where the 5′-nucleotidase activity, an established plasma membrane marker, was found. The supernatant from the 900 *g* spin was centrifuged at 10,000 *g* for 10 min yielding a pellet containing the mitochondrial fraction. The second supernatant was ultra-centrifuged at 100,000 *g* for 40 min (Beckman Ultra L2-652, SW40 Rotor) yielding the microsomal fraction in the pellet. The fractions were suspended in 1·5 ml of a low Na^+ ATPase-assay buffer (91 mM Tris, 58 mM Na^+, 0·1 mM EDTA, pH 7·0) and dialysed in the cold overnight.

ATPase assay. ATPase activity was assayed by a method as described by Bonting, Simon & Hawkins (1961). 0·05 ml samples of dialysed and lightly sonicated ATPase preparations were aliquoted into microtitre plates and allowed to react with the Tris–ATP substrate (20 μmol/well) with the appropriate ions and inhibitors for 3 hr at 37°C in a reaction volume of 0·2 ml. Following incubation, proteins were precipitated by adding 0·1 ml of 25% trichloroacetic acid. The plates were centrifuged at 900 *g* for 10 min, following which 0·2 ml aliquots of the samples were transferred into 0·3 ml of the molybdate complexing reagent described by Bonting *et al.* (1961). Absorbances, at 800 nm, of the phosphomolybdate complexes were determined. Estimates of inorganic phosphate concentrations were obtained by utilizing orthophosphate standards with appropriate concentrations of Ca^{2+} and Mg^{2+}. For optical densities between 0·1 and 1·0, absorbance *vs* phosphate concentration plots were essentially linear. The plasma membrane fraction from sixteen rats (10^7 mast cells) aliquoted into a thirty-sample experiment, with a full complement of ions and no inhibitors, yielded an optical density of approximately 1·0 for a 1 cm optical path. This corresponded to a reaction-well inorganic phosphate concentration of approximately 700 nmol/ml. Blanks lacking ATPase yielded 10% or less of the maximum activity.

Histamine release. For histamine-release experiments, peritoneal mast cells were harvested from rats which had been previously (25–30 days) immunized with 3000–4000 larvae (s.c.) of the nematode *Nippostrongylus brasiliensis* (Ogilvie, 1964, 1967). The cells were washed twice and suspended in a Tris-buffered (pH 7·0) solution which was 120 mM Na^+, 25 mM Tris, 5 mM K^+, 0·1 mM Mg^{2+}, 1 mM Ca^{2+} and 0·03% gelatine. Worm antigens of *N. brasiliensis* were obtained from a saline extract of the adult worms (Kojima *et al.*, 1974). The protein content of the worm extract was determined by the method of Lowry *et al.* (1951). Inhibitors and cells were added together at 37°C and allowed to pre-incubate for 3 min. The cells were then challenged with worm antigen (10 μg/ml) and allowed to incubate for 30 min in a final reaction volume of 0·6 ml. The tubes were subsequently centrifuged and the supernatants decanted for the histamine assay.

Histamine assay. The histamine was analysed by an automated (Siraganian, 1974) spectrofluorometric technique (Shore, Burkhalter & Cohn, 1959). The percentage histamine was calculated in excess of the blank. The total quantity of histamine in a sample tube was 1000–1500 ng of histamine base per ml. Blank tubes lacking antigen released less than 5% of the total histamine content.

Analysis of data. All experimental data points were performed in triplicate and from the mean value a mean deviation was calculated. In all the graphs of this report, the mean deviation is shown about the mean.

RESULTS

ATPase activity in the plasma membrane, mitochondrial and microsomal fractions

Purified mast cells were subjected to a hypotonic sucrose solution, homogenized and fractionated by differential centrifugation in order to determine the proportion of detectable ATPase activity in the microsomal, mitochondrial and plasma membrane fractions. The obtained fractions were equated by suspending them in equal volumes of buffer and then tested for ATPase activity in the presence of a full complement of ions (1 mM Mg^{2+}, 1 mM Ca^{2+}, 5 mM K^+ and 58 mM Na^+). Although ATPase activity can be demonstrated in all three of the cellular homogenate fractions, it can be seen from bar (a) of Fig. 1 that the major portion of the detectable ATPase activity (76%) is in the plasma membrane fraction.

Ion dependence of plasma membrane ATPases

The ion dependence of the ATPase activity in the plasma membrane fraction is displayed in Fig. 2. The percentage activity is calculated relative to the amount of ATPase action demonstrated in the presence of a full complement of ions (bar a). Bar (b) of Fig. 2 shows the relative ATPase activity in the absence of K^+ and illustrates that the major portion (90%) of the plasma membrane ATPases are Ca^{2+}–Mg^{2+}-activated. Bar (d) of Fig. 2 presents the relative ATPase activity which is Mg^{2+}-activated (74%) and bar (f) exhibits the level of Ca^{2+}-dependent ATPases (62%). Since the degree of Ca^{2+}–Mg^{2+}-activated ATPases (bar c) is less than the sum of the Mg^{2+}- activated and Ca^{2+}-activated (bars d and f), there is an inference that some ATPases in the plasma membrane fraction can be activated by either Ca^{2+} or Mg^{2+}. A comparison of bar (c) to bar (d) or bar (e) to bar (f) indicates that at least 10% of the detectable ATPase activity is K^+-dependent. However, bar (g) and bar (h) of Fig. 2 indicate that K^+ does not have the capacity to activate or enhance the plasma membrane ATPases without the presence of Ca^{2+} or Mg^{2+}.

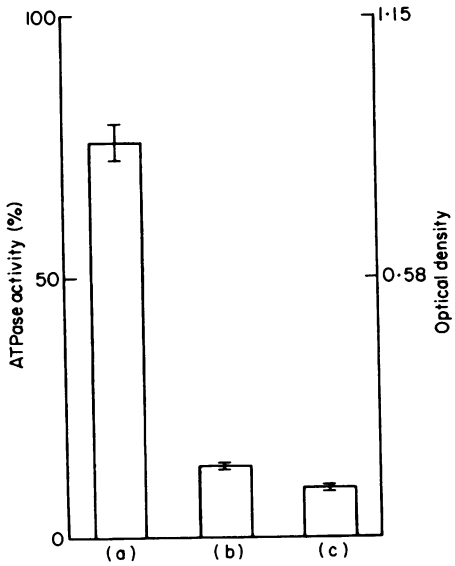

FIG. 1.

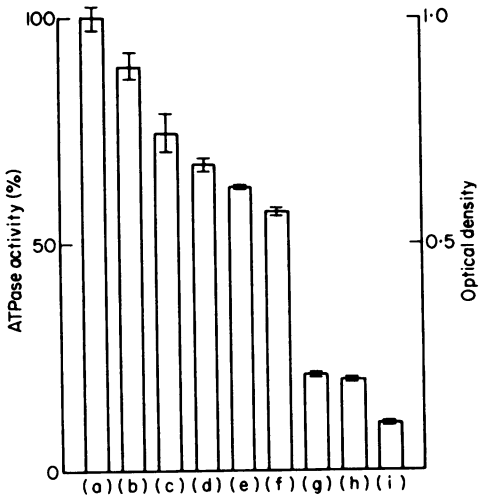

FIG. 2.

FIG. 1. Distribution of detectable ATPase activity from rat mast cell homogenate. Fractions obtained by differential centrifugation were tested for ATPase activity with a full complement of ions (1 mM Mg^{2+}, 1 mM Ca^{2+}, 58 mM Na^+ and 5 mM K^+). Bar (a), plasma membrane fraction; bar (b), mitochondrial fraction; bar (c), microsomal fraction.

FIG. 2. Ion dependence of ATPase activity in plasma membrane fraction of rat mast cell homogenate. ATPase activity in the plasma membrane fraction in the presence or absence of 1 mM Mg^{2+}, 1 mM Ca^{2+}, 5 mM K^+. All reactions were carried out in 58 mM Na^+. Bar (a), Ca^{2+}, Mg^{2+}, K^+; bar (b), Ca^{2+}, Mg^{2+}; bar (c), Mg^{2+}, K^+; bar (d), Mg^{2+}; Bar (e), Ca^{2+}, K^+; bar (f), Ca^{2+}; bar (g), no ions; bar (h), K^+; bar (i) Ca^{2+}, Mg^{2+}, K^+, without ATPase.

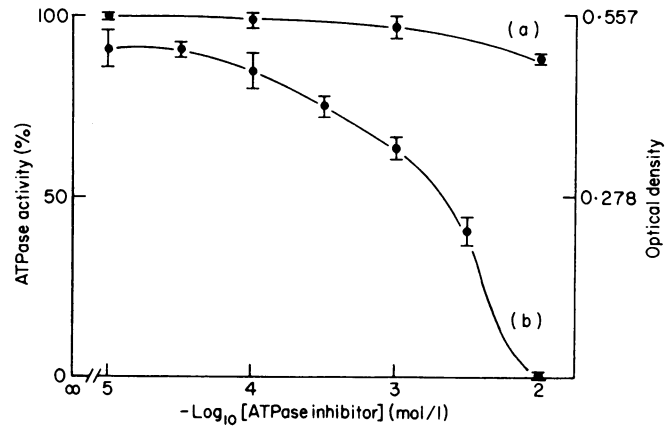

FIG. 3. Effect of ouabain and ethacrynic acid upon ATPase activity in the plasma membrane fraction. The effect of increasing concentrations (abscissa) of ouabain (curve a) and ethacrynic acid (curve b) upon ion-activated (1 mM Ca^{2+}, 1 mM Mg^{2+} and 5 mM K^{+}) ATPase activity of the plasma membrane fraction.

Effect of ATPase inhibitors upon plasma membrane ATPases

Fig. 3 demonstrates the ability of the two ATPase inhibitors ouabain (curve a) and ethacrynic acid (curve b) to inhibit the plasma membrane ATPase activity in the presence of a full complement of ions. It can be seen from curve (a) of Fig. 3 that ouabain is relatively ineffectual in its capacity to inhibit the ATPases. The data of curve (a) are not surprising when considered with the circumstances that ouabain is an inhibitor specific for Na^{+}–K^{+}-activated ATPases and that the majority of the demonstrable ATPases in the plasma membrane fraction were shown (Fig. 2) to be K^{+}-independent. Conversely, the data of curve (b) of Fig. 3 show that increasing concentrations of ethacrynic acid do totally inhibit the plasma membrane ATPases. Duggan & Noll (1965) have reported that ethacrynic acid inhibition of renal cortex membrane ATPases requires the presence of the olefinic bond in the ethacrynic acid molecule. The reaction of ethacrynic acid with cysteine forms an ethacrynic acid–cysteine adduct which is a way of saturating the olefinic bond. In order to determine whether saturating the olefinic bond prevents inhibition of mast cell ATPases, ethacrynic acid was reacted with cysteine. Fig. 4 illustrates the results of reacting an inhibitory dose (5×10^{-3} mol) of ethacrynic acid with increasing concentrations of cysteine

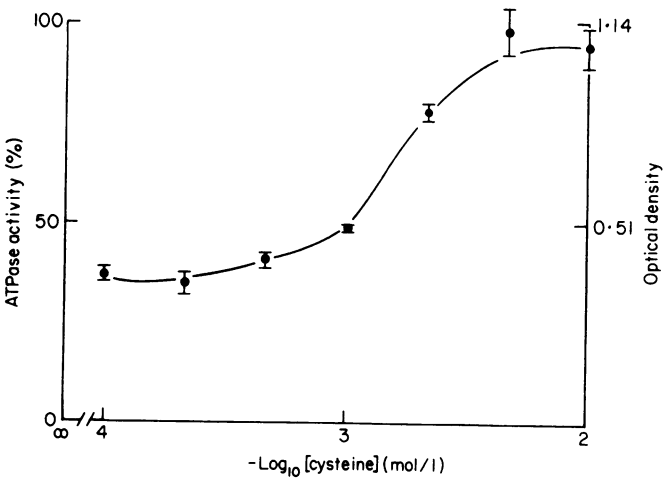

FIG. 4. Effect of cysteine upon ethacrynic acid inhibition of ATPase activity. The effect of increasing concentrations (abscissa) of cysteine upon ethacrynic acid's (5×10^{-3} mol) ability to inhibition-activated (1 mM Ca^{2+}, 1 mM Mg^{2+} and 5 mM K^{+}) ATPase activity of the plasma membrane fraction.

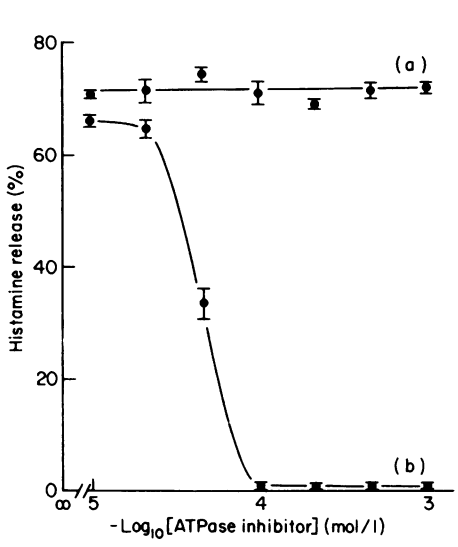

FIG. 5.

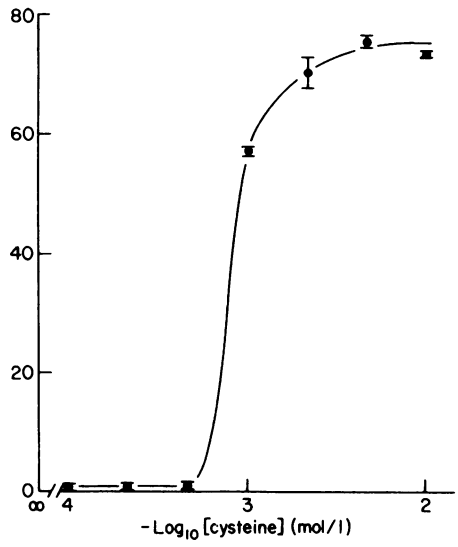

FIG. 6.

FIG. 5. Effect of ouabain and ethacrynic acid upon histamine release from rat mast cells. The effect of increasing concentrations (abscissa) of ouabain (curve a) and ethacrynic acid (curve b) upon the quantity of histamine released (70%) from rat mast cells challenged with *N. brasiliensis* worm antigen (10 μg/ml). The release was potentiated by phosphatidyl serine (10 μg/ml).

FIG. 6. Effect of cysteine upon ethacrynic acid inhibition of histamine release from rat mast cells. The effect of increasing concentrations (abscissa) of cysteine upon ethacrynic acid's (10^{-3} mol) ability to inhibit histamine released (70%) from rat mast cells challenged with *N. brasiliensis* worm antigen (10 μg/ml). The histamine release was potentiated by phosphatidyl serine (10 μg/ml).

(abscissa). It can be seen that as the concentrations of cysteine approach the concentration of ethacrynic acid the ability to inhibit the mast cell ATPases is progressively impeded. As the quantity of cysteine becomes sufficient to completely form the ethacrynic acid–cysteine adduct, the capacity of ethacrynic acid to inhibit the mast cell ATPases is entirely lost.

Effect of ATPase inhibitors upon histamine release

The data of Fig. 5 show how *N. brasiliensis*-induced histamine release from rat mast cells is affected by increasing concentrations of the ATPases inhibitors ouabain (curve a) and ethacrynic acid (curve b). It can be seen from the data of curve (a) that increasing concentrations of ouabain have no inhibitory effect upon the quantity of histamine released by the fixed amount of worm antigen. The data of curve (b), however, show that increasing concentrations of ethacrynic acid effectively inhibit the quantity of histamine released. The reactions were carried out in the presence of phosphatidyl serine in order to potentiate the histamine release (Goth, Adams & Knoohuizen, 1971). Although phosphatidyl serine improved about three-fold the percentage histamine release, the presence or absence of phosphatidyl serine did not alter the inhibitory behavior of either ouabain or ethacrynic acid. Fig. 6 demonstrates that inhibition of histamine release from rat mast cells by ethacrynic acid requires the olefinic bond in the ethacrynic acid molecule. As with ATPase inhibition, the ability of the molecule to inhibit the histamine release is completely prevented by saturating the olefinic bond.

DISCUSSION

Although Cooper & Stanworth (1976) have taken the position that the ATPase activity associated with the plasma membrane of rat mast cells is an ecto-ATPase, the possibility that a major portion of the ATPase activity is due to myosin-type ATPases on the cytoplasmic side of the plasma membrane cannot

be discounted. Orr *et al.* (1972) have suggested a system for Ca^{2+} coupling in histamine-releasing cells involving actomyosin-like contractile microfilaments which are Ca^{2+}-activated; it is deduced that the microfilaments play an important role in the exocytosis of histamine-containing granules by supplying the motive power for granule extrusion. Differing electron micrograph interpretations have put forward the two conflicting opinions that granules are encapsulated by perigranule membranes separate from the plasma membrane (Chi, Lagunoff & Koehler, 1975), and that granules are not separate from, but retained within, invagenations of the plasma membrane. For a contractile process to accomplish either an opening of invagenations in the plasma membrane, or to supply motive power for a migration of granules toward the plasma membrane, contractile microfilaments would have to be in some way anchored to the plasma membrane. The presence of actin in non-muscle cells has long been established (Tilney & Mooseker, 1971; Yang & Perdue, 1972; Probst & Lüscher, 1972). Localization of actin in non-muscle has been accomplished by indirect immunofluorescence utilizing highly specific antibodies directed against actin. A fluorescent visualization of the intracellular distribution of actin demonstrated actin to be associated with fibers that span the length of the cell which often converge to 'focal points' closely associated with the plasma membrane (Lazarides & Weber, 1974). Utilizing rat mast cells, Röhlick (1975) was able to obtain electron micrographs of arrowhead shapes which are characteristic of actin-heavy meromyosin binding. Röhlick (1975) also observed many of the filaments to be attached to the cytoplasmic side of the plasma membrane. If there are submembraneous actomyosin-like microfilaments in rat mast cells which are adhered in some way to the plasma membrane, since ATPase activity associated with actomyosin is known to be Ca^{2+}–Mg^{2+}-activated, the data in this present report could be considered supportive of Orr *et al.*'s (1972) postulate.

The influx of Ca^{2+} being a requirement for the exocytosis of histamine has directed attention to the phenomenon of Ca^{2+} transport and its relevance to histamine release. The release of histamine is currently viewed as a secretory process and the highest levels of membrane ATPase activity are found in secretory and excitatory tissue having the greater capacity for active cation transport (Bonting, Caravaggio & Hawkins, 1962). It has long been recognized that activated ATPases are part of, or identical to, the cation transport system (Schatzmann, 1953; Skou, 1965). Whether Ca^{2+} influx in histamine-releasing cells is a mediated process involving membrane carriers (Pressman, 1968) whose energy requirements are met by activated ATPases, or whether it is a non-mediated, passive process due to conformational changes at the cell membrane resulting in increased permeability to Ca^{2+}, is not yet clear. Divalent cation-activated ATPases which control Ca^{2+} concentration gradients across membranes of vesicles of the endoplasmic reticulum from muscle cells have been observed (Ebashi & Lipmann, 1962). The data of Fewtrell & Gomperts (1977), which show that restrictors of sarcoplasmic reticulum membrane transport ATPases also inhibit histamine release, implicate transport ATPases in histamine secretion. In the belief that the ATPases involved in ion transport are usually modulated and activated by the transported ion, these data here, which show that ethacrynic acid inhibits both histamine release and Ca^{2+}-activated ATPases in the membrane fraction of histamine-releasing cells, are not in conflict with the concept of an energy-requiring mediated Ca^{2+} transport system.

The plasma membrane fraction demonstrates a high level of Mg^{2+}-activated ATPases. It is known that when actin complexes with myosin, the actin confers Mg^{2+} sensitivity to the myosin ATPase. Whether this is the source of the Mg^{2+}-activated ATPases in histamine-releasing cells is not clear. To release histamine from rat mast cells the presence of Mg^{2+} is not essential, but high concentrations of Mg^{2+} can be inhibitory; lower concentrations of Mg^{2+} (0·1–1 mM) do slightly enhance the quantity of histamine released when added in combination with 1 mM Ca^{2+}. It has been demonstrated that in some cell types Mg^{2+} is necessary, but not sufficient, for histamine release. The incapability of substituting magnesium for calcium in the activation of anaphylactic histamine release raises the question of why such a high level of Mg^{2+}-activated ATPases were found in the mast cell homogenate. The answer may lie in the fact that Ca^{2+} can accomplish ATPase activation in addition to serving in some other essential role, such as fusion of perigranule and plasma membranes as postulated by Mongar *et al.* (1973). This would account for Ca^{2+} having the capacity of being both necessary and sufficient for histamine release in rat mast cells. Also, high-dose Mg^{2+} inhibition indicates that although Mg^{2+} cannot totally

substitute for Ca^{2+}, there is some mode of action in which Ca^{2+} and Mg^{2+} can be competitive, and possibly it involves the ATPases which can be activated by either Ca^{2+} or Mg^{2+}.

Contrary to the recent publication of Okazaki *et al.* (1976), the data in both this present investigation and the previous reports (Fewtrell & Gomperts, 1977; Magro, 1977) show that ouabain has no inhibitory effect upon the quantity of histamine released from antigen-challenged cells. This is in keeping with the fact that ouabain is an inhibitor specific for Na^+–K^+-activated ATPases and that a high level of Na^+–K^+ ATPase activity was not demonstrable in the mast cells, and that the degree of ouabain-inhibitable ATPase activity in the mast cells was found to be negligible (Cooper & Stanworth, 1976). Ethacrynic acid, on the other hand, was demonstrated to be an effective inhibitor of antigen-induced histamine release which was in correlation with the data showing ethacrynic acid to be an inhibitor of the mast cell ATPases. Although the concentrations of ethacrynic acid required to inhibit histamine release are lower than those required to inhibit the ATPase activity of the mast cell homogenate, effective blocking of histamine release may not require inhibition of all the cellular ATPases. In fact, at those concentrations of ethacrynic acid where the ATPases of the homogenate are totally inhibited (10^{-3} M to 10^{-2} M) the ethacrynic acid becomes 'toxic' (not shown) to the cell in that the total histamine content of the cell is released. In addition, the cysteine adduct experiments showed that histamine-release inhibition was reversed in an identical manner to ATPase inhibition when the olefinic bond in the ethacrynic acid molecule was saturated. It should be pointed out that we are presently investigating in excess of 100 analogues of ethacrynic acid. Structure activity correlations utilizing these analogues indicate that effective inhibition is not simply a property of the olefinic bond's sulfhydryl binding capacity, but rather involves several structural features of the molecule.

The ability to obtain a highly purified population of rat mast cells was put to advantage in this present report in order to investigate the effect of ATPase inhibitors upon the ATPase system of histamine-releasing cells. It was determined that the detectable ATPase activity in a crude plasma membrane fraction of the mast cell homogenate, which is primarily divalent cation-activated, is ethacrynic acid-inhibitable. This is not to suggest that the crude plasma membrane fraction is totally free of intracellular membranes, organelles or debris. In particular, no attempt was made to separate the perigranule membranes which encapsulate the histamine-containing granules. Also, it is not unlikely that existing subcellular structures connecting plasma and perigranule membranes would be found in the crude plasma membrane fraction. These considerations aside, the data does serve to point up that Ca^{2+}-activated ATPases, which are probably in the proximity of, or in some way associated with, the plasma membrane may play a crucial role in calcium's ability to link stimulus and secretion in histamine-releasing cells.

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