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# Blocking of histamine release from human basophils *in vitro* by the ATPase inhibitor, ethacrynic acid

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SUMMARY

Ethacrynic acid, a known inhibitor of both $Na^+$–$K^+$ and $Mg^{2+}$-activated ATPases, effectively inhibits histamine release from antigen-challenged human basophils *in vitro*. Ouabain, an inhibitor specific for $Na^+$–$K^+$-activated ATPases, shows no effect upon the quantity of histamine released from the antigen-challenged basophils. Ethacrynic acid also effectively inhibits $Ca^{2+}$–ionophore A23187-induced release, implying it inhibits the $Ca^{2+}$-dependent secretory stage of the histamine-release process. Inhibition of ATPases and histamine release by ethacrynic acid both require the presence of the olefinic bond in the ethacrynic-acid molecule. Possible utilization of analogues of ethacrynic acid as anti-allergic drugs and as a device to investigate the ATPase system of histamine-releasing cells is suggested.

## INTRODUCTION

Sensitized human basophils derived from a population of peripheral leucocytes have the capacity to release histamine when challenged with allergens *in vitro*. The release of histamine is an energy-requiring process which can be inhibited by various metabolic blockers, one of which is 2-deoxyglucose. The necessity of an intact glycolytic system addresses the question of whether the energy requirements are linked to the individual stages in the glycolytic pathway or whether it is the adenosine triphosphate (ATP), produced by the overall glycolytic reaction, which is the major source of energy for histamine release.

The requirement of an ion-activated adenosine triphosphatase (ATPase) to make the energy of ATP available was first investigated by Skou (1957). Skou (1960) was able to demonstrate a $Mg^{2+}$-activated ATPase and a $Na^+$–$K^+$-activated ATPase where the latter, but not the former, could be inhibited by the cardiac glycoside ouabain. The diuretic agent, ethacrynic acid,† a broader ATPase inhibitor than ouabain, has been shown to inhibit both the $Na^+$–$K^+$-dependent ouabain-sensitive (Duggan & Noll, 1965) and the $Mg^{2+}$-dependent ouabain-insensitive ATPases (Proverbio, Robinson & Whittembury, 1970).

Regulation of the release of histamine induced by allergens from basophils and mast cells has been an important subject of investigation for more than two decades. The development of *in vitro* techniques (Mongar & Schild, 1962), the identification and characterization of IgE (Ishizaka & Ishizaka, 1967), and the utilization of spectrofluorometric-assay systems (Shore, Burkhalter & Cohn, 1959) has added great impetus to these studies. The understanding derived from these investigations has provided a rationale for studying the allergic response on a molecular and cellular level and has produced a well-defined system for further exploration into the basic parameters of mast cell and basophil activation. The possible role of ATPases has been of theoretical interest and, although investigators have hypothesized an

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† Ethacrynic acid = [2,3 dichloro-4-(2-methylenebutyryl)-phenoxy]acetic acid.

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ATPase system, various ATPase inhibitors have been unsuccessful in their ability to affect the histamine-release process.

The premise that histamine release can be controlled by inhibiting cellular ATPases prompted this investigation. A comparison is made of the two ATPase inhibitors, ouabain and ethacrynic acid, in their effect upon antigen-challenged human basophils *in vitro*. Some of the characteristics of ethacrynic acid's ability to block histamine release are explored. This report is the preliminary study in promoting the concept that analogues of ethacrynic acid can serve as potent anti-allergic agents.

## MATERIALS AND METHODS

*Buffers and cell preparations.* Human leucocytes were obtained from allergic donors who were bled by venipuncture. After sedimentation of the red cells in dextran and EDTA, the leucocyte-rich supernatant was centrifuged to remove platelets and plasma. The isolated leucocytes were washed twice and then resuspended in a Tris-buffered (pH 7·4) solution, which was 120 mM $Na^+$, 25 mM Tris, 5 mM $K^+$, 1 mM $Mg^{2+}$, 0·7 mM $Ca^{2+}$ and 0·03% human serum albumin.

*Inducing agents.* The purified protein Antigen-E, derived from ragweed pollen (King & Norman, 1962) was supplied by the National Institute of Health. The $Ca^{2+}$-ionophore A23187 (Reed & Lardy, 1972) was provided by Lilly. The ionophore was dissolved in dimethyl sulfoxide and appropriately diluted from a 1 mg/ml stock solution.

*ATPase inhibitors.* Ethacrynic acid in the monosodium form was obtained from Merck, Sharp and Dohme. Quabain octahydrate was purchased from Sigma.

*Molar concentrations.* The molar concentrations were estimated based on a mol. wt. of 37,000 daltons for Antigen-E, 523 daltons for ionophore A23187, 303 daltons for ethacrynic acid, and 729 daltons for ouabain octahydrate.

*Reaction mixtures.* The sequence of additions was dependent upon the type of experiment. For most of the experiments, reagents and inducing agents were added followed by the cells all at 4°C. The additions were made into 12 × 75 mm Falcon plastic tubes (2052) to a final volume of 0·6 ml. Typically, there were $1–2 \times 10^6$ leucocytes per tube yielding a total quantity of histamine equivalent to 50–100 ng of histamine base per ml.

*Histamine release.* Following the addition of inducing agent, the reaction mixtures were passed to a 37°C bath and incubated for 60 min. The tubes were then centrifuged and the supernatants decanted and analysed for histamine. Total histamine content was obtained by analysing the quantity of histamine in the supernatants from tubes in which the cells were lysed by incubating in 0·3 N perchloric acid. Blank tubes lacking inducing agent released less than 5% of the total histamine content.

*Histamine assay and analysation of data.* The histamine was analysed by an automated (Siraganian, 1974) spectrofluorometric technique. The percentage of histamine released was calculated in excess of the blank as follows:

$$\text{Per cent release} = 100 \times \frac{\text{sample blank}}{\text{complete blank}}.$$

Experiments were performed in either duplicate, triplicate, or quadruplicate data points. No data points were omitted and a mean value, $\bar{x}$, was calculated from the number of data points, N. A mean deviation, m.d., was calculated as follows: $\text{m.d.} = \sum_{j=1}^{j=N} \frac{|x_j - \bar{x}|}{N}$ where $x_j$ is an individual data point and $|x_j - \bar{x}|$ is the absolute value of the deviation of $x_j$ from the mean. In all the graphs of this report, the values of ± m.d. are shown about $\bar{x}$. For histamine release in excess of 10%, the parameter of m.d./$\bar{x}$ was always less than 0·05, which implies that for this representative data, the precision is within ± 5% about the mean for release in excess of 10%.

## RESULTS

### *The effect of ethacrynic acid and ouabain upon Antigen-E-induced histamine release*

Heuristically, the histamine-release process can be divided into two stages: an inducing agent-dependent activation stage and a $Ca^{2+}$-dependent secretory stage (Mongar & Schild, 1958). Following antigen challenge, an analysis of the quantity of histamine released from the cells is a measure of the overall two-stage response.

The *in vitro* IgE-mediated release of histamine from human basophils follows a dose–response curve where the quantity of histamine released ascends to a maximum and then descends with increasing concentrations of inducing agent. Fig. 1 (inset) shows the dose-response curve for an Antigen-E (AgE) sensitive donor, J.C., and the arrow shows the position on the dose–response curve of the concentration of AgE ($10^{-10}$ M) used to obtain the data of curves (a) and (b). The data (Fig. 1) show how the overall two stages of the release of histamine, induced by a fixed amount of AgE, are affected by increasing concentrations of the ATPase inhibitors ouabain (curve a) and ethacrynic acid (curve b). As an ATPase

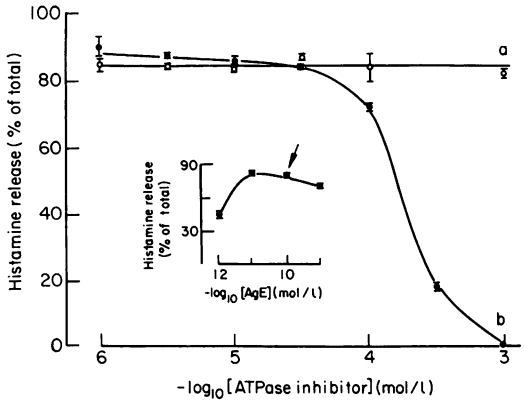


FIG. 1

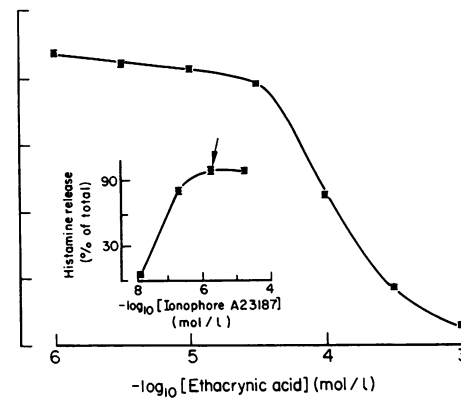


FIG. 2

FIG. 1. Effect of ouabain and ethacrynic acid on Antigen-E-induced histamine release. Inset: dose–response curve of ragweed-sensitive donor, J.C., for AgE-induced histamine release. The arrow indicates the position on the dose–response curve of the concentration of AgE ($10^{-10}$ M) used to obtain the data of the other two curves. Main curves: the effect of increasing concentrations (abscissa) of ouabain (curve a) and ethacrynic acid (curve b) upon the quantity of histamine released from the cells of J.C. by the fixed amount of AgE.

FIG. 2. Effect of ethacrynic acid upon calcium–ionophore A23187-induced histamine release. Inset: dose–response curve of allergic donor, S.S., for $Ca^{2+}$–ionophore A23187-induced histamine release. The arrow indicates the position on the dose–response curve of the concentration of ionophore ($1{\cdot}9 \times 10^{-6}$ M) used to obtain the data of the main curve. Main curve: the effect of increasing concentrations (abscissa) of ethacrynic acid upon the quantity of histamine released from the cells of S.S. by the fixed amount of ionophore A23187.

inhibitor, ouabain begins to show activity at concentrations in excess of $10^{-6}$ M. It can be seen from the data of curve (a) (Fig. 1) that concentrations increasing from this range have no effect upon the quantity of histamine released by the fixed amount of AgE. The data of curve (b) show that increasing concentrations of ethacrynic acid effectively inhibit the quantity of histamine released by the fixed amount of AgE.

*The effect of ethacrynic acid upon calcium–ionophore A23187-induced histamine release*

The activation stage of IgE-mediated histamine release will decay if the cells are challenged with inducing agent in the absence of $Ca^{2+}$ (Mongar & Schild, 1958). Following decay of the activation stage, the histamine-release sequence will not progress to the $Ca^{2+}$-influx secretory stage upon subsequent challenging with an IgE-mediated inducing agent. However, ionophores which have the capacity to initiate $Ca^{2+}$ transport across cell membranes have the ability to release histamine (Foreman, Mongar & Gomperts, 1973) through a bypass of the IgE-mediated activation stage and will initiate the histamine-releasing $Ca^{2+}$-dependent stage, regardless of whether the activation stage has decayed. Ionophore A23187, a transporter of $Ca^{2+}$, has the capacity to initiate histamine release and Fig. 2 (inset) shows the dose–response curve for the cells of the allergic donor, S.S. The arrow indicates the concentration of ionophore A23187 ($1{\cdot}9 \times 10^{-6}$ M) used for the main curve. Since the ionophore induces histamine release through a bypass of the activation stage, the data of the main curve (Fig. 2) show the effect of ethacrynic acid upon the $Ca^{2+}$-dependent secretory stage of the histamine release process. It can be seen that increasing concentrations of ethacrynic acid effectively inhibit the quantity of histamine released by the fixed dose of ionophore.

*Requirement of thiol reactivity for ethacrynic acid to inhibit ATPases and histamine release*

One of the chemical properties of ethacrynic acid which enables it to inhibit ATPases is the capacity of the olefinic bond within the ethacrynic acid molecule to react with sulphhydryl groups. Reversal of ATPase inhibition can be accomplished by saturating the olefinic bond or by previously reacting the molecule with competing sulphhydryl groups. The rate and degree at which ethacrynic acid inhibits ATPases correlates with the rate and degree of its thiol reactivity (Duggan & Noll, 1965). To ascertain if

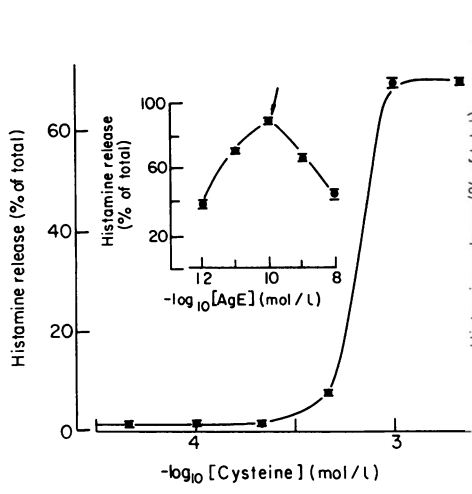


FIG. 3.

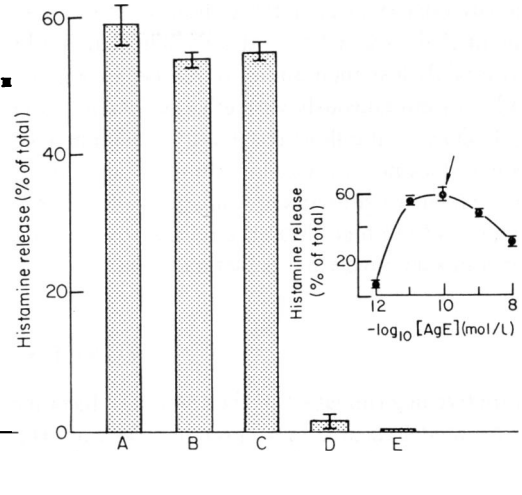


FIG. 4.

FIG. 3. Effect of cysteine upon ethacrynic acid inhibition of Antigen-E-induced histamine release. Inset: dose–response curve of ragweed-sensitive donor, C.S., for AgE-induced release. The arrow indicates the position on the dose–response curve of the concentration of AgE ($10^{-10}$ M) used to obtain the data of the main curve. Main curve: the effect of increasing concentrations (abscissa) of cysteine when added simultaneously with an inhibitory dose of ethacrynic acid ($10^{-3}$ M) upon the quantity of histamine released from the cells of C.S. challenged with AgE.

FIG. 4. Irreversibility of ethacrynic acid's inhibition of Antigen-E-induced histamine release. Inset: dose–response curve of ragweed-sensitive donor, H.W., for AgE- induced release. The arrow indicates the position on the dose–response curve of the concentration of AgE ($10^{-10}$ M) used to obtain the bar data. Bars A–E: Cells of H.W. were incubated at 37°C with various combinations of reagents for 3 min. The cells were subsequently washed then challenged with the AgE and incubated an additional 60 min at 37°C.

Bar A: Cells incubated at 37°C for first 3 min with buffer. Bar B: Cells incubated at 37°C for first 3 min in reaction tubes which were $5\times10^{-2}$ M in cysteine. Bar C: Cells incubated at 37°C for first 3 min in reaction tubes which were simultaneously made $10^{-3}$ M in ethacrynic acid and $5\times10^{-2}$ M in cysteine. Bar D: Cells incubated at 37°C for first 3 min in reaction tubes which were $10^{-3}$ M ethacrynic acid. Bar E: Cells incubated at 37°C for first 3 min in reaction tubes which were $10^{-3}$ M ethacrynic acid. The tubes were subsequently made $5\times10^{-2}$ M in cysteine and incubated an additional 3 min before washing.

ethacrynic acid inhibits histamine release by a similar mechanism, experiments were performed to determine whether increasing concentrations of competing sulphhydryl groups could reverse the histamine release-blocking ability of ethacrynic acid. Increasing concentrations of the sulphhydryl source, cysteine, were added simultaneously to a concentration of ethacrynic acid ($10^{-3}$ M) which effectively inhibits histamine release. A near-maximum dose of AgE ($10^{-10}$ M) was added, followed by the cells, and the reaction mixtures were passed to the 37°C bath and incubated. The data (Fig. 3) show that the capacity of ethacrynic acid to inhibit histamine release is reversed by increasing concentrations of cysteine. It can be seen that the inhibition due to $10^{-3}$ M ethacrynic acid remains unaltered until the concentrations of cysteine approach the concentration of ethacrynic acid.

*The effects of washing and cysteine addition following incubation of cells with ethacrynic acid*

The data of Fig. 3 indicate that the olefinic bonds in the ethacrynic acid molecule must be free to bind in order to inhibit histamine release. However, a modification of the tertiary structure of the ethacrynic acid molecule by the reactive cysteine could be an alternative explanation of the loss of its ability to inhibit. To determine if the olefinic bond is actually reacting during inhibition of histamine release, experiments were performed using cells which were previously reacted for a short time (three min) with ethacrynic acid in the presence and the absence of cysteine. The cells were then washed and subsequently challenged with inducing agent. It can be seen from bar D (Fig. 4) that the washing of cells, previously

reacted with ethacrynic acid in the absence of cysteine, does not restore the cells' ability to respond to the subsequent challenge of $10^{-10}$ M AgE. The data imply that cells reacted for a short time with ethacrynic acid irreversibly lose their ability to release histamine. As in Fig. 3, bar C of Fig. 4 shows that cysteine, when added simultaneously with ethacrynic acid, reverses the inhibitory activity of ethacrynic acid. Bar E (Fig. 4) shows that following a 3 min interaction of cells and ethacrynic acid, cysteine addition followed by washing does not reverse the inhibitory activity of ethacrynic acid in that the ability of the washed cells to respond to subsequent antigen challenge is not restored. Bar E (Fig. 4) not only shows that ethacrynic acid inhibits histamine release irreversibly, but implies that ethacrynic acid reacts with the cells via a linkage involving its olefinic bond.

## DISCUSSION

A quantitative determination of the amount of histamine released from human basophils is recognized as an *in vitro* model capable of accurately measuring the anaphylactic response. This *in vitro* system was utilized here to determine whether agents known to inhibit ATPases can affect the anaphylactic response in humans. The data show that ouabain, an inhibitor specific for $Na^+$–$K^+$-activated ATPases, has no inhibitory effect upon the quantity of histamine released from antigen-challenged basophils. Ouabain's inability to inhibit histamine release is in agreement with the idea that the histamine-release process is relatively insensitive to extracellular concentrations of $Na^+$ and $K^+$; human basophils are known to respond to antigen challenge unhampered in $Na^+$–$K^+$-free buffers such as isotonic choline chloride. Ethacrynic acid, a broader ATPase inhibitor than ouabain, does effectively inhibit histamine release. Inhibition of histamine release by ethacrynic acid suggests the possibility that $Ca^{2+}$–$Mg^{2+}$-activated ATPases have a role in the anaphylactic response in humans. The presence of an ATPase system in human leucocytes (Block & Bonting, 1964) and the $Ca^{2+}$–$Mg^{2+}$ requirements for histamine release from human basophiles are supportive of a $Ca^{2+}$–$Mg^{2+}$-activated ATPase system. Unfortunately, the limited number of basophils (1%) in human peripheral leucocytes does not lend itself to obtaining unequivocal evidence of ATPase involvement. However, if it were known *a priori* that the energy requirements for the exocytosis of histamine were met by $Ca^{2+}$–$Mg^{2+}$-activated ATPases in human basophils, the data in this report would not be contradictory in that the data show ethacrynic acid inhibits the $Ca^{2+}$–$Mg^{2+}$-dependent stage of the histamine-release process. In addition, the behavior of ethacrynic acid toward histamine release correlates with its inhibitory effects upon ATPases in that inhibition of histamine release and ATPases both require the presence of the olefinic bond in the ethacrynic acid molecule.

In considering the pharmacological control of the allergic response, ethacrynic acid as a commercially available approved drug could offer opportunities for the treatment of allergies and allergy-related diseases. As a diuretic, structure-activity relationships of derivatives of the aryloxy-acid series, of which ethacrynic acid is a member, have been extensively studied (Schultz *et al.*, 1962, 1976; Bicking *et al.*, 1976a, b; Woltersdorf *et al.*, 1976). These investigations have correlated the structures of nearly 100 analogues with their uricosuric, diuretic and saluretic effects. At high doses many of them have favourable uricosuric properties with negligible or no diuretic–saluretic activity. Currently, we are investigating the capacity of these analogues to inhibit histamine release and whether the structural requirements for histamine inhibition parallel the structural requirements for diuresis.

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