

IONOPHORE A-23187 INDUCED HISTAMINE RELEASE FROM RAT MAST CELLS AND RAT BASOPHIL LEUKEMIA (RBL-1) CELLS

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Ionophore A-23187 releases histamine from normal mast cells apparently by promoting Ca^{++} influx (Foreman et al, *Nature* 245: 249, 1973). In our hands at concentrations of $>0.2 \mu\text{g/ml}$ release occurs in 1 to 2 min, is blocked by metabolic inhibitors, and is unaccompanied by cytotoxicity (trypan-blue uptake, lactic dehydrogenase (LDH) release). At higher doses ($0.5 \mu\text{g/ml}$) histamine release is followed by significant cytotoxicity, but again Ca^{++} is required. In parallel studies, we examined cultured rat basophilic leukemia (RBL-1) cells. These cells, which apparently have normal surface receptors for IgE, contained $\sim 700 \text{ ng}$ histamine/ 10^6 cells but did not release histamine when IgE-mediated release was looked for. They do not respond to doses of ionophore which would be expected to give non-cytotoxic histamine release. At higher doses histamine release is preceded by progressive LDH release: LDH release is 75% complete at 5 min whereas 10 min are required for 75% maximal histamine release. This reaction requires Ca^{++} and is temperature dependent but is not inhibited by metabolic poisons (2-deoxyglucose, dinitrophenol, CN^-). These studies suggest that either Ca^{++} does not enter into these cells normally or that one or more mechanisms which are ordinarily triggered by the changes in Ca^{++} flow are unresponsive in the RBL-1 cells. These studies also underline the importance of ruling out cytotoxicity in ionophore-induced phenomena.

A unique rat leukemia has been discovered by Eccleston *et al.* (1). The rat basophilic leukemia cells (RBL-1)³ resemble mammalian basophilic leukocytes in many respects; they contain basophilic granules, histamine, serotonin, eosinophilic chemotactic factor (1, 2) and cell surface receptors for IgE (3). The cells can be easily cultured, so that large numbers of homogeneous cells can be readily obtained; consequently, they may serve as a useful model for studying the structural and functional properties of basophils. One of these functions is non-cytotoxic histamine release and we have explored the capacity of the RBL-1 cells to perform this function. Initial studies utilized cells "sensitized" with reaginic (IgE) antibodies or myeloma IgE and reacted with antigen or anti-IgE, respectively (Kulczycki, Siraganian, and Metzger, unpublished studies). Despite a substantial number of experiments in

which the doses of reactants were varied widely and several useful additives (heavy water, serum, and phosphatidyl serine) were added, no histamine release could be elicited.

It has recently been reported that the Ca^{++} binding ionophore A-23187 (4, 5) can induce a variety of biologic phenomena (6-8), including histamine release from normal rat mast cells (9, 10) and human basophils (11). It was therefore of interest to use this reagent in order to compare its effect on the RBL-1 cells with its effect on normal mast cells.

MATERIALS AND METHODS

Buffers and media. A modified Tris buffer was used containing 25 mM Tris, 123 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, and 0.1% gelatin, pH 7.4, at 37°C. CaCl_2 , MgCl_2 and ethylenediaminetetraacetic acid (EDTA) (pH 7.4) as 0.1 M solutions were added to the Tris buffer to prepare the solutions indicated in *Results*. An isotonic sucrose buffer free of sodium was prepared using 270 mM sucrose, 1.0 mM CaCl_2 , 0.4 mM MgCl_2 , and 0.1% gelatin. Eagle's minimal essential medium contained 4 mM L-glutamine, 100 units/ml penicillin and 100 $\mu\text{g/ml}$ of streptomycin (all from the NIH media unit) and 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) at pH 7.1. RPMI 1640 (from Grand Island Biological Co.) was made 10 mM with HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) to which was added 0.03% human serum albumin (A. G. Behring, Somerville, N. J.) and 1.0 mM Ca^{++} .

Compounds. Ionophore A-23187 (donated by Eli Lilly and Co., Indianapolis, Ind.), was dissolved in methanol or dimethylsulfoxide at a concentration of 2.5 mg/ml and stored refrigerated. The ionophore was diluted into the experimental buffers immediately before use. The concentration of methanol or dimethylsulfoxide in the diluted buffers never exceeded 0.2%. Controls with these concentrations added to cells did not induce histamine or lactic dehydrogenase (LDH) release and had no effect on the histamine and LDH assays.

NaF, KCN, 2-deoxyglucose, and phosphatidyl serine were obtained from Sigma Chemicals, St. Louis, Mo. Chlorphenesin was a gift from Carter Wallace Laboratories, New York, N. Y. and Hetrazan (diethylcarbamazine) was generously provided by Lederle Laboratories, Pearl River, N. Y.

RBL-1 cells. RBL-1 cells were grown in Falcon flasks or spinner culture in Eagle's minimal essential medium supplemented with fetal calf serum (10 to 12% for flask cultures, 15% for spinner cultures), 4 mM glutamine, 100 units/ml of penicillin, and 100 $\mu\text{g/ml}$ streptomycin. Cells were harvested by centrifugation at $50 \times G$ for 10 min at room temperature and routinely washed once in the experimental buffer unless otherwise indicated. Viability was determined by the exclusion of trypan blue.

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³Abbreviations used: RBL-1, rat basophilic leukemia cells; LDH, lactic dehydrogenase; EDTA, ethylenediaminetetraacetic acid.

Rat mast cells. Peritoneal cells were obtained by previously described methods (12); the mast cells were isolated by centrifugation through a bovine serum albumin solution as described and were over 96% pure. The cells were washed first with the Tris buffer containing 4 mM EDTA, centrifuged at $200 \times G$ for 8 min at 4°C and then rewashed with Tris buffer, recentrifuged, and finally suspended in the experimental buffer.

Reaction mixtures. Several concentrations of ionophore were added in 0.25 ml of buffer or medium to 12- x 75-mm plastic tubes in an ice bath. Cells were added to all tubes in a volume of 0.25 ml and the racks transferred to a 37°C bath. When studying the effect of inhibitors, 0.2 ml each of cells, ionophore, and inhibitor were used in the reaction mixture. The reaction was stopped by transferring the tubes to an ice bath followed by centrifugation at $700 \times G$ for 10 min at 4°C . In time course experiments larger reaction volumes were used, and samples were removed into EDTA containing buffers as described in Results.

Histamine and LDH assays. Histamine was assayed by the automated fluorometric technique developed by Siraganian (13). In each experiment perchloric acid at a final concentration of 3% was added to some samples ("completes"), and the contents of the tube were vortexed, incubated 30 min at 37°C , and centrifuged to obtain the total histamine content of the cells. LDH was measured by an automated fluorometric method (14). Total LDH content of the cells ("completes") was measured after incubation in 0.15% Brij-35 solution for 30 min at 37°C , vortexed and then centrifuged, and centrifuged.

The percentage of release of histamine and LDH were both calculated using the formula:

$$\% \text{ release} = \frac{\text{Experimental tube} - \text{blank}}{\text{Complete} - \text{blank}} \times 100$$

(Blank = release in the presence of buffer alone; complete = total content in cell suspension).

RESULTS

Studies with peritoneal mast cells. Mast cells were exposed to varying concentrations of ionophore A-23187 and the release of histamine as a function of time was assessed. LDH release was measured simultaneously as a sensitive indicator of cytotoxic effects.

We observed that over a very narrow range of ionophore concentration histamine release could be induced in the absence of detectable LDH release. A representative experiment in which $0.2 \mu\text{g/ml}$ of ionophore was used is illustrated by the open symbols in Figure 1. Approximately 80% of the total cellular histamine was released within 2 min with no further release noted over the next 38 min. At $0.1 \mu\text{g/ml}$ of ionophore (not shown), 40% of the histamine was released within 4 min without further release over the subsequent 38 min. No LDH was released throughout this period. At $0.5 \mu\text{g/ml}$ (solid symbols, Fig. 1) the histamine release was similar to that observed at $0.2 \mu\text{g/ml}$ but now was accompanied by progressive LDH release (solid symbols, Fig. 1). The values at $1.0 \mu\text{g/ml}$ of ionophore were superimposable with the data obtained at $0.5 \mu\text{g/ml}$. At higher concentrations of ionophore (e.g., 5 to $100 \mu\text{g/ml}$) there was progressive decrease in the histamine release and an increase in the LDH release. Repetitive challenge of the same mast cell suspension with low doses of ionophore demon-

strated that the cells still responded to a second challenge despite incomplete release after the first challenge.

The A-23187 induced histamine release was completely inhibited by 1 mM EDTA in the absence of added cations. The maximal histamine release was with a concentration of 1 mM of Ca^{++} and Mg^{++} ; in the absence of added cations the release was 56% of the maximal release. Adding Mg^{++} alone slightly increased the histamine release. In contrast, at optimal Ca^{++} levels, Mg^{++} decreased the release or had no effect.

The metabolic inhibitors 2-deoxyglucose and KCN blocked the ionophore-induced non-cytotoxic histamine release from the mast cells. The results of such studies are shown in Table I. When CN^- was tested at a concentration of $1 \times 10^{-3} \text{ M}$ the histamine release induced by cytotoxic levels of ionophore (0.5 to $10 \mu\text{g/ml}$) was more than 70% inhibited. The ionophore-induced histamine release was not enhanced by the addition of phosphatidyl serine (1 to $100 \mu\text{g/ml}$).

Studies with RBL-1 cells. At $0.1 \mu\text{g/ml}$ ionophore, a dose which led to 40% histamine release and no LDH release from mast cells, 5 to 10% of the cellular histamine and ~5% of the LDH was released from the RBL-1 cells during 60 min of observation. The data were too scattered to allow an accurate determination of the time dependence of the reaction. Typical data obtained at $0.2 \mu\text{g/ml}$ are shown in Figure 2 (open symbols). Only a modest percentage of histamine was released accompanied by a somewhat smaller amount of LDH release. At $0.5 \mu\text{g/ml}$ of ionophore (solid symbols, Fig. 2), more substantial (up to 80%) histamine was released, but this was preceded by a similar degree of LDH release. Notably, although the LDH release from the RBL-1 cells was, if anything, greater and more rapid than that observed for the mast cells, the release of histamine was considerably slower; the half-time was ~6 min for the RBL-1 cells compared to <1 min for the mast cells. The results obtained at 1 or $2 \mu\text{g/ml}$ of A-23187 were very similar to those shown for $0.5 \mu\text{g/ml}$.

Several variations in the experimental conditions were introduced in an attempt to dissociate the release of histamine

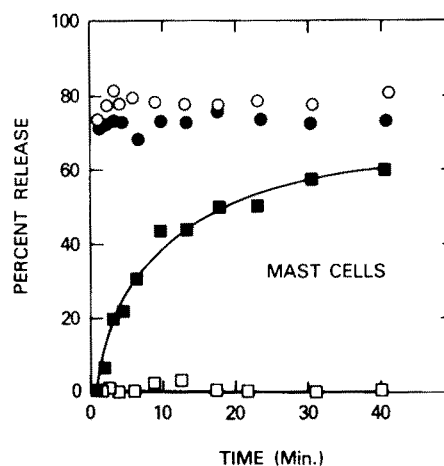


Figure 1. Time course of histamine and LDH release from rat mast cells. Prewarmed rat mast cells and ionophore solutions in Tris buffer, 1.0 mM Ca^{++} , 0.4 mM Mg^{++} were mixed and incubated at 37°C . Aliquots of 0.1 ml from the reaction mixtures were transferred at the indicated times to 0.7 ml of Tris buffer containing 4 mM EDTA at 0°C and centrifuged immediately. Final cell concentration in the reaction mixture was $3.9 \times 10^5/\text{ml}$. The histamine values are shown in circles, LDH results in squares. Ionophore concentrations were: $\square$, $0.2 \mu\text{g/ml}$; $\blacksquare$, $0.5 \mu\text{g/ml}$. Release in the absence of ionophore was < 10%.

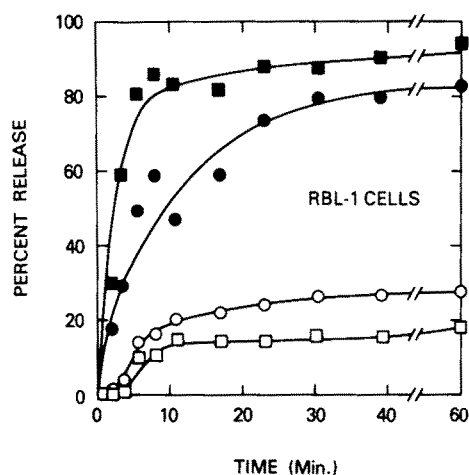


Figure 2. Time course of histamine and LDH release from RBL-1 cells. Reaction was at 37°C in Tris buffer 1.9 mM Ca^{++} , 0.9 mM Mg^{++} . Histamine values are shown in circles, LDH results in squares. Ionophore concentrations were 0, $\square$: 0.2 $\mu\text{g}/\text{ml}$; $\bullet$, $\blacksquare$: 0.5 $\mu\text{g}/\text{ml}$. Final cell concentration was $4.1 \times 10^5/\text{ml}$. Release in the absence of ionophore was 4 to 8%. See Figure 1 for details of reaction conditions.

from the cytotoxic effect of A-23187. In one experiment the temperature was varied (18, 25, 32 and 37°C), LDH release preceded histamine release at all temperatures studied. Variations in Ca^{++} and Mg^{++} concentrations produced more complicated effects which can be summarized as follows: in the absence of added Ca^{++} and Mg^{++} the cells released 34% of their total histamine and 17% of their total LDH during the 40 min of observation (65% of the maximal histamine release). When 0.1 to 3 mM Mg^{++} was added in the absence of Ca^{++} , release of both constituents averaged only 5 to 10%. With 0.1 to 0.2 mM Ca^{++} substantial histamine and LDH were released at 0 to 1 mM Mg^{++} . Some reduction was observed at 3 mM Mg^{++} . At 0.5 to 2 mM Ca^{++} , 40 to 60% of the histamine was released, added Mg^{++} having little effect. The effects on LDH release were similar, although with 2 mM Ca^{++} the LDH release was somewhat depressed at intermediate doses of Mg^{++} . The addition of excess EDTA (1 mM) completely inhibited the A-23187-induced histamine release from the RBL-1 cells.

Since more complete media enhance the viability of RBL-1 cells, tissue culture media were used in the histamine release reaction. The A-23187-induced histamine release was not dissociated from LDH release by the use of Eagle's media or by

RPMI 1640 with or without fetal calf serum. The addition of ionophore to RBL-1 cells incubated in Ca^{++} and Mg^{++} containing isotonic sucrose caused histamine and LDH release, indicating that sodium influx is not required (10).

Several metabolic inhibitors failed to inhibit the effect of the ionophore on the RBL-1 cells (Table I). In fact, high concentrations of CN^- and chlorphenesin enhanced both the histamine and LDH release. Time course experiments showed no difference in the rate of the ionophore-induced release in the presence or absence of CN^- of 2-deoxyglucose.

The addition of excess EDTA before the reaction of RBL-1 cells with ionophore completely inhibited both the histamine and LDH release. However, the addition of excess EDTA after the initiation of the ionophore reaction caused more variable results. At 37°C when excess EDTA was added at various times after the start of the ionophore reaction, the release of both LDH and histamine was abrogated. If the EDTA was added to cells which had been transferred to 0°C, the histamine and LDH release continued for some time. These results would suggest that the membrane defect caused by ionophore in the RBL-1 cells is reversed faster at 37°C than 0°C by EDTA.

DISCUSSION

The current concept of antigen-induced IgE-mediated histamine release is that the reaction is closely related to the "stimulus-secretion coupling" mechanism which is characteristic of such systems as neurotransmitter release and hormone-induced zymogen granule secretion by a variety of cells. The dependence on Ca^{++} and on energy metabolism as well as the resemblance of the ultrastructural events are some of the salient similarities among these systems.

It is thought that one of the earliest events in stimulus-secretion coupling is a change in transmembrane Ca^{++} flux. Such changes have recently been demonstrated in several systems including the rat mast cell (9). The lipophilic ionophore A-23187 is thought to facilitate diffusion of cations, particularly Ca^{++} across membranes (15, 16). Thus its ability to mimic the effects of specific stimuli in a variety of situations lends credence to the critical role of Ca^{++} flux in these systems.

Our own results with rat peritoneal cells are in close agreement with the results of others on these cells as well as on human basophils (11). Over a narrow dose range the ionophore led to rapid release of histamine without concomitant LDH release. Although added Ca^{++} was not absolutely required, the response was optimized by 1 mM Ca^{++} and Mg^{++} in the

TABLE I
Effect of metabolic inhibition on ionophore-induced histamine release^a

Inhibitor	Concentration Range Tested	Concentration for 50% Inhibition	
		Rat mast cells	RBL-1 cells
<i>M</i>			
NaF	1×10^{-5} – 5×10^{-3}	No inhibition	No inhibition
KCN	1×10^{-5} – 5×10^{-3}	3×10^{-5}	No inhibition ^b
2,4-Dinitrophenol	1×10^{-5} – 1×10^{-3}	NT ^c	No inhibition
2-Deoxyglucose	1×10^{-5} – 5×10^{-3}	43% at 5×10^{-4}	No inhibition
Chlorphenesin	1×10^{-5} – 5×10^{-3}	NT ^c	No inhibition ^b
Diethylcarbamazine	1×10^{-5} – 5×10^{-3}	NT ^c	7×10^{-4}

^a RBL-1 or rat mast cells were suspended in the Tris buffer free of glucose. The cells were incubated with the inhibitors at 37°C for 30 min, and the reaction was started by the addition of ionophore (0.3 $\mu\text{g}/\text{ml}$ for rat mast cells, 1.0 $\mu\text{g}/\text{ml}$ for RBL-1 cells). The rat mast cell reaction was stopped after 10 min and the RBL-1 after 60 min. Release in the absence of inhibitors was 50 to 70%.

^b At 1.5×10^{-3} M these compounds enhanced both histamine and LDH release.

^c NT, not tested.

medium and was abrogated in the presence of excess EDTA. Similarly, metabolic inhibitors blocked the reaction.

At higher doses of ionophore the rapid histamine release was followed by more slowly developing signs of cytotoxicity (increasing LDH release as well as trypan blue positivity). Such cytotoxic effects of higher doses of ionophore A-23187 have been noted in other systems and may result from effects on mitochondrial metabolism (17).

The results with the RBL-1 cells were notably different. At no dose of ionophore was rapid histamine release observed. Instead, only at doses similar to those which produced cytotoxicity in the mast cells was there a slow release of histamine clearly preceded by LDH release. These responses could not be inhibited by metabolic poisons but showed the same Ca^{++} dependence observed with the mast cells. The faster release of LDH than histamine suggests that the effect of ionophore on the RBL-1 cells is initially to damage the cell membrane which allows the escape of LDH followed by a secondary action on the histamine-containing granules.

It thus appears that the RBL-1 cells' unresponsiveness to IgE-mediated and ionophore-mediated stimulation is caused by a defect in one or more functions required for non-cytotoxic histamine release. The nature of this defect is of course unknown. In view of our interest in the early events of secretion ("triggering"), it will be important to distinguish between two major possibilities. The first is that the mechanisms by which IgE and ionophore stimulate changes in Ca^{++} flux are abnormal in these cells. The second is that the changes in Ca^{++} flux occur normally but that the system lacks one or more of the mechanisms which respond to these perturbations in Ca^{++} flow. Direct measurements of Ca^{++} fluxes could be helpful in distinguishing between these possibilities and such studies are being attempted.

Since the nature of the defect is unclear it is pointless to expound at length on its origin. It is worth noting, however, that the nature of the RBL-1 cells is uncertain. Although originally described as containing $\sim 20 \mu\text{g}$ of histamine per 10^6 cells (1) (similar to the value observed with peritoneal mast cells (18, 19)), our assays, performed both on tumor suspensions and cultured cells have revealed only about 700 ng of histamine/ 10^6 cells. These levels progressively decreased over the $\sim 1\frac{1}{2}$ years we have studied these cells at this writing. This relatively low concentration would be consistent with their being basophil-like cells since at least in humans and monkeys circulating basophils contain only about 5% of the histamine observed in the mast cells of the same species (20, 21). The RBL-1 cells were also considered to be more like basophils than mast cells on the basis of their presence in the peripheral blood during the leukemic stage and of their staining characteristics (1). However, it is noteworthy that there is considerable doubt that a peripheral basophil exists in rats at least under normal conditions (22, 23). Thus it is difficult to rule out the possibility that these cells may represent an arrested stage of mast cell development.

These considerations make a meaningful comparison be-

tween the results obtained on the RBL-1 cells and peritoneal mast cells difficult at this time. It is known that basophils and mast cells may differ markedly in their histamine-releasing response in respect to kinetics of release, inducibility by such reagents as 48/80, and inhibition or stimulation by a variety of drugs. Thus the differences we have observed could be partially caused by the nature of the cell (basophil *vs* mast cell), the stage of differentiation, or the fact that the cells are neoplastic. Elucidation of the differences may be helpful in understanding the nature of the mechanisms involved in normal cells.

These results emphasize the importance of excluding cytotoxic phenomena in ionophore-induced reactions. Inhibition by metabolic inhibitors may not be sufficient evidence; we found that the release of histamine from normal mast cells was substantially inhibited with CN^- even though cytotoxic levels of ionophore were employed. It is also noteworthy that the effective non-cytotoxic dose range can be exceedingly narrow.

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