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INHIBITION OF HISTAMINE RELEASE FROM RAT MAST CELLS BY CYTOCHALASIN A AND OTHER SULFHYDRYL REAGENTS *

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Abstract—We adduce several lines of evidence to indicate that the inhibition of histamine release from rat mast cells by cytochalasin A (Cyto A) depends on its reactivity with mast cell sulfhydryl (SH) groups: (1) Cyto A reacts with cysteine, glutathione and protein SH groups; (2) prior reaction of Cyto A with glutathione eliminates its inhibitory action; and (3) 2,2′-dithiodipyridine (DTDP), a SH reagent with greater specificity for SH groups and very different structure from Cyto A, has parallel inhibitory effects on mast cell secretion. Inhibition of histamine release by Cyto A or by DTDP is independent of depression of cell ATP or cell glutathione. Inhibition of release induced by the calcium ionophore A23187 and polymyxin suggests that the two SH reagents act to block the release mechanism subsequent to a putative increase in cytoplasmic Ca^{2+} concentration. In contrast, *N*(7-dimethylamino-4-methylcoumarinyl)maleimide (DACM), *p*-chloromercuribenzene sulfonate (PCMBS) and *N*-ethylmaleimide (NEM) ($< 10^{-5}$ M) appear to react with a SH group required for polymyxin B but not A23187-induced release. The effect of NEM at high concentrations (5×10^{-5} M) is attributable to depression of cell ATP.

Orr *et al.* [1] in 1972 observed a marked difference between the effects of cytochalasins A (Cyto A) and B (Cyto B) on mast cell secretion. In their experiments, a 100 times greater molar concentration of Cyto B than of Cyto A was required for equivalent inhibition of histamine release induced by compound 48/80. In related experiments, Cyto B was noted to be considerably more effective as an inhibitor of release by antigen than by compound 48/80. In consideration of the evidence that Cyto A [2–4] is far more reactive with sulfhydryl (SH) groups than Cyto B [5], we undertook experiments to test the hypothesis that Cyto A inhibits mast cell histamine release by virtue of its reaction with SH groups. Preliminary results consistent with this hypothesis have been reported previously [6]. Recently, Nemeth and Douglas [7] have reported further observations in accord with the proposal that Cyto A inhibits mast cell secretion through a reaction with SH groups; they draw somewhat different inferences than we do as to the site of action of Cyto A.

MATERIALS AND METHODS

Peritoneal cells were obtained from adult male rats by the method described previously [8]. A balanced salt solution (BSS) containing 9 g/liter of NaCl, 0.2 g/liter of KCl, 0.1 g/liter of $CaCl_2 \cdot 2H_2O$, 0.57 g/liter of Na_2HPO_4 and 0.37 g/liter of KH_2PO_4, pH 7.2, was used throughout as the medium for the cells. All solutions were prepared in BSS unless otherwise indicated. Mast cells for most of the experiments were separated from the other peritoneal cells, to 85 per cent or greater purity, by centrifugation through a solution of 27.2% albumin [8]. The rats used as a source of mast cells were purchased from Tyler Laboratories (Bellevue, WA) whose stock derives from Hilltop Sprague–Dawley animals.

Cyto A, 2,2′-dithiodipyridine (DTDP, "Aldrithiol-2") and 6,6′-dithiodinicotinic acid (DTDN) were purchased from the Aldrich Chemical Co., Milwaukee, WI. *N*-ethylmaleimide (NEM), *p*-mercuribenzene sulfonate (*p*-chloromercuribenzene sulfonate, PCMBS), polymyxin B sulfate, firefly lanterns, ATP, dinitrophenol (DNP) and *o*-phthalaldehyde (OPT) were purchased from the Sigma Chemical Company, St. Louis, MO. *N*-(7-dimethylamino-4-methylcoumarinyl)maleimide (DACM) [9,10] was purchased from Polysciences, Inc., Warrington, PA. Albumin (Pathocyte-4) was purchased from Miles Laboratories, Inc., Elkhart, IN. The calcium ionophore A23187 was generously provided by Dr. Robert L. Hamill, Lilly Research Laboratories, Indianapolis, IN.

The standard protocol for testing the SH reagents was to preincubate mast cells for 10 min at room temperature in 0.2% albumin in BSS with the agent. Poorly aqueous soluble reagents were prepared as stock solutions in ethanol (Cyto A, 5×10^{-3}; DTDP, 10^{-2}; DACM, 2×10^{-4}, and DNP, 0.1 M) and added to the BSS so that the final concentration of ethanol in the initial incubation media did not exceed 0.5%, a level at which no effect on ATP or histamine release was measurable. The secretagogue was added and the cells were incubated for an additional 10 min at room temperature. The stock solution of A23187 was prepared in ethanol at 10^{-4} M and added to the mast cell suspension to give a final concentration of 10^{-6} M and 1.5% ethanol. Polymyxin B was dissolved in BSS and added to the cells to give a final concentration of 1.4 μM. After incubation with the secretagogue, the cells were centrifuged in a refrigerated centrifuge, and the supernatant fraction and precipitate assayed for histamine by the OPT method [8]. Dose–response curves for inhibition of histamine release were analyzed by the Logit method [11] using a Hewlett–Packard 9830A calculator.

Mast cell ATP was normally determined after the initial incubation but before the addition of secretagogue. The cells were centrifuged, and ATP was meas-

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Table 1. Inhibition of histamine release by SH reagents

Inhibitor*	ID_{50}† (95% confidence interval) Polymyxin B‡, (1.4 μM)	A23187‡ (1 μM)
Cytochalasin A	2.4×10^{-7} (1.8–3.3)	1.7×10^{-7} (0.9–3.2)
N-Ethylmaleimide	2.7×10^{-6} (1.9–3.8)	4.4×10^{-5} (2.8–6.9)
N-(7-Dimethylamino-4-methylcoumarinyl)maleimide	2.3×10^{-7} (1.2–4.2)	$> 10^{-6}$
2,2′-Dithiodipyridine	1.0×10^{-6} (0.72–1.5)	1.5×10^{-6} (0.9–2.4)
p-Mercuribenzene sulfonate	1.2×10^{-4} (1.0–1.4)	$> 5 \times 10^{-4}$

* Mast cells were incubated at varied concentrations of inhibitor for 10 min at room temperature in the presence of 0.17% albumin.

† ID_{50}'s, the concentrations required for 50 per cent inhibition of histamine release, were determined using the Logit transformation [11]. The 95 per cent confidence interval was also determined and is indicated within the parentheses. Each value was calculated from at least three determinations in duplicate at at least four concentrations of inhibitor.

‡ Secretagogue was added and histamine release in 10 min, also at room temperature, was measured.

ured on the cell pellet using the luciferin–luciferase assay [12] as described previously [13]. With polymyxin B, release is complete within a minute and there is less than a 10 per cent drop in ATP during a 10-min incubation after the addition of polymyxin. With A23187 (1 μM), release requires several minutes, and mast cell ATP falls as low as 10 per cent of the initial value. Glutathione levels were also measured prior to the addition of polymyxin B or A23187. The method was a modification of the method of Tietze [14]. After initial incubation, mast cells were washed twice in 2 ml BSS. The final pellet was brought up in 0.25 ml of 0.01 M HCl, 0.1% Triton X-100 and sonicated for 30 sec in a bath-type sonicator. The lysate was centrifuged at 500 *g* for 5 min and 0.2 ml of the supernatant fraction was assayed for glutathione, according to the macro variant of Brehe and Burch [15]. Appropriate tests were performed to exclude inhibition of glutathione reductase by free SH reagents carried over in the cell lysate. The procedure measures total glutathione, but reduced glutathione in mast cells constitutes at least 95 per cent of the total glutathione (cf. Ref. 16).

RESULTS

Cyto A was an effective inhibitor of histamine release induced by either polymyxin B or A23187 (Table 1). The inhibition of histamine release by Cyto A could not be reversed by two washes in medium lacking Cyto A (Table 2). The inhibitory effects of Cyto A were eliminated by prior reaction of Cyto A with an excess of glutathione (Table 2). Addition of glutathione, after mast cells were incubated for 10 min with Cyto A, did not reverse the inhibition of histamine release (Table 2.)

The unsaturated carbon–carbon bond at position 10-11 flanked by a ketone and a lactone in Cyto A bears a strong resemblance to the maleyl group. Therefore, we compared the inhibitory effects of NEM and DACM with those of Cyto A. NEM inhibited histamine release by both polymyxin B and A23187, but concentrations 10 times and 250 times those of Cyto A, respectively, were required for equivalent inhibition (Table 1). DACM, a fluorescent, *N*-substituted maleimide, was as effective as Cyto A in inhibiting release by polymyxin B but did not inhibit release by A23187 at the highest

Table 2. Effects of glutathione and washing on the inhibitory action of cytochalasin A

Incubation* Primary	Secondary	Fraction of control† Histamine release
Cyto A	Mast cells	0.001 ± 0.001‡
Cyto A + GSH	Mast cells	1.005 ± 0.030
Cyto A + mast cells	GSH	0.001 ± 0.001
Cyto A + mast cells	[Wash × 2]	0.0003 ± 0.0003

* Cytochalasin A (10^{-6} M) was incubated in (a) BSS, (b) BSS + glutathione 10^{-4} M or (c) directly with mast cells in BSS for 10 min at 22°. Mast cells were added to the first two for an additional 10 min at 22°. Glutathione (10^{-4} M) was added to cells preincubated with Cyto A alone and incubated for 10 min at 22°, or else the cells exposed to Cyto A were washed two times with BSS by centrifugation.

† At the completion of the second incubation, polymyxin B was added to a final concentration of 1.4 μM, the cells were incubated for another 10 min at 22°, and histamine release was measured. Background histamine release in the absence of polymyxin B in the final incubation was consistently below 0.05, while release from mast cells not exposed to Cyto A was 84.3 ± 6.5 per cent for the three experiments.

‡ Each value is the mean ± S.E. for three experiments with duplicate determinations in each experiment.

Table 3. Lack of ATP depression in mast cells by concentrations of SH reagents which inhibit histamine release

SH reagent*	Concn (M)	Fraction of control Histamine release†	ATP‡
Cytochalasin A	2.5×10^{-6}	0.003 ± 0.002	1.02 ± 0.03
2,2′-Dithiodipyridine	5.0×10^{-6}	0.01 ± 0.01	0.97 ± 0.04
p-Mercuribenzene sulfonate	5.0×10^{-4}	0.08 ± 0.01	1.10 ± 0.11
N-(7-Dimethylamino-4-methyl-coumarinyl)maleimide	10^{-6}	0.06 ± 0.004	0.99 ± 0.01
N-Ethylmaleimide	1.0×10^{-3}	0.02 ± 0.01	0.97 ± 0.04

* Mast cells were incubated for 10 min at 22° with the indicated concentrations of SH reagent. Aliquots of cells were centrifuged, washed once in BSS and assayed for ATP. To other aliquots, polymyxin B (1.4 μM) was added and histamine release measured after 10 min at 22°.

† Histamine release was determined on mast cells that were incubated in the absence of SH reagents. The secretory stimulus was polymyxin B (1.4 μM). Values are the means ± S.E. from at least three experiments with duplicate determinations in each experiment. Mean histamine release was 67.5 per cent (S.D. = 10.6).

‡ Mean ATP was 0.833 μg/10^6 mast cells (S.D. = 0.175). Values are the means ± S.E. from at least three experiments with duplicate determinations in each experiment.

concentration (10^{-6} M) we could test under standard conditions. Three other SH reagents, DTDP, DTDN and PCMBS, were examined for inhibitory effects on histamine release. The concentrations of DTDP and PCMBS required for 50 per cent inhibition of release are presented in Table 1. DTDN did not inhibit release by either A23187 or polymyxin B at 10^{-4} M, the highest concentration tested.

Agents reactive with SH groups are capable of interfering with a variety of enzymes, including some essential for ATP generation [17]. Since permissive levels of ATP are essential for histamine release by either polymyxin B [18] or A23187 [19], the possibility was examined that the inhibitory effects of the SH reagents on histamine release are attributable to a depression of cell ATP. Neither DACM nor PCMBS reduced cell ATP significantly at the highest concentrations tested (10^{-6} and 5×10^{-4} M respectively). Cyto A at a sufficiently high concentration (10^{-5} M) did depress mast cell ATP; however, it completely inhibited histamine release at concentrations which did not lower cell ATP levels (Table 3). On the basis of parallel results, the inhibition of histamine release by DTDP cannot be attributed to a reduction of mast cell ATP (Table 3).

In the case of NEM, the relationship between inhibition of mast cell ATP and of histamine release is different for the two secretagogues used in those experiments. With polymyxin B-stimulated cells, the depression of ATP cannot account for the inhibition of histamine release (Table 3), whereas for secretion elicited with A23187, inhibition of histamine release was very

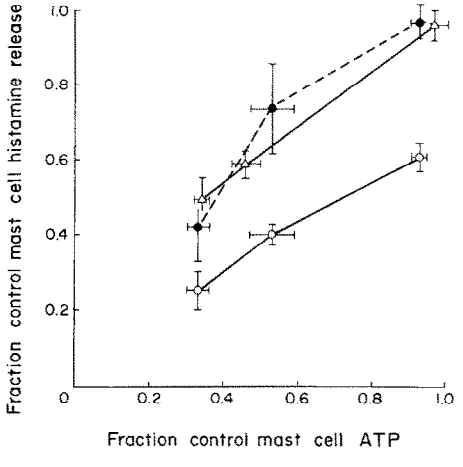

Fig. 1. Regression of histamine release on mast cell ATP levels. The experimental protocol was as indicated for Table 3. Each point represents the mean of at least three separate experiments. The arms indicate the standard error on the parallel axis. All values are expressed as a fraction of the control values obtained in the absence of inhibitor. Key: (○——○) DNP, A23187, 1 μM; (●– – –●) DNP, polymyxin B, 1.4 μM; and (△——△) NEM, A23187, 1 μM. Mean control ATP was 0.848 μg/10^6 mast cells (S.D. = 0.119). Mean histamine release in the absence of inhibitor was 44.1 per cent (S.D. = 11.1) for A23187 and 55.6 per cent (S.D. = 2.4) for polymyxin B.

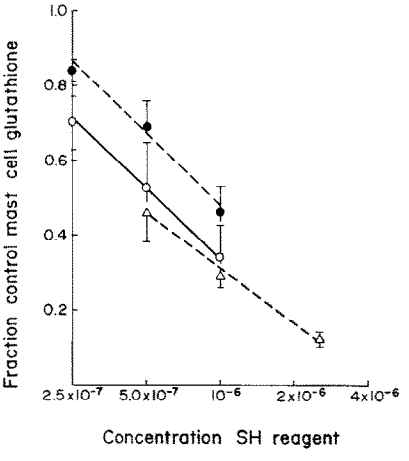

Fig. 2. Effect of sulfhydryl reagents on mast cell glutathione levels. Mast cells were exposed to the SH reagents for 10 min at 22°, washed two times, and glutathione was extracted. Key: (○——○) Cyto A; (●– – –●) DACM; and (△– – –△) NEM. Each value is the mean ± S.E. for at least three experiments with duplicate determinations in each experiment. Mean control glutathione was 168 ng/10^6 mast cells (S.D. = 26).

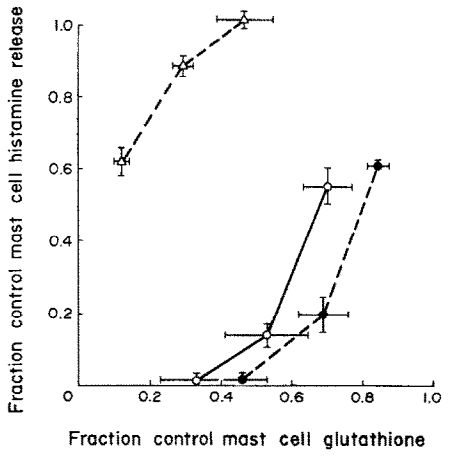

Fig. 3. Regression of histamine release on glutathione levels. Mast cells were exposed to varying concentrations of SH reagents for 10 min at 22° and aliquots were taken for glutathione determinations. To other aliquots, polymyxin B was added to a final concentration of 1.4 μM, and histamine release measured after 10 min at 22°. Key: (○——○) Cyto A; (●– – –●) DACM; and (△– – –△) NEM. Each point represents the mean for at least three experiments with duplicates assayed in each experiment. The arms indicate S.E. for the values on the parallel axis. Mean control glutathione was 168 ng/10^6 mast cells (S.D. = 26). Mean histamine release in the absence of any inhibitor was 69.4 per cent (S.D. = 6.3).

close to that observed with DNP for an equivalent reduction in ATP (Fig. 1). The difference in effectiveness of DNP in inhibiting secretion elicited by A23187 and polymyxin B (Fig. 1) suggests that DNP may influence release by A23187 by an action in addition to the depression of cell ATP.

The possibility that inhibitory SH reagents were acting by depressing the concentration of non-protein SH groups was examined by measuring glutathione levels in mast cells treated with Cyto A, NEM and DACM. Cyto A and NEM were roughly equivalent in depressing glutathione levels; DACM was about half as effective (Fig. 2). Since NEM was only $\frac{1}{10}$ as effective as Cyto A or DACM in inhibiting histamine release, the effects of the latter two agents are effectively dissociated from their effects on glutathione (Fig. 3).

DISCUSSION

Several observations are consistent with the proposal that Cyto A inhibits histamine release by virtue of its reactivity with SH groups: (1) Cyto A reacts avidly with SH groups [2–4], sluggishly with amino groups and not at all with imidazole groups [2]; (2) prior reaction of Cyto A with SH groups blocks its inhibition of histamine release; and (3) NEM and DACM, *N*-substituted maleimides with SH reactive sites resembling those of Cyto A, and DTDP, a reagent with a structure very different from that of Cyto A but with the common property of reacting avidly and specifically with SH groups [20], effectively inhibit histamine release.

The simplest explanation for the observation that Cyto A and DTDP inhibit histamine release induced by A23187 and polymyxin B equivalently is that these two SH reagents act at a step in the secretory mechanism common to the two secretagogues and presumably subsequent to an increase in cytoplasmic Ca^{2+} concentration. Unfortunately, reliable techniques to measure Ca^{2+} influx are not available to exclude the unlikely alternative that Cyto A and DTDP interfere with the increase in cell calcium induced by A23187, with dose–response relationships fortuitously parallel to those for inhibition of histamine release by polymyxin B. Depression of cell ATP as a mechanism for inhibition of histamine release by Cyto A and DTDP is excluded by our results.

While NEM is significantly less effective than Cyto A as an inhibitor of histamine release by polymyxin B, it nonetheless seems to inhibit by a mechanism independent of an effect on cell ATP. NEM inhibition of histamine release by A23187, however, may well depend entirely on its depression of mast cell ATP content. We are led by the difference in sensitivity of release by A23187 and polymyxin B to propose that NEM acts to inhibit the secretory mechanism initiated by polymyxin B at a different site than does Cyto A or DTDP; we suggest the site may be a component of the postulated mechanisms for increasing cell Ca^{2+}. DACM is a more effective inhibitor than NEM with an ID_{50} very close to that of Cyto A when the secretagogue is polymyxin B; but, as with NEM, DACM has a significantly smaller effect on release by A23187.

Cyto A and NEM possess similar second-order rate constants for their reactions with glutathione [2]; similar intracellular availability of the two reagents is indicated by the parallel reduction of glutathione levels. Yet Cyto A is 10 times more effective than NEM in inhibiting histamine release by polymyxin B and 300 times more effective in inhibiting release by A23187. The 0.2% albumin routinely included in the BSS provides potentially reactive SH groups 10 times the total number of molecules of Cyto A required for 100 per cent inhibition, and 30 per cent of intracellular glutathione remains unreacted when release is completely inhibited by Cyto A. Thus, the sensitivity of mast cell secretion to Cyto A inhibition seems best attributed to an unusually high reactivity with one or more protein SH groups critical for the secretory mechanism.

Nemeth and Douglas [7] have proposed that the Cyto A-sensitive SH groups in mast cells are on the outer surface of the cell membrane. They base this suggestion on two experimental observations: (1) glutathione, which does not enter normal cells readily, prevents inhibition by Cyto A; and (2) PCMBS, a SH reagent, which permeates cells poorly, inhibits 48/80-induced secretion. The first observation of Nemeth and Douglas does not specify an extracellular locus of action since it can be explained by the rapid and irreversible reaction of glutathione with Cyto A before Cyto A enters the cell. As for the second argument, we find that, while PCMBS does inhibit histamine release by polymyxin B, it does not inhibit release by A23187. We propose that there are two loci of SH groups essential for secretion: one, in agreement with Nemeth and Douglas, is on the cell surface and is required for secretion elicited by polymyxin B or 48/80 but not by A23187; the other is inaccessible to PCMBS yet inhibitable by Cyto A and required for release stimulated by both polymyxin B and A23187. This second site may well be intracellular.

Comparison of the effects of the several inhibitors on A23187 and polymyxin B-induced release that we have tested leads us to assign the action of Cyto A and DTDP to the second site and that of PCMBS, DACM and low concentrations of NEM to the cell surface site. Any possible specific effect of NEM at higher concentrations on the postulated second site is obscured by depression of cell ATP.

The results of Orange [21] from studies of the effect of Cyto A on histamine release from human lung fragments by antigen or anti-IgE are quite different from our results and those of Nemeth and Douglas [7] with rat mast cells. Orange found that Cyto A inhibited SRS-A release but stimulated histamine release if the secretory stimulus was anti-IgE. When release was initiated by antigen, Cyto A at low doses enhanced the release of both substances, but at high concentrations SRS-A release was inhibited and histamine release augmented [21].

Cyto A, DACM and DTDP seem to us to be of more than passing interest as inhibitors of rat mast cell secretion because of the likelihood of selective action on a restricted range of cell SH groups; neither GSH nor proteins involved in ATP generation are as reactive. Such selectivity offers the possibility of using these or related compounds as markers for protein(s) essential for secretion. The potential usefulness of Cyto A as a therapeutic agent is clouded by the observations of Orange [21] with human tissue but probably warrants further study, in view of the finding of Magro [22] that ethacrynic acid, which, like Cyto A, NEM and DACM, has a ketone-activated unsaturated bond highly reactive with SH groups, is an effective inhibitor of histamine release from basophils.

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