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EFFECTS OF CHYMOTRYPSIN AND TRYPSIN ON RAT PERITONEAL MAST CELLS*

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Abstract—Alpha-chymotrypsin was demonstrated to release histamine from rat peritoneal mast cells. Release of histamine was shown by electron microscopy not to be cytotoxic. Release was temperature dependent, required Ca^{2+} and cell ATP for optimal effect, and involved the direct action of α-chymotrypsin on the mast cells. Trypsin was far less active in releasing histamine, but exposure of the cells to trypsin inhibited the subsequent release of histamine by chymotrypsin. The relationship between the action of exogenous α-chymotrypsin and an endogenous "activatable" esterase could not be established from this set of experiments because the release of histamine by polymyxin B was only slightly inhibitable by the esterase inhibitor, diisopropylfluorophosphate, and, therefore, did not apparently require the participation of the endogenous esterase.

The large store of chymotrypsin-like enzyme within the mast cells [1, 2] led to the hypothesis that this enzyme was involved in the secretion of histamine. The hypothesis was disproved by the evidence that virtual complete inhibition of the intracellular enzyme with diisopropylfluorophosphate (DFP) did not interfere with histamine release [3, 4]. Subsequently, Becker and Austen [5] provided evidence that activation of an enzyme precursor was necessary for normal mast cell secretory activity. Since this enzyme resembled α-chymotrypsin in its reactivity with two series of phosphonate inhibitors [6, 7], it seemed reasonable to examine the mechanism of action of exogenous chymotrypsin as a possible model for the action of an intrinsic esterase in the secretory mechanism.

Although the release of histamine from mast cells by α-chymotrypsin was reported from this laboratory in an abstract in 1959 [8], difficulties in establishing the reproducibility of the effect *in vitro* prevented us from definitive publication at that time. Independently, Uvnäs *et al.* [9–11] had undertaken a study of the effects of a wide range of enzymes on mast cell degranulation and found that α-chymotrypsin had substantial activity. Further studies of the effect of α-chymotrypsin on histamine release or degranulation have since been reported by Keller [12], Saeki [13], Rothschild *et al.* [14] and Bach *et al.* [15]. In several of these studies, the effect of trypsin on mast cells has also been examined [12, 14, 15]. Improved methods have allowed us to extend our studies of the action of α-chymotrypsin and trypsin on rat peritoneal mast cells.

MATERIALS AND METHODS

Peritoneal mast cells were obtained from adult male CD rats purchased directly from Charles River Inc. or bred in our laboratory from CD stock. Procedures for harvesting the cells, separating mast cells from other peritoneal cell types by centrifugation through concentrated albumin and measuring histamine release using the *o*-phthalaldehyde assay were identical to those previously described [16]. Balanced salt solution (BSS) used as suspending media for the mast cells consisted of NaCl, 0·90; KCl, 0·02; $CaCl_2$–$2H_2O$, 0·01; Na_2H–PO_4, 0·057; and KH_2PO_4, 0·037 g/100 ml, pH 7·2. The techniques utilized for electron microscopy with 2% glutaraldehyde fixation and Epon embedding are also those that have been used and described before [16]. ATP and polymyxin B sulfate were purchased from Sigma Chemical Corp. and 48/80 from Burroughs/Wellcome Ltd. Alpha-chymotrypsin, the diisopropyl phosphoryl derivative of α-chymotrypsin (DP-chymotrypsin), trypsin, TPCK-trypsin [a preparation of trypsin in which the chymotrypsin contaminant is inhibited by L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone] and DP-trypsin were purchased from Worthington Biochemical Corp. Diisopropylphosphofluoridate (DFP) was purchased from Aldrich Chemical Corp. and diluted immediately before use to 1 M in methanol, prior to a final dilution in balanced salt solution. DFP was periodically tested for its activity in inhibiting α-chymotrypsin. Alpha-chymotrypsin and trypsin were assayed essentially as described in the Worthington Manual [17], using a Gilford recording spectrophotometer. Dinitrophenol (DNP) was prepared as a 0·1 M stock solution in ethanol which was diluted directly in BSS to give the desired final concentration. Control cells were exposed to the same concentration of ethanol. Ethanol concentrations, 1% v/v or below, had no effect on histamine release. ATP levels in isolated mast cells were measured by the method of Holmsen *et al.* [18].

RESULTS

Highly purified bovine α-chymotrypsin released histamine from peritoneal mast cells. The concentration dependence for release was little different when measured with mixed peritoneal cells of which mast cells

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constitute about 2 per cent or with mast cells purified to 90 per cent or better by centrifugation through albumin (Fig. 1). Histamine release induced by chymotrypsin was temperature dependent. In five experiments, at 27°, 200 μg/ml of α-chymotrypsin released 53·7 ± 2·9 per cent of cell histamine in 5 min compared with 5·8 ± 1·1 per cent at 9°. The comparable values for 2 μg/ml of polymyxin B were 79·4 ± 6·9 and 1·9 ± 0·3 per cent. Dinitrophenol at a concentration of 10^{-3} M depleted mast cell ATP from a mean of 1·07 ± 0·04 μg ATP/10^6 cells to 0·07 ± 0·03 in three experiments. This depletion was partially reversible, since washing the cells with 0·55 M glucose in BSS or BSS alone restored the ATP levels to 0·51 ± 0·01 and 0·50 ± 0·02 respectively. DNP under these conditions reversibly inhibited histamine secretion (Table 1). Exclusion of Ca^{2+} from the medium had an inhibitory effect (Table 2). DP-chymotrypsin neither released histamine from mast cells nor affected the release by active chymotrypsin (Table 3).

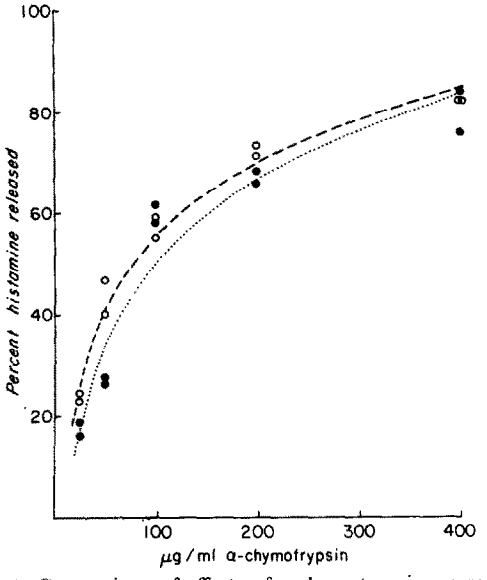

Fig. 1. Comparison of effects of α-chymotrypsin on mast cell histamine in mixed peritoneal cells (O——O) and isolated mast cells (●·····●). Cells were incubated at 37° for 5 min. Mixed peritoneal cells and isolated mast cells were obtained from the same pool of peritoneal cells. Per cent histamine released was corrected for spontaneous release from cells incubated in BSS. The drawn lines were calculated from linear regression analysis of normal-log plots of the data.

The ultrastructural appearance of mast cells treated with chymotrypsin closely resembled that of cells treated with polymyxin B and other non-cytotoxic histamine-releasing agents [19–23]. The granules were extruded into the extracellular space, but the mast cell cytoplasm and nucleus were not apparently affected by the process (Figs. 2 and 3).

The closely related proteolytic serine esterase, trypsin, was tested for its effect on mast cells. Trypsin caused clumping of mixed peritoneal cells and some release of mast cell histamine which was extremely variable (Table 4). When cells were treated with trypsin and then washed free of the enzyme, the release of histamine by chymotrypsin was inhibited (Tables 5 and 6). TPCK-trypsin, in which trypsin activity is intact but residual chymotrypsin activity is abolished, was as effective as trypsin in inhibiting the release of histamine caused by chymotrypsin; trypsin completely inactivated with DFP (DP-trypsin) was significantly less inhibitory (Table 6). Mast cells treated with trypsin showed little or no change in their secretory response to polymyxin B (Table 5).

Because of the variation in the effects previously reported [4, 5, 24, 25], the action of DFP in inhibiting histamine release was re-examined using polymyxin B as the secretory stimulus. Peritoneal cells were incubated for 15 min with 1·0 mM DFP; polymyxin B, 2 μg/ml, was added and the cells were incubated at 21° for 15 min. In five experiments, the mean inhibition by DFP was 17·3 ± 4·3 per cent.

Table 1. Reversible inhibition of histamine release by dinitrophenol*

α-Chymotrypsin concn (μg/ml)	Control	Dinitrophenol (10^{-3} M)	Dinitrophenol ↓ wash ↓ glucose 0·055 M	Dinitrophenol ↓ wash ↓ no glucose
	Per cent release of histamine			
200	93·1	1·8	73·7	
200	84·4	3·3	43·2	28·9
200	66·9	0·0	52·4	43·8
200	58·1	1·0		
500	78·0	1·6		
1000	88·4	3·8		
1000	60·2	2·1		
Mean ± S.E.	75·6 ± 5·3	1·9 ± 0·5	56·4 ± 9·0	36·4

*Cells were preincubated in dinitrophenol 10^{-3} M for 10 min at 21° or 27° and the α-chymotrypsin was added to the cell suspension, or else the cells were washed twice in balanced salt solution at 0–4° with or without glucose 0·055 M and resuspended in the same solution to which α-chymotrypsin was added. Incubation with chymotrypsin was at 21° or 27° for 30 min. Values for per cent histamine released were corrected for spontaneous release of histamine in control samples not exposed to α-chymotrypsin. A modest increase of spontaneous histamine release was observed in cells exposed to 10^{-3} M DNP. This rarely exceeded 15 per cent of total histamine.

Table 2. Effect of Ca^{2+} on histamine release by chymotrypsin*

Preincubation and incubation medium	No preincubation		Preincubation	
	Chymo + Ca^{2+}	Chymo − Ca^{2+}	Chymo + Ca^{2+}	Chymo − Ca^{2+}
	Per cent histamine released			
Ca^{2+} free + EDTA	85·6	23·2	22·4	9·3
Ca^{2+} free + EGTA	65·8	26·0	47·7	28·2
Ca^{2+} free	44·0	25·6	28·7	5·8

* Peritoneal cells were initially collected and washed in balanced salt solution lacking Ca^{2+} or lacking Ca^{2+} with 10^{-3} M EDTA or 10^{-3} M EGTA as indicated. Cells were preincubated in Ca^{2+}-free medium. In each experiment, aliquots were incubated with chymotrypsin for 5 min at 21° in the absence of Ca^{2+} (− Ca^{2+}) or in the presence of 0·7 mM Ca^{2+} (+ Ca^{2+}) for 5 min at 21°. Values for per cent histamine released were corrected for spontaneous release from cells not treated with chymotrypsin. Values are means of two determinations in a single experiment.

DISCUSSION

Proteases acting on intact cells have been shown to affect a variety of functions including cell adhesion [26, 27], pinocytosis [28], phagocytosis [29, 30], hormone effects on cell metabolism [31, 32], growth characteristics of cells in culture [33] and agglutinability of cells by lectins [34]. The working assumption is that the proteases are acting on cell membrane components. Direct assessment of the effects of proteolytic enzymes on components of the cell membrane in the case of intact red blood cells or right-side-out red blood cell ghosts indicates that a limited number of membrane proteins are accessible to attack [35, 36]. In the case of platelets, of seven surface proteins accessible to iodination by lactoperoxidase, only three are hydrolyzed by trypsin [37].

Results obtained in the present study confirm previous reports [1, 9–15] that bovine-α-chymotrypsin causes mast cell secretion. Our results confirm those of Uvnäs and Antonsson [11] and Saeki [13], indicating that maximal secretion induced by exogenous chymotrypsin requires permissive levels of intracellular ATP and exogenous Ca^{2+}. Our ultrastructural studies support the contention that the secretory process stimulated by chymotrypsin is not cytotoxic; the morphologically evident events are entirely comparable to those observed with a number of other stimuli. Since release by chymotrypsin of histamine from mast cells largely separated from other peritoneal cells is the same as the release from mixed peritoneal cells (Fig. 1), the possibility that chymotrypsin acts only indirectly on the mast cell is eliminated.

The failure of DP-chymotrypsin to release histamine from mast cells establishes that the action of α-chymotrypsin depends on its enzyme activity rather than on a non-enzymatic action, such as that manifest by polyamines such as 48/80 or polymyxin B. Further evidence that the interaction of chymotrypsin with the cell involves the enzyme's active site is provided by the negative results of attempted competition experiments between chymotrypsin and DP-chymotrypsin. If a cell–enzyme complex that did not require the proteolytically active site of the enzyme were an essential step in the reaction sequence, a large excess of DP-chymotrypsin would be expected to block chymotrypsin-induced histamine release by competitive binding to the cell.

Both Keller [12] and Bach *et al.* [15] have reported little or no release of histamine with trypsin treatment, while Rothschild *et al.* [14] found that trypsin released significant amounts of histamine. A considerable, unexplained variation in the release of histamine by trypsin occurred in our experiments, and this variability may be the basis for the discrepancy among

Table 3. Effect of DP-chymotrypsin on histamine release from mast cells*

Experiment	Treatment	Per cent histamine released
A	Chymotrypsin (100 μg/ml)	81·0
	DP-chymotrypsin (500 μg/ml)	4·9
	DP-chymotrypsin (500 μg/ml) + chymotrypsin (100 μg/ml)	75·5
B	Chymotrypsin (25 μg/ml)	35·3
	DP-chymotrypsin (25 μg/ml)	0·9
	DP-chymotrypsin (100 μg/ml)	1·4
C	Chymotrypsin (5 μg/ml)	26·8
	Chymotrypsin (200 μg/ml)	93·0
	DP-chymotrypsin (5 μg/ml)	0·0
	DP-chymotrypsin (200 μg/ml)	3·7

* Cells were incubated with chymotrypsin, DP-chymotrypsin or both for 10 min at 21°. The per cent of histamine released was corrected for spontaneous release. Values are the means of two determinations in a single experiment.

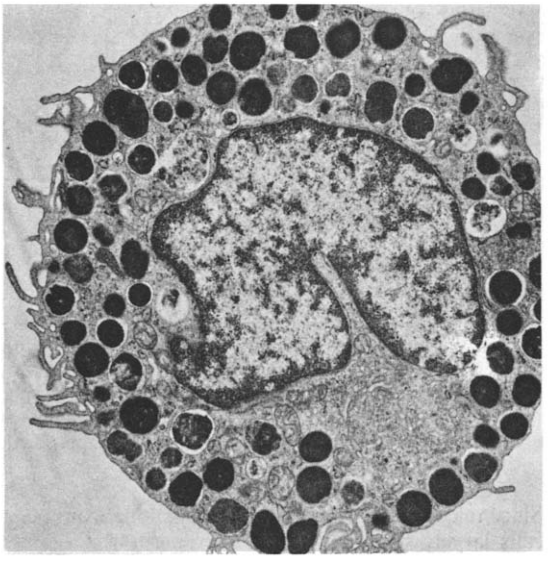

Fig. 2. A rat mast cell treated with α-chymotrypsin, 200 μg/ml, at 9° for 5 min. This cell is indistinguishable from an untreated cell. There is no evidence of granule secretion. A well developed Golgi apparatus and typical microvilli are evident. Glutaraldehyde-fixed. Uranyl acetate, lead hydroxide stained (12,500).

the several previous reports. Interestingly, Rothschild *et al.* [14] provided evidence that the release of histamine by trypsin, unlike that caused by chymotrypsin, was not inhibited by an enzyme substrate and that DP-trypsin was as active as unmodified trypsin.

While trypsin in our experiment had only a limited ability to release histamine, it was very effective in preventing histamine release by chymotrypsin. Although some inhibitory effect remained in the enzymatically inactive DP-trypsin, the inhibitory effect of trypsin required active enzyme for complete expression and did not depend on contamination with small amounts of chymotrypsin.

The common requirement for cell ATP [24, 25, 38, 39] and Ca^{2+} [40, 41] and the similar ultrastructural correlates of secretion when rat mast cells are stimulated by a variety of non-cytotoxic secretagogues, including 48/80, polymyxin B and chymotrypsin, make it likely that there are terminal steps in the secretory mechanism common to these agents. Since histamine secretion induced by chymotrypsin but not by 48/80 or polymyxin B is inhibited by treatment of the cells with trypsin, it appears that chymotrypsin invokes the participation of one or more steps not involved in the process stimulated by the polyamines. The experimental results leave open the question of the relationship between the action of the "activatable" esterase and exogenous α-chymotrypsin, because the secretion of histamine induced both by polymyxin B and 48/80 [4] is not substantially inhibited by high concentrations of DFP. DFP has been reported to inhibit effectively rat peritoneal mast cell secretion induced by rabbit neutrophil cationic protein [24] and rabbit anti-rat Fab [25], so that the effect of trypsin pretreatment on the release of histamine by these latter two agents would be expected

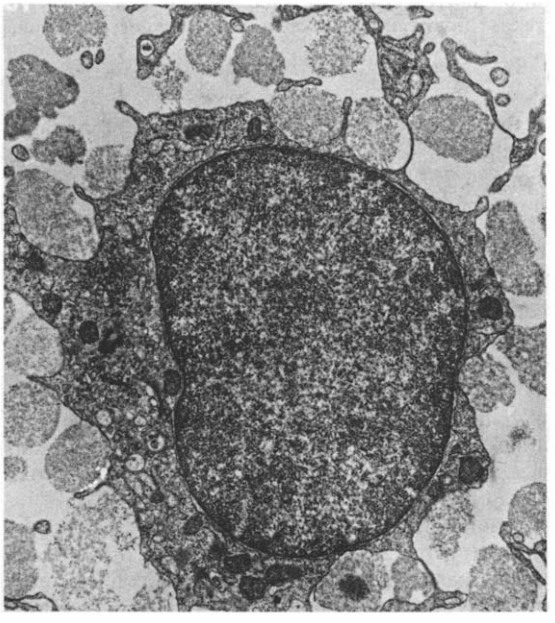

Fig. 3. A rat mast cell treated with α-chymotrypsin, 200 μg/ml, at 27° for 10 min. This cell exhibits ultrastructural features characteristic of mast cell secretion induced by other non-cytotoxic agents *in vitro*: the formation of channels into the cell harboring modified granules, the persistence of a number of unaffected granules near the nucleus, and intact intergranular cytoplasm. A centriole, Golgi apparatus, mitochondria, various sized vesicles, a small amount of rough endoplasmic reticulum and the nucleus show no abnormalities. Glutaraldehyde-fixed. Uranyl acetate, lead hydroxide stained (8500).

Table 4. Effect of trypsin on the release of histamine from mast cells*

Trypsin concn 25 μg/ml	50 μg/ml	100 μg/ml	Mean
Per cent histamine released			
20·4	23·9	32·3	25·3 ± 3·50
3·7	5·1	4·5	4·4 ± 0·40
0·4	0·4	0·5	0·4 ± 0·03
12·0	12·2	11·9	12·0 ± 0·09
9·1 ± 4·5†	10·4 ± 5·1†	12·3 ± 7·1†	

* Cells were incubated with the enzyme for 15 min at 20°. Per cent histamine released was corrected for spontaneous release. Mean and S. E. were calculated from the results of four experiments.

† Mean ± S.E.

Table 5. Effect of treatment of cells with trypsin on release of histamine by polymyxin B and chymotrypsin*

Chymotrypsin			Polymyxin B			48/80		
	Trypsin			Trypsin			Trypsin	
Control % Release	% Release	% Inhibition	Control % Release	% Release	% Inhibition	Control % Release	% Release	% Inhibition
58·0	2·7	95·3	70·6	63·4	10·2			
65·6	1·1	98·3	32·4	25·9	20·1			
47·5	1·6	96·6						
78·3	11·7	85·1	62·4	61·9	0·8	51·2	54·0	0·0
54·0	2·8	94·8	58·1	46·6	19·8	48·0	42·1	12·3
Mean ± S. E.		94·0 ± 2·3	Mean ± S. E.		12·7 ± 4·6	Mean		6·2

* Cells were treated with trypsin, 50 μg/ml, at 20° for 15 min, washed in BSS, resuspended in BSS containing polymyxin B or α-chymotrypsin and incubated for 5 min at 27°. Per cent release was calculated on the basis of total histamine present after the wash and corrected for spontaneous release.

Table 6. Effect of trypsin on the release of histamine by chymotrypsin*

BSS-control	Trypsin		DP-trypsin		TPCK-trypsin	
% Release	% Release	% Inhibition	% Release	% Inhibition	% Release	% Inhibition
72·1	3·2	95·6	35·9	50·2		
79·6	4·4	94·5	44·4	44·2		
64·9	2·4	96·3	32·0	50·7		
80·2	1·2	98·5	32·9	59·0	3·6	95·5
31·5	5·0	84·5			3·8	87·9
	Mean ± S. E.	93·8 ± 2·5	Mean ± S. E.	51·0 ± 3·0	Mean	91·7

* Cells were exposed to 0·05 mg/ml or 0·1 mg/ml trypsin, DP-trypsin or TPCK-trypsin in BSS for 15 min at 20°, washed and treated with 0·2 mg/ml of chymotrypsin for 5 min at 37°. Values for per cent histamine released at 37° were corrected for spontaneous release of histamine during the 5-min period at 37°. No release in excess of spontaneous release occurred in the preincubation with trypsin, DP-trypsin or TPCK-trypsin in these experiments.

to provide additional evidence needed for defining the relationship between the action of a possible endogenous esterase and exogenous α-chymotrypsin.

Whatever the case, chymotrypsin and trypsin are both highly purified enzymes with well-characterized esterolytic and proteolytic specificities. If the proximate initiator of secretion stimulated by chymotrypsin is either an enzymatically modified cell component or a stable complex between α-chymotrypsin and a cell surface molecule, the identification of the modified membrane component seems a feasible next step. Similarly, the molecular site of the inhibitory effect of trypsin should also be identifiable.

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