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TRIGGERING ACTION OF PHOSPHATIDASE A AND CHYMOTRYPSINS ON DEGRANULATION OF RAT MESENTERY MAST CELLS

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Abstract—The degranulating action of phosphatidase A and chymotrypsins was studied on rat mesentery mast cells *in situ*. In spite of the hydrolytic character of the two enzymes, their degranulating action was blocked by several inhibitors, among them metabolic inhibitors. Also anoxia blocked their degranulating action. Glucose had a preventive action on this block. The conclusion is drawn that the two enzymes only trigger the degranulation process, as do polymer histamine releasers such as compound 48/80, antigens, extracts from *Ascaris* and *Cyanea*, etc. The possible rôle of the two enzymes as triggering enzymes in the degranulation process is discussed.

PHOSPHATIDASE A has been reported by Högberg and Uvnäs[1] to degranulate rat mesentery mast cells. Since then the enzyme has been shown to release histamine and serotonin from isolated rat mast cells.[2] Recently another enzyme, chymotrypsin, has been observed to attack isolated mast cells, producing morphological changes and histamine release.[2]

The present article describes the degranulating action of phosphatidase A and chymotrypsin and the influence of enzyme inhibitors, anoxia and glucose on the degranulation. The observations show that the two enzymes do not act merely by hydrolysis of their substrates, thereby increasing the permeability of mast cell membranes. They seem to act by triggering an energy-requiring process, similar to or identical with that initiated by compound 48/80 and antigens.

METHODS

Observations on rat mesentery mast cells were performed as described by Högberg and Uvnäs.[4] The incubation medium (pH 7·4) contained $1{\cdot}54 \times 10^{-1}$ M NaCl, $2{\cdot}68 \times 10^{-3}$ M KCl, 9×10^{-4} M $CaCl_2$ (anhydrous) and 10% Sörensen phosphate buffer ($6{\cdot}7 \times 10^{-2}$ M Na_2HPO_4, $2H_2O$ + $6{\cdot}7 \times 10^{-2}$ M KH_2PO_4). When inhibitors were investigated the mesentery was preincubated for 15 min together with the inhibitor before the degranulating substance was added.

Phosphatidase A was prepared from bee venom.[5] Chymotrypsin was supplied by California Corporation for Biochemical Research, Los Angeles, Cal.: α-chymotrypsin (pancreas) B-grade, activity 9100 ATEE units/mg; β-chymotrypsin B-grade, 2 × crystallized; γ-chymotrypsin (bovine pancreas), B-grade, activity > 6000 ATEE units/mg; α-chymotrypsin (bovine pancreas) crystallized A-grade, activity 7·8 units/mg.

When not otherwise stated, the enzymes and other degranulating agents were used in submaximal doses (calculated from dose response curves to give 80–90% degranulation).

RESULTS

Dose-response curves

The dose-response curves of the different enzyme preparations used appear from Fig. 1. The phosphatidase A preparation showed a considerably higher degranulating power than the chymotrypsin preparations, even the purest ones. Parallel determination of chymotrypsin action on n-acetyl-L-tyrosine ethylester at pH 7.9 in accordance with Schwert, Neurath, Kaufman and Snoke,[6] and on mast cells showed a good correlation between the peptide splitting and the degranulating effects of the four different chymotrypsin preparations.

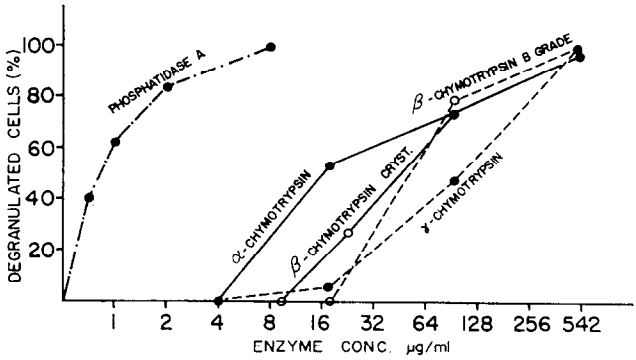

FIG. 1. Degranulating power of phosphatidase A and chymotrypsins

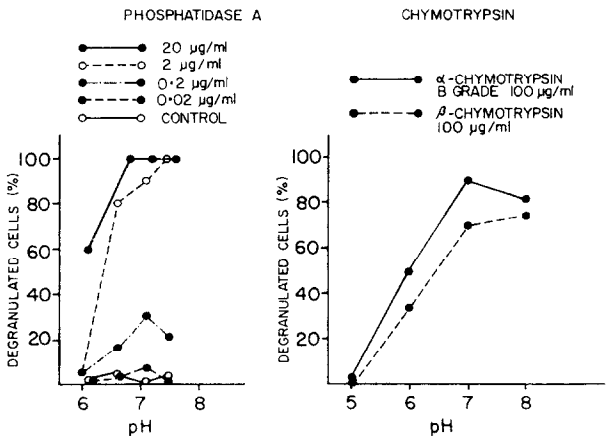

FIG. 2. Influence of pH on degranulating action of phosphatidase A and chymotrypsins

Influence of pH and temperature

Below pH 7 the degranulating action of the enzymes declined rapidly with increasing acidity (Fig. 2). In accordance with the known heat stability of phosphatidase A, its degranulating action withstood 2 hr boiling at pH 5. In contrast the chymotrypsins used were rapidly inactivated by heat, as was their mast cell degranulating power, which was extinguished by heating to the boiling point at the same or neutral pH.

Prewarming of the mast cells at 45°C for 5 min before their exposure to the enzymes at 37°C made the cells insensitive to the degranulating action of the enzymes.

In a previous paper[4] it was stated that the degranulating action of phosphatidase A remained even after heating the mast cells to 50 and 60°. However, in those experiments the mast cells were heated to these temperatures in the presence of phosphatidase A.

Influence of Ca^{2+} and Mg^{2+} ions

Both enzymes required Ca^{2+} ions for their degranulating action (Fig. 3), at least under aerobic conditions, while degranulation seemed to proceed freely in the absence

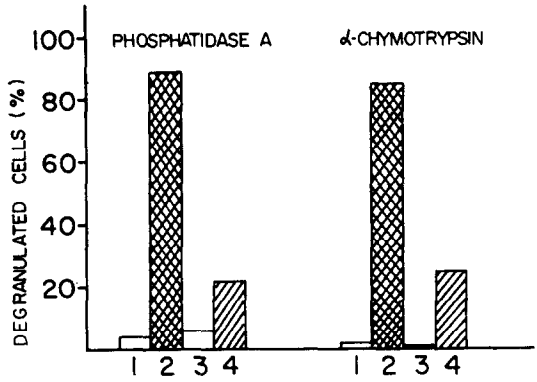

FIG. 3. Influence of calcium lack on degranulating action of phosphatidase A and α-chymotrypsin

(left) 1. Control. No phosphatidase A; with $CaCl_2$ 9×10^{-4} M
2. Phosphatidase A 2 μg/ml; with $CaCl_2$ 9×10^{-4} M
3. Control. No phosphatidase A; no $CaCl_2$
4. Phosphatidase A 2 μg/ml, no $CaCl_2$

(right) 1. Control. No α-chymotrypsin; with $CaCl_2$ 9×10^{-4} M
2. α-chymotrypsin 100 μg/ml; with $CaCl_2$ 9×10^{-4} M
3. Control. No α-chymotrypsin; no $CaCl_2$
4. α-chymotrypsin 100 μg/ml; no $CaCl_2$

of Mg^{2+} ions in the incubation fluid. An influence from Mg^{2+} ions on the degranulating action of chymotrypsin appeared, however, under anaerobic conditions, the depressing action of anoxia being considerably counteracted by the presence of such ions (Fig. 4). No such influence from Mg^{2+} ions was seen under anoxic conditions, when phosphatidase A or compound 48/80 were used as degranulating agents.

Influence of enzyme inhibitors

In Table 1 are listed a number of substances observed to inhibit the degranulating action of the two enzymes. Among the inhibitors were metal ions, agents supposed to block NH_2 and SH groups and substances known to interfere with oxydative energy generating processes.

DFP (diisopropylfluorophosphate) and Mipafox (bismonoisopropylamidophosphorylfluoride) which block some esterases, had no inhibitory effect in concentrations up to 10^{-4} M. Phenol and skatole had some inhibitory action, while indole had none in

concentrations below 10^{-2} M. Several chymotrypsin substrates were tried as competitive inhibitors. Unfortunately most of them had a degranulating action of their own, making an evaluation of their inhibitory abilities impossible. Only two substances—n-acetyl-L-tyrosine ethylester and n-acetyl-L-tryptophane ethylester—showed a possible inhibitory action. This inhibitory action applied not only to chymotrypsin but also to phosphatidase A and compound 48/80.

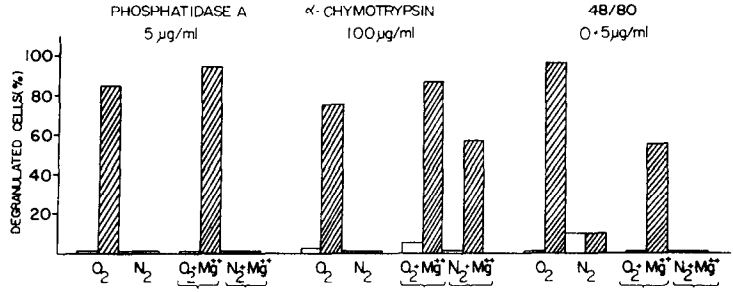

FIG. 4. Preventive action of Mg^{2+} ions on inhibitory effect of anoxia on degranulation caused by α-chymotrypsin. No preventive action of Mg^{2+} ions on degranulation caused by compound 48/80 and phosphatidase A

Control. No releaser. With releaser

TABLE 1. ACTION OF ENZYME INHIBITORS ON DEGRANULATION DUE TO PHOSPHATIDASE A, CHYMOTRYPSIN AND COMPOUND 48/80.

Inhibitor	Molar concentrations of inhibitors exerting 50 per cent inhibition on degranulation caused by				
	Phosphatidase	Chymotrypsin	48/80	Remarks	
$PbNO_3$	10^{-6}–10^{-5}		10^{-6}–10^{-5}		
$PbCl_2$		10^{-5}–10^{-4}			
$ZnCl_2$	10^{-5}	10^{-5}	10^{-5}		
Allicin	10^{-4}–10^{-3}	10^{-5}–10^{-4}	10^{-4}–10^{-3}		
Sodium arsenite	10^{-5}–10^{-4}	10^{-4}	10^{-4}–10^{-3}		
Sodium azide	10^{-3}–10^{-2}	10^{-4}–10^{-3}	10^{-4}–10^{-3}		
Dinitrophenol	10^{-5}–10^{-4}	10^{-5}–10^{-4}	10^{-5}–10^{-4}		
Dinitrophenol + glucose	$>10^{-3}$	10^{-3}	$>10^{-3}$		
Sodium cyanide	10^{-4}–10^{-3}	10^{-3}	10^{-4}–10^{-3}		
Diisopropyl-fluorophosphate (DFP) in propyleneglycol 1%	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$		
Mipafox	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$		
Phenol	10^{-3}–10^{-2}	10^{-3}–10^{-2}	$>10^{-2}$		
Indole	$>10^{-2}$	$>10^{-2}$	$>10^{-2}$		
Skatole	10^{-3}	10^{-4}–10^{-3}	10^{-3}		
L-tyrosine ethyl ester				Degranulation	10^{-4}–10^{-3}
n-acetyl-L-phenyl alanine ethyl ester				Degranulation	10^{-6}–10^{-5}
L-phenyl alanine ethyl ester				Degranulation	10^{-5}–10^{-4}
L-phenyl alanine methyl ester				Degranulation	10^{-5}–10^{-4}
L-tryptophan ethyl ester		$>10^{-4}$		Degranulation	10^{-4}–10^{-3}
n-acetyl-L-tyrosine ethyl ester	10^{-3}	10^{-4}–10^{-3}	5×10^{-3}	Degranulation	$>10^{-3}$
n-acetyl-L-tryptophan ethyl ester	10^{-4}–10^{-3}	10^{-4}–10^{-3}	10^{-4}	Degranulation	$>10^{-5}$–10^{-4}
L-leucine ethyl ester	$>10^{-3}$	$>10^{-3}$		Degranulation	$>10^{-3}$

Influence of anoxia

Preincubation of the mast cells under anaerobic conditions (N_2-gas) made the cells unresponsive to the degranulating action of the enzymes (Fig. 4). As to chymotrypsin, the presence of Mg^{2+} ions counteracted the depressive action of anoxia, but as mentioned above, these ions had no preventive action when phosphatidase A or compound 48/80 were used as degranulating agents. On the other hand, when glucose was

TABLE 2. INHIBITORY ACTION OF ANOXIA ON DEGRANULATION DUE TO COMPOUND 48/80, ANTIGEN (ALBUMIN), PHOSPHATIDASE A, AND CHYMOTRYPSIN. PREVENTIVE ACTION OF GLUCOSE (1000 μg/ml)

Degranulating agent	Degranulated cells (percent) Preincubation in air 15 min		Preincubation in N_2 15 min		Preincubation in N_2 30 min	
	No glucose	Glucose	No glucose	Glucose	No glucose	Glucose
Phosphatidase A	83	91	4	85	4	87
Chymotrypsin	84	—	0	82	8	74
Compound 48/80*	81	—	0	68	3	79
Albumin	64	79	7	46	0	45

* From Diamant and Uvnäs 1961[15]

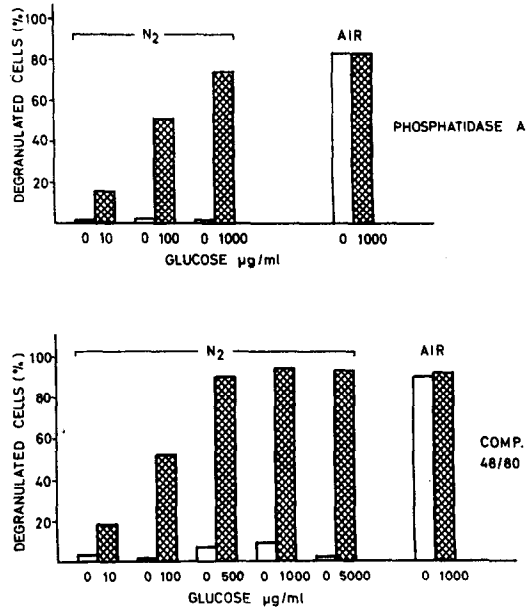

FIG. 5. Preventive action of glucose on inhibitory effect of anoxia on degranulation caused by phosphatidase A (2 μg/ml) and compound 48/80 (0·5 μg/ml)
□ Releaser without glucose. ▨ Releaser in presence of glucose

present in the incubation fluid anoxia was unable to depress the reactivity of the mast cells to any of the degranulators presented in Table 2—chymotrypsin, phosphatidase A, compound 48/80 and antigen (albumin). The concentrations of glucose observed to protect the mast cells appear from Fig. 5.

DISCUSSION

Phosphatidase A and chymotrypsin are hydrolytic enzymes with quite different points of attack. Phosphatidase A splits off an unsaturated fatty acid from phospholipids such as lecithin, forming a lysocompound. Chymotrypsin attacks peptide bonds, specially aromatic bonds, as well as esters and amides of such amino acids. Ca^{2+} ions activate both enzymes. Heavy metal ions are reported to inhibit them. However, a glance at Table 1 shows that many other inhibitors, including those interfering with metabolic processes, block the degranulating action of the two enzymes. The hydrolytic action of the enzymes is independent of anoxia, and yet they do lose their degranulating power in the absence of oxygen, provided that glucose is not present. These observations clearly indicate that the two enzymes, phosphatidase A and chymotrypsin, by their hydrolytic action merely trigger the degranulation process, which later in its course depends on energy-yielding processes. Some step in these processes is very sensitive to heat, since the degranulation process is prevented by warming the mast cell up to as little as 45°.

The degranulating action of compound 48/80, agents from *Cyanea* and *Ascaris* and antigens have been reported to be influenced by the same factors as have been found to be instrumental in this paper, e.g. calcium ions, pH, temperature, enzyme inhibitors, oxygen, glucose, etc.[7] It therefore seems reasonable to assume that the degranulation process evoked by these agents is essentially the same as that initiated by phosphatidase A and chymotrypsin. The question then arises whether or not these two enzymes may be involved in the initial steps of the degranulation of mast cells induced by the polymer agents mentioned. Both types of enzymes seem to be present in rat mast cells.

As is evident from observations with the Cartesian diver technique, isolated mast cells contain a phosphatide-splitting factor reminiscent of phosphatidase A.[7] The localization in the cell of this enzyme has not been established. The isolation procedure may damage the cells sufficiently to permit the escape of small amounts of enzyme-bearing material. However, even mast cells taken directly from the peritoneal wash fluid—without centrifugation—showed lecithin-splitting activity, whereas the fluid itself was devoid of such activity. Probably therefore the enzymatic activity is localized to the surface of the mast cells.

Activation of phosphatidase A should lead to the appearance of unsaturated fatty acid(s). On histamine release from tissue as well as from a mast cell suspension, there occur spasmogenic lipids suggestive of unsaturated fatty acids. There is thus indirect evidence that agrees with a phosphatidase A activity during the degranulation process.

A chymotrypsin-like enzyme is also reported to be present in mast cells.[8] The enzyme is stated to be localized to the granules.[9] If so, it seems difficult to allow to it a role as trigger of the degranulation process, since to fulfil this function it ought to be situated in the mast cell membrane. However, the triggering action of chymotrypsin observed in the present experiments has to be accounted for. A highly speculative explanation is as follows. It has been suggested on the basis of phosphorylation and acetylation procedures[1] that the triggering enzyme on the mast cell surface requires—as does phosphatidase A—free NH_2 groups for its activity. According to the working hypothesis of these authors, the membrane enzyme is unable in the normal mast cell to attack the membrane phospholipids since its NH_2 groups are masked by an acid inhibitor. Chymotrypsin is able to unmask NH_2 groups by breaking peptide or amide

bonds. Whether this mechanism of action of chymotrypsin on mast cells holds true remains to be proven.

An increased protease activity has been reported by many authors as occurring concomitantly with histamine release both when antigen and compound 48/80 have been used as releasers.[10] The origin of such protease activity is not known but part of it may emanate from mast cells, since these cells have been observed by histochemical techniques to harbour several such enzymatic activities (trypsin-like enzyme,[11] leucine aminopeptidase,[12] fibrinolysin[13]). The cellular localization of these enzymes is unknown but some of them may occur on the cell surface. Since proteases are stated to be activated by polymers, one could conceive of polymer releasers as inducing protease activity on the mast cell surface, thereby unmasking blocked NH_2 groups and leading to activation of the hypothetical membrane phospholipase A. The degranulation process could consequently be set off. Needless to say the triggering mechanism outlined is speculative and needs experimental verification.

REFERENCES

1. B. Högberg and B. Uvnäs, *Acta physiol. scand.* **41**, 345 (1957).
2. N. C. Moran, B. Uvnäs and B. Westerholm, *Acta physiol. scand.* **56**, 26 (1962).
3. R. Keller, *Biochem. Pharmacol.* **8**, 152 (1961).
4. B. Högberg and B. Uvnäs, *Acta physiol. scand.* **48**, 133 (1960).
5. B. Fredholm, B. Högberg and B. Uvnäs, *Biochem. Pharmacol.* **5**, 39 (1960).
6. G. W. Schwert, H. Neurath, S. Kaufmand and J. E. Snoke, *J. biol. Chem.* **172**, 221 (1948).
7. B. Uvnäs, *Proc. Int. Union Physiol. Sci. XXII Int. Congr. Leiden* 1962, p. 106.
8. B. Fredholm, E. Giacobini, G. Sedvall and B. Uvnäs (1962). To be published.
9. E. P. Benditt and M. Arase, *J. exp. Med.* **110**, 451 (1959).
10. D. Lagunoff and E. P. Benditt, Conference on Mast Cells and Basophils, *N.Y. Acad. Sci.* (1962). In press.
11. G. Ungar and E. Damgaard, *J. exp. Med.* **101**, 1 (1955).
12. G. G. Glenner and L. A. Cohen, *Nature, Lond.* **185**, 846 (1960).
13. O. Braun-Falco and K. Salfeld, *Nature, Lond.* **183**, 51 (1959).
14. N. Ende and J. V. Auditore, *Nature, Lond.* **189**, 593 (1961).
15. B. Diamant and B. Uvnäs, *Acta physiol. scand.* **53**, 315 (1961).