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STUDIES ON A MAST CELL DEGRANULATING FACTOR IN BEE VENOM*

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Abstract—Bee and snake venoms containing phosphatidase A are known to cause degranulation of mast cells and histamine release. This effect has been ascribed to their phosphatidase content. Recent studies have shown that a factor in bee venom, not related to phosphatidase A, releases histamine from rat peritoneal mast cells. In the present study semipurified bee venom phosphatidase A was subjected to separation by gel filtration and the fractions tested for degranulating effect on rat mesentery mast cells. The results show that this effect was due to a factor which was not phosphatidase A and not identical with the earlier demonstrated histamine releasing principle in bee venom.

THE HISTAMINE releasing effect of bee and certain snake venoms was first observed in 1937 by Feldberg and Kellaway, when they perfused lung tissue from guinea pig, cat, dog and monkey.[1, 2] In 1957 Högberg and Uvnäs showed that phosphatidase A preparations from bee and several snake venoms had a degranulating effect on rat mesentery mast cells, and that this effect could be blocked by enzyme inhibitors.[3] Later, the bee venom phosphatidase A was shown to release histamine and 5-hydroxytryptamine from isolated rat peritoneal mast cells.[4] A mast cell degranulating factor, considered to be phosphatidase A, has also been reported in extracts from ox pancreas.[5]

The mechanism of action for the mast cell degranulating effect of these agents has not been understood. It has been assumed that in rat mesentery mast cells degranulation is preceded by an activation of an energy requiring process and not due to a simple lysis of the cells.[6] On the other hand, the effect on guinea pig mast cells does not seem to involve an enzymatic mechanism in the cell.[7]

When working with phosphatidase A preparations from other sources than those mentioned it was noted that the enzymatic activity was not invariably accompanied by the mast cell degranulating effect (Fredholm, B., unpublished). This finding indicates either that there exist different types of phosphatidase A, or that the degranulating effect is due to some other factor present in the enzyme preparations. This latter suspicion gained strong support when Rothschild[8] and Habermann[9] reported that they had been able to separate a histamine releasing factor from venoms containing phosphatidase A.

The present work describes a method to separate a semipurified bee venom phosphatidase A into one enzymatically active and one mast cell degranulating fraction.

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METHODS AND MATERIALS

Fractionation of the bee venom preparation

The fractionation was performed by gel filtration on the polysaccharide Sephadex.[10] After appropriate pretreatment the gel was packed into 20 × 265–20 × 330 mm columns. The columns were equilibrated with 0·05 M tris-HCl buffer, pH 7·7, and the same buffer was used both as a solvent for the bee venom samples (usually 5 mg in 1·0 ml) and for elution. The elution was performed under a pressure of 30 cm H_2O, and the temperature was kept at +4°C. The fractions (6 ml each) were either tested immediately or stored in the refrigerator overnight, which evidently did not influence their activity.

Observations on mast cells

Rats of both sexes (Sprague–Dawley strain), weighing 200–300 g were used. The mast cell degranulating activity was determined on the mesentery mast cells as described in a previous paper.[5] The incubation medium contained $1{\cdot}54 \times 10^{-1}$ M NaCl, $2{\cdot}68 \times 10^{-3}$ M KCl, 9×10^{-4} M $CaCl_2$ and 10% Sörensen phosphate buffer ($6{\cdot}7 \times 10^{-2}$ M $Na_2HPO_4 + 6{\cdot}7 \times 10^{-2}$ M KH_2PO_4). The pH of the incubation fluid was 7·0 unless otherwise stated. A total of 200 mast cells were counted in each sample. The spontaneous degranulation (5·5 ± 1·2 per cent) was not deducted from the values.

Determination of enzyme activity

Phosphatidase A activity was measured by following the decrease in acyl ester bonds during lecithin hydrolysis. This technique was a modification of that described by Magee and Thompson.[11] The reaction system was composed of: (1) 1·0 ml lecithin, emulsified in 0·4 ml of a water solution containing $1{\cdot}54 \times 10^{-1}$ M NaCl and $6{\cdot}25 \times 10^{-3}$ M $CaCl_2$; (2) 0·1 ml 2% sodium desoxycholate; (3) 0·5 ml of the enzyme solution to be tested (in 0·05 M tris-HCl). The incubation was carried out under continuous shaking at 37°. During incubation a slight decline in pH was observed, never exceeding 0·2 units after 30 min, and it was probably due to the release of free fatty acids. The reaction was stopped by adding 2·0 ml ethanol and 1·0 ml of hydroxylamine reagent (1 M hydroxylamine hydrochloride in 1·75 M NaOH). Colour development was performed according to Stern and Shapiro[12] and the colour yield determined in a Hilger Biochem colorimeter at 520 mμ. The solutions obeyed Beer's law over the concentration range used.

Protein measurement

The protein content was determined with the Folin–Ciocalteu phenol method according to Lowry *et al.*[13]

Materials

Bee venom phosphatidase A. Prepared with ion exchange chromatography on Amberlite IRC-50 (Högberg, B. Unpublished) was supplied by Dr. B. Högberg, AB Leo, Hälsingborg, Sweden.

Lecithin (egg). Supplied by Dr. A. Wretlind, National Institute of Public Health, Stockholm, Sweden.

Other substances. Were obtained from standard commercial sources.

RESULTS

Properties of the unfractionated preparation

The hydrolysis of lecithin by bee venom was found to proceed rapidly in tris-HCl

buffer. The reaction velocity was considerably higher in alkaline than in acid solution, although there was no defined pH-optimum. The lowest concentration of bee venom, in which hydrolytic activity could be accurately determined was about 1·0 μg/ml. Figure 1 shows the time course of lecithin hydrolysis by different concentrations of bee venom in tris-HCl, pH 7·5.

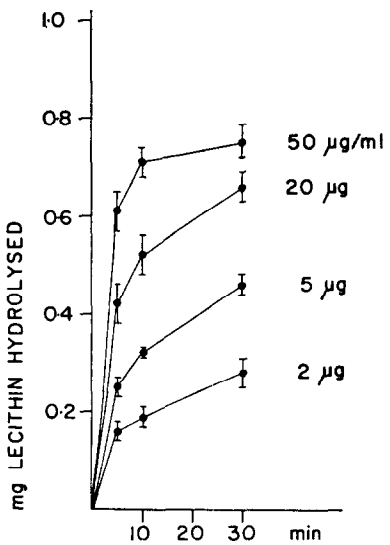

FIG. 1. Time course of lecithin hydrolysis induced by different concentrations of bee venom phosphatidase A in tris-HCl buffer, pH 7·5. Each point represents the mean of four to five experiments. Vertical bars represent standard errors.

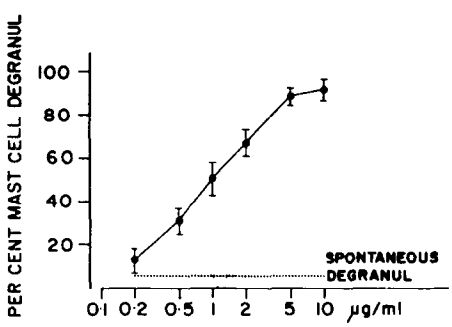

FIG. 2. Dose-response curve of mast cell degranulation induced by bee venom phosphatidase A. Phosphate buffer, pH 7·0. Each point represents the mean of four to nineteen experiments. Vertical bars represent standard errors.

The mast cell degranulating activity had a pH-optimum with maximal effect between pH 6·5 and 8. Threshold concentration for degranulation at pH 7·0 was about 0·2 μg/ml, and degranulation of 100 per cent of the cells was obtained with 5–10 μg/ml (Fig. 2).

Separation of the effects

Several types of Sephadex with different degrees of cross linkage (G10–G200) were tried. G50 was found to be the most suitable and was used in the majority of the experiments. Figure 3 shows a typical experiment with 5 mg of the bee venom preparation run on a 20 × 265 mm column in 0·05 M tris-HCl. Void vol. was 26 ml and the flow rate about 1·0 ml/min. Sixteen fractions of 6 ml were collected, and samples

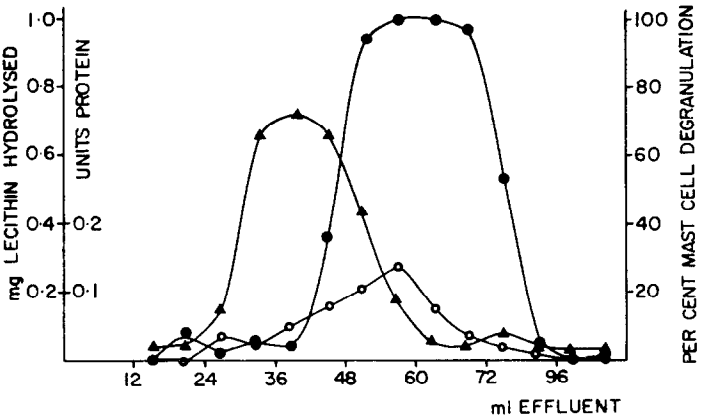

FIG. 3. Gel filtration of 5 mg bee venom phosphatidase A on a 20 × 265mm Sephadex G50 column. Elution was made with 0·05 M tris-HCl buffer, pH 7·7. ▲——▲——▲, phosphatidase A activity (10-min values); ●——●——●, mast cell degranulating activity; ○——○——○, protein content (arbitrary units).

were taken from these for assay of hydrolytic and mast cell degranulating activity, and for protein measurement. The degree of reproducibility was very high, and in three replicate experiments, the elution patterns were nearly identical. As can be seen from Fig. 3 the phosphatidase A activity was eluted first (immediately after the void vol.), followed closely by, but clearly distinguished from, the mast cell activity. Assay of the hydrolytic and mast cell degranulating effects were routinely performed at pH 7·5 and 7·0, respectively. Shifting the pH to 6·5 resulted only in an overall decrease in activities.

The recovery was approximately 50 per cent of the phosphatidase A activity, and 80 per cent of the degranulating activity. If the height of the column was increased to 330 mm there were no overlapping of the two peaks, but the recovery was reduced.

In experiments with Sephadex gels with differing degree of cross linkage it was found that the phosphatidase A was not retained even on G200, which is reported to retain molecules with a molecular weight up to 200,000. The mast cell degranulating factor was retained on all of the gels used except G10 (retention limit about 1000), from which it was at least partly excluded.

The bulk of proteins (and peptides) was eluted within the mast cell activity peak. There was, however, no relationship between the amount of protein and the degree of mast cell degranulating activity in the fractions.

Properties of the fractionated material

Quantitative assay was performed on pooled fractions. Tubes containing phosphatidase A activity were combined and called F I, and mast cell active fractions were pooled and called F II.

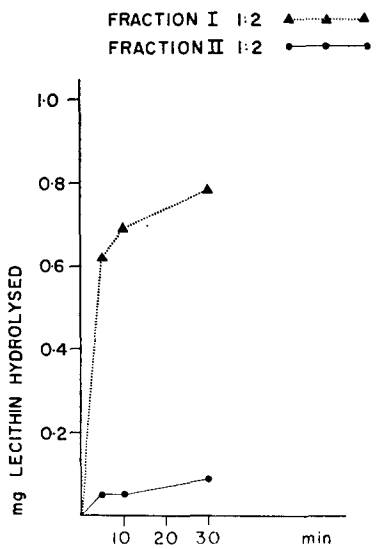

FIG. 4. Time course of lecithin hydrolysis induced by F I (Fraction I) and F II (Fraction II). Tris-HC buffer. Each point represents the mean of four experiments.

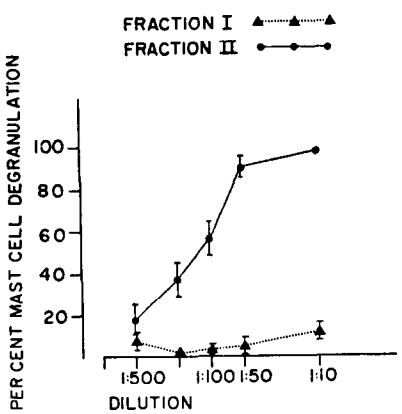

FIG. 5. Concentration–response curves of mast cell degranulation induced by F I (Fraction I) and F II (Fraction II). Phosphate buffer, pH 7·0. Each point represents the mean of four to eight experiments. Vertical bars represent standard errors.

The time course for lecithin hydrolysis by F I is shown in Fig. 4. The activity/unit protein (specific activity) was calculated to be about twice that of the unfractionated material. The mast cell degranulating activity was practically zero (Fig. 5).

The lecithin splitting activity of F II was less than one tenth of that in the original bee venom preparation. A time curve for the F II-induced hydrolysis is shown in

Fig. 4. The mast cell degranulating effect of F II is illustrated in Fig. 5. The activity was, on a protein basis, about twice that of the unfractionated material.

The pH dependency of the F II-induced mast cell degranulation was similar to that of the original bee venom preparation. Maximum effect was obtained between pH 7 and 8, and above pH 8·5 no degranulation occurred.

The susceptibility of the mast cell degranulating process initiated by F II to enzyme inhibitors was tested with dinitrophenol and N-ethylmaleimide. Both of them were found to produce a powerful blocking of the degranulation (Table 1).

TABLE 1. EFFECT OF ENZYME INHIBITORS ON THE MAST CELL DEGRANULATION INDUCED BY F II 1:100

Inhibitor	Per cent degranulation
0	62 ± 5·0
2,4-dinitrophenol 10^{-5} M	4 ± 0·9
10^{-4} M	4 ± 1·1
N-ethylmaleimide 10^{-5} M	5 ± 1·9
10^{-4} M	9 ± 3·1

The mesentery pieces were incubated with inhibitor for 15 min at 37° before addition of releaser. Values are means and standard errors of three experiments.

DISCUSSION

The best known sources for phosphatidase A are several snake venoms and bee venom. Preparations of these venoms have, in addition to their phosphatide splitting activity, an ability to cause degranulation of mast cells and release of histamine in various species. This effect has been assumed to be due to phosphatidase A.

The results of the present work show that the degranulation of rat mesentery mast cells induced by bee venom is not due to phosphatidase A but to some other factor, present in the preparation. This factor, which is of protein nature, has not yet been identified. An estimation of its molecular weight can be made from elution patterns from Sephadex gels with differing degree of cross linkage. The reported molecular weight limits for complete exclusion from Sephadex G10 is about 1000 and from G25 about 5000. Since F II is excluded from G10 but not from G25, its molecular weight should lie between these values.

A histamine releasing peptide, melittin, has been isolated from bee venom by Habermann and co-workers (for references see Habermann, 1963[9]). Although the mast cell degranulating factor demonstrated in this work might be assumed to be identical with melittin, it apparently is not. Melittin liberates 5-hydroxytryptamine from rabbit platelets,[14] while F II does not,[15] and melittin is "directly" hemolysing,[16] while F II lacks this property (Fredholm, B., unpublished).

The mast cell degranulation experiments in this work have all been conducted with rat mesentery mast cells. The results obtained, together with earlier reports on the effect of unfractionated bee venom phosphatidase A[6, 17] indicate that this is an energy-requiring, enzyme-dependent process, possibly of the same type as that induced by compound 48/80. However, in guinea pig mast cells, bee venom apparently exerts its effect by inducing a lysis of the cell membrane without involvement of other

enzymes and without the need for energy.[7] It is probable that such species differences can be related to different components in the venom preparation. Work is in progress to elucidate these matters.

During the preparation of this manuscript A. M. Rothschild[18] reported that melittin, but not phosphatidase A, caused degranulation of rat mesentery mast cells. Dinitrophenol had no inhibitory effect on the action of melittin, and he concluded that the histamine releasing mechanism of melittin had no resemblance to that of compound 48/80. This finding thus lends further support to the view that F II is not identical with melittin.

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