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The capacity of basic peptides to trigger exocytosis from mast cells correlates with their capacity to immobilize band 3 proteins in erythrocyte membranes

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The effect of mast-cell-triggering peptides on the rotational properties of band 3, a protein component of the human erythrocyte membrane, was measured by observing flash-induced transient dichroism of the triplet probe eosin maleimide. In the presence of melittin, polylysine and five synthetic peptides, varying degrees of retardation in the rotational motion of band 3 were produced. When placed in order of band 3 immobilizing activity, the peptides formed a series identical with their order of efficacy in releasing 5-hydroxytryptamine from rat peritoneal mast cells. The correspondence in the abilities to immobilize band 3 in the erythrocyte and trigger mast cells is significant because structure–activity analyses of the peptides show both processes to have the same cationic, hydrophobic and stereochemical requirements. Probably, the immobilization of band 3 proteins reflects an ability of the basic peptides to aggregate anionic surface moieties, and therefore a similar mechanism is implied in mast-cell triggering.

The exocytotic secretion of histamine and other mediators of inflammation from mast cells is known to require the association of IgE–receptor complexes in the cell membrane. Normally, secretion occurs in response to challenge with a specific antigen against which the IgE is directed, but mast cells can also be triggered by other IgE-directed ligands; such as anti-IgE antibody, or independently of IgE by a variety of naturally occurring and synthetic basic peptides (Johnson & Erdös, 1973; Jasani *et al.*, 1973, 1979). In previous studies, it was established that an active peptide should possess a cluster of basic residues (arginine or lysine), thereby giving a site of high positive charge. The presence of hydrophobic residues adjoining such a region enhances activity, while acidic residues abrogate it (Johnson & Erdös, 1973; Jasani *et al.*, 1973, 1979).

The primary structure of the first human IgE heavy chain to be sequenced (Bennich *et al.*, 1976) was found to incorporate a peptide sequence within the Cε4 domain which showed the essential features for triggering of mast cells, as defined in the earlier studies. Peptides comprising this sequence, spanning residues 496–506 of the ε-chain, were subsequently synthesized and shown to be potent stimulators of secretion from rat mast cells (Stanworth *et al.*, 1979).

In the present study we have looked at a range of mast-cell-triggering peptides for effects on the rotational properties of band 3, a major protein component of the erythrocyte membrane (Fairbanks *et al.*, 1971). This work was prompted by the finding that the histamine-releaser melittin, a pharmacologically active polypeptide from honey bee (*Apis mellifera*) venom, was also a potent immobilizer of band 3 protein (Dufton *et al.*, 1984). In terms of structure, melittin is 26 residues in length and is notable for a marked division between a hydrophobic *N*-terminal segment and a highly positively charged *C*-terminal segment (Habermann, 1972). There are no acidic residues present and the *C*-terminus is amidated. Structure–activity studies on the band 3 immobilization phenomenon have revealed that a reduction in the basicity of the melittin reduces the effect, while the introduction of acidic groups abolishes it entirely (Dufton *et al.*, 1984). Thus, there seemed to be a correlation in the structure–activity requirements for band 3 immobilization and triggering of mast cells that demanded further investigation.

Abbreviation used: IgE, immunoglobulin E.

Materials and methods

Peptides

Synthetic peptides were prepared by the solid phase method and purified and characterized as described in detail elsewhere (Stanworth *et al.*, 1984). Purified phospholipase-free melittin was kindly given by Dr. R. C. Hider of the University of Essex. Analysis by h.p.l.c. showed the melittin to be contaminated with two minor components (probably isotoxins) which together comprised less than 10% of the mixture. Poly(L-lysine) hydrobromide (average M_r 2000, 10 residues) was from Sigma, Poole, Dorset, U.K. The amino acid sequences of melittin, polylysine, and the synthetic peptides are shown in Table 1.

Measurement of band 3 rotation

The transient dichroism technique used to measure band 3 rotation has been described in detail elsewhere (Cherry, 1979; Nigg & Cherry, 1979, 1980). Briefly, band 3 was selectively labelled by incubating intact human erythrocytes for 45min at room temperature with the triplet probe eosin-5-maleimide (Molecular Probes Inc.). The eosin:band 3 monomer stoichiometry was approx. 1:1. The rotational diffusion of band 3 was measured in ghosts by observing the transient dichroism of ground state depletion signals arising from the excitation of the probe by a linearly polarized light pulse from a Nd-YAG laser (JK Lasers). Excitation was at 535nm and absorbance changes were recorded at 515nm for light polarized parallel and perpendicular with respect to the polarization of the exciting flash. The signals were collected and averaged by a Datalab DL 102A signal averager. Typically, 512 signals were collected in an individual experiment and the recorded result was the average of four separate measurements. Data were analysed by calculating the absorption anisotropy $r(t)$, given by:

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes at time t after the flash for light polarized parallel and perpendicular, respectively, relative to the polarization of the exciting flash.

The experimental decay curves were then fitted by a double exponential equation:

$$r(t) = r_1 \exp\left(\frac{-t}{T_1}\right) + r_2 \exp\left(\frac{-t}{T_2}\right) + r_3 \quad (2)$$

This resolution of the experimental decay of $r(t)$ into two exponential terms yields two time constants, T_1 and T_2, which may be regarded as order of magnitude relaxation times characterizing the rotation of rapidly and slowly rotating species of band 3 (Nigg & Cherry, 1979). The coefficients r_1 and r_2 are related to the fractional contribution of rapidly and slowly rotating populations. A second equation:

$$r(t) = r_1 \exp\left(\frac{-t}{T_1}\right) + r_3 \quad (3)$$

was used when the experimental decay became minimal at high peptide concentrations and it proved impossible to fit eqn. (2). For both equations, $\%r_3$ is defined as $100r_3/r_0$ where r_0 is the anisotropy at zero time.

Release of 5-hydroxytryptamine and lactate dehydrogenase from mast cells

The methods for isolation of rat peritoneal mast cells and for measurement of release of granule-incorporated 5-hydroxy[^{3}H]tryptamine have been described in detail previously (Stanworth *et al.*, 1984). In experiments in which release of lactate

Table 1. *Peptide sequences*

Peptide	Sequence
Melittin	Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2
Polylysine	Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys
76*	Lys-Thr-Lys-Gly-Ser-Gly-Phe-Phe-Val-Phe-NH_2
74†	Lys-Thr-Lys-Gly-Ser-Gly-Phe-Phe-Val-Phe-NH_2
89‡§	Lys-Thr-Lys-Trp-Ser-Gly-Phe-Phe-NH_2
90‡	Lys-Thr-Lys-Gly-Ser-Gly-Phe-Ala-NH_2
66	Val-Phe-Ser-Arg-Leu-Glu-Val-Thr-Arg-Ala-Glu

* Corresponds to segment 496–506 of the Cε4 domain of human IgE.
† First three residues are of the D configuration.
‡ *N*-Terminus is acetylated.
§ Tryptophan is of D configuration.

dehydrogenase was assayed (for method see Riches & Stanworth, 1981), mast cells were purified by isopycnic sedimentation through metrizamide (Stanworth *et al.*, 1984).

Results

Rotational mobility of band 3

The decay of absorption anisotropy arising from eosin-labelled band 3 in the erythrocyte membrane, both in the absence and presence of the peptides, is shown in Fig. 1. Curve (i) shows the experimental $r(t)$ obtained for untreated ghosts. The anisotropy decay reflects the rotational motion of band 3 proteins, which probably occurs only about an axis perpendicular to the plane of the membrane (Cherry, 1979). Peptides 74, 89 (results not shown), 76, polylysine and melittin produced increasing degrees of retardation in the rate of the decay, thereby implying successive decreases in the rotational motion (curves iii–vi of Fig. 1). Peptide 90 (curve ii) and peptide 66 (results not shown) did not produce significant changes in the rate of decay. Regarding the concentrations chosen, work on melittin (Dufton *et al.*, 1984) has shown that a melittin concentration of 1.0×10^{-7} mol/mg of ghost protein effectively abolishes anisotropy decay. In order to measure the relative efficacy of the synthetic peptides, however, concentrations around 2.0×10^{-7} mol/mg of ghost protein were required to bring the differences within the resolving power of the experiment. For polylysine, the most effective of the synthetic peptides, and peptide 90, which produced the smallest observable effect, concentrations were below and above, respectively, 2.0×10^{-7} mol/mg of ghost protein.

When attempts were made to fit these curves, it was apparent that no very clear information was obtainable from the variation of the parameters r_1, r_2, T_1 and T_2. This is in part because the curves showing marked decay retardation can no longer be fitted to eqn. (2) and in part because of the well known complications of fitting multiple exponential decays. The most useful parameter is $\%r_3$, which is related to the fraction of band 3 molecules which are immobile over the experimental time range of 2ms. Table 2 shows $\%r_3$ as a function of peptide type and concentration, and demonstrates that the order of efficacy is melittin > polylysine > peptides $76 > 89 \approx 74 > 90 \approx 66 \approx$ control.

In view of known differences in the manner of mast cell triggering by melittin and the synthetic

Table 2. *Band 3 immobilizing activity*

Peptide	$10^7 \times$ Concentration (mol/mg of ghost protein)	$\%\ r_3$
Melittin	1.0	100
Polylysine	1.6	90
76	1.9	78
89	2.0	58
74	1.9	56
90	2.7	43
66	2.0	40
–	0	42

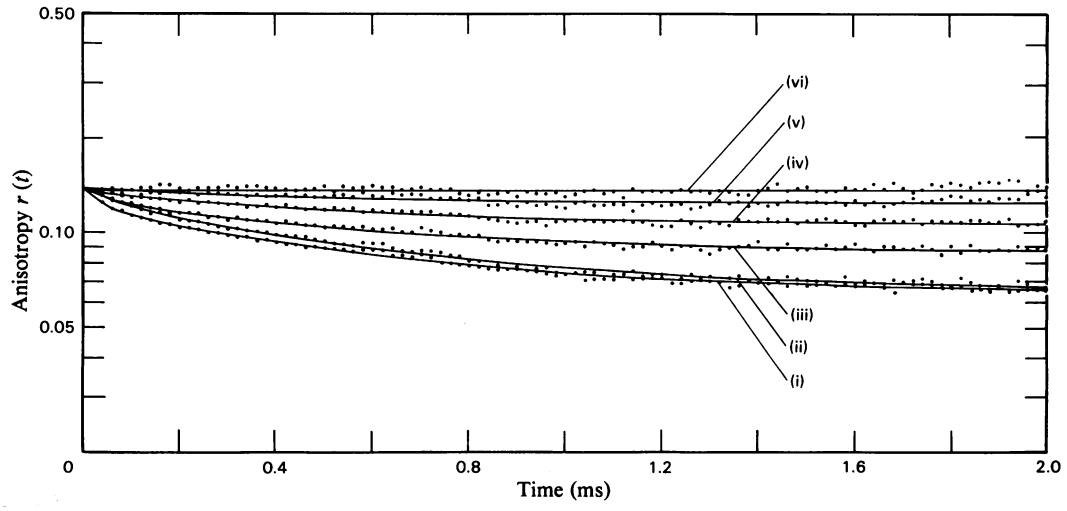

Fig. 1. *Anisotropy decay curves for eosin-labelled band 3 in the presence of melittin, polylysine and peptides 76, 74 and 90*
$r(t)$ was measured at 37°C with membranes suspended in 5mM-sodium phosphate buffer, pH7.4. For peptide concentrations, see Table 2. (i) Control, (ii) peptide 90, (iii) peptide 74, (iv) peptide 76, (v) polylysine, (vi) melittin. Ghost protein concentration was 0.75mg/ml.

Table 3. *Reversibility of band 3 immobilization*

Peptide	Concentration (mol/mg of ghost protein)	% r_3 Pre-wash	% r_3 Post-wash
Melittin	5.2×10^{-8}	74	69
Peptide 76	2.5×10^{-7}	91	44
Control	-	40	40

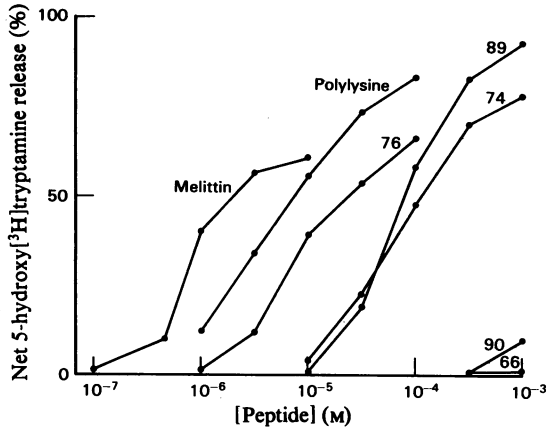

Fig. 2. *Concentration–response curves showing secretion of 5-hydroxy[^3H]tryptamine from rat peritoneal mast cells challenged with the peptides*

Each curve represents the mean of three experiments, each performed in duplicate.

peptides (see the Discussion), an experiment was performed in which ghosts treated with either melittin or peptide 76 were washed to determine the reversibility of the band 3 immobilization. After two washings in the suspension buffer, the peptide 76-induced immobilization was reversed, but the melittin-induced immobilization remained (Table 3).

Peptide-induced secretion from mast cells

Fig. 2 shows concentration–response curves for peptide-induced release of 5-hydroxy[^{3}H]-tryptamine from rat peritoneal mast cells. The peptides tested could be assigned the following rank-order of potency: melittin > polylysine > peptides 76 > 74 = 89 ≫ 90 > 66.

Melittin, polylysine and peptides 74, 76 and 89 all triggered a marked release of 5-hydroxy[^{3}H]-tryptamine at the concentrations tested (up to 10^{-3} M), while peptide 90 was only weakly active at 10^{-3} M and peptide 66 was totally inactive. Of the five active releasing peptides, only melittin was found to cause cell lysis, monitored as release of the cytoplasmic marker lactate dehydrogenase.

Discussion

A comparison of the relative abilities of the peptides to release 5-hydroxytryptamine from rat mast cells and immobilize band 3 protein in the erythrocyte membrane reveals a close correlation. Clearly, the greater the capacity of a peptide to effect exocytosis, the more able it is to immobilize band 3.

Before seeking to account for these results, consideration must be given to differences between the properties of melittin and those of the other peptides. With the sample of melittin used, the release of 5-hydroxytryptamine was accompanied by cell lysis (as witnessed by the release of lactate dehydrogenase). This dual action has been noted with previous melittin samples and other synthetic peptides in which substantial hydrophobic character is present (Jasani *et al.*, 1979; Stanworth *et al.*, 1979). If the hydrophobic *N*-terminal segment of 16 residues is removed from the melittin, the lytic activity is lost while the selective histamine-releasing activity is retained (Jasani *et al.*, 1973, 1979). Evidently, some additional membrane-penetrative ability is present in the whole melittin which is absent from the other peptides tested. This difference has further expression in the results of an experiment in which ghosts were washed after treatment with melittin or peptide 76 at concentrations sufficient to produce pronounced immobilization of band 3 (Table 3). After two washings it was clear that the melittin-induced immobilization had only been reversed to a minor extent. In contrast, the washings did completely reverse the immobilization induced by peptide 76. This confirms therefore, that melittin is the more firmly bound to the membrane.

Turning to the significance of the effects on band 3, the immobilization itself is considered to arise from a change in the aggregation state of the band 3 molecules. A trend towards more slowly rotating species of band 3 is interpreted as being indicative of the formation of higher aggregates (Nigg & Cherry, 1979, 1980). A previous study on the structural requirements of melittin-induced band 3 aggregation has shown that decreases in cationic character produce decreases in aggregative ability (Dufton *et al.*, 1984). To complement this finding, electron microscopy of erythrocyte ghost membranes has also demonstrated that cations such as polylysine, basic proteins, Ca^{2+} and Mg^{2+} will produce aggregation of intramembrane particles (Elgsaeter *et al.*, 1976). Acridine Orange, a cationic dye, will produce the same phenomenon in intact erythrocytes, where only the outer membrane surface is readily accessible (Lelkes *et al.*, 1983).

It is therefore evident that in the requirement for cationic substances, there is a similarity between the activation of mast cells and the aggregation of band 3 protein in erythrocyte membranes. Significantly, the cationic peptides tested in this study showed the same rank order of potency in both experimental systems. More importantly, however, there are some subtler properties, other than cationicity, governing mast-cell-triggering ability which also apply to the mechanism of band 3 aggregation. For instance, although peptides 76 and 74 are identical in terms of charge and hydrophobicity, the latter contains three D-amino acids. Since peptide 74 is less active in both assays, both mechanisms are stereoselective. Similarly, peptide 89, which is active in both systems, and peptide 90, which is inactive, have identical charges, but 90 has two replacement residues of lower hydrophobicity. Hence degree of hydrophobicity is a third mutual requirement. In view of these smaller differences being readily distinguished by the two assays, the correspondence in the order of peptide potency becomes most significant.

The major anionic moieties present in the ghost membrane are the intracellular portions of band 3 (Kaul *et al.*, 1983) and the extracellular portions of glycophorin. It has been postulated that mutual electrostatic repulsion between the anionic glycophorin molecules is a major reason for its even distribution across the surface of the membrane (Elgsaeter *et al.*, 1976; Gahmberg *et al.*, 1978), and also that an interaction between glycophorin and band 3 exists (Nigg *et al.*, 1980). This being the case, the band 3 aggregating tendencies of cationic substances can be readily understood.

It would seem, therefore, that basic peptides activate the secretory mechanism of mast cells by aggregation of an anionic cell membrane protein, which is as yet unidentified, and which is presumably dispersed in unactivated cells by mutual electrostatic repulsion between negative charges. Previous studies with neuraminidase (Coleman, 1982; Foreman & Lichtenstein, 1980) and the cationic detergent benzalkonium chloride (Coleman, 1982) have provided evidence for the involvement of negatively charged cell surface components in basic peptide-induced secretion. The mast cell membrane component which we implicate is unlikely to be IgE or the IgE receptor, since secretion induced by peptides differs in kinetic, and certain pharmacological, aspects from secretion associated with cross-linking of IgE (Foreman & Lichtenstein, 1980; Coleman *et al.*, 1981). In addition, anti-IgE-induced secretion is inhibited by the putative inhibitor of phospholipid methylation, 3-deaza-adenosine plus L-homocysteine thiolactone (Ishizaka, 1981), whereas peptide-induced secretion is not inhibited by this treatment (J. W. Coleman, unpublished work). This implies that there are differences in the biochemical features underlying the mechanism of cell activation resulting from these two types of stimulus.

In conclusion, this study suggests that basic peptides that trigger secretion from mast cells do so by aggregating protein components within the membrane. The identification of these proteins awaits further investigation, but the similar subtleties in the structure–activity requirements of the mast-cell-activating and band-3-aggregating phenomena may show that a similar membrane moiety is involved.

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