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The influence of melittin on the rotation of band 3 protein in the human erythrocyte membrane

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Abstract. The rotational mobility of band 3, a protein constituent of the human erythrocyte membrane, was measured by observing the flash-induced transient dichroism of the triplet probe eosin maleimide. In the presence of melittin, a pharmacologically active polypeptide from honey bee (*Apis mellifera*) venom, a dose-dependent loss of rotational mobility was detected. With acetylated melittin, the ability to immobilise is reduced. Succinylated melittin, however, is devoid of immobilising activity.

The possible relevance of these findings to the normal mode of action of melittin was examined by comparing the relative abilities of the native, acetylated and succinylated melittins to lyse erythrocytes and synergise with phospholipase A_2, another constituent of bee venom. For both these properties, the order of effectiveness is native melittin > acetyl melittin > succinyl melittin = 0, the same as their order of effectiveness in immobilising band 3.

A mechanism is proposed in which melittin is anchored in the membrane by its hydrophobic N-terminus, while its cationic C-terminal moiety binds to negatively charged residues on membrane proteins. This leads either directly or indirectly to protein aggregation and hence loss of mobility. From a detailed comparison of the different effects of the melittin derivatives, it is concluded that melittin may function in vivo by aggregating membrane proteins in order to allow phospholipase A_2 to gain access to the membrane bilayer and commence catalysis.

Key words: Band 3, melittin, phospholipase A_2, erythrocyte, transient dichroism, rotational diffusion

Introduction

Melittin is the major constituent of Honey Bee (*Apis mellifera*) venom and is largely responsible for the inflammation and tissue damage that follows a sting from this insect. Despite its powerful pharmacological properties, the structure of melittin is relatively simple, and for this reason, it has attracted much interest as to its mode of action. It is a peptide of 26 residues and its sequence is notably composed of mostly apolar and basic amino acids which are localised towards the N- and C-termini respectively (Habermann 1972). Its secondary, tertiary, and quarternary structures depend on concentration, the presence of certain counterions and solution dielectric constant. At high concentrations and in the presence of divalent anions, a tetrameric form of melittin predominates in which the monomers adopt a largely α-helical form and pack together symmetrically. At low concentrations and without the benefit of counterions, dissociation into monomers takes place with some concomitant loss of the regular helical secondary structure (Faucon et al. 1979; Yunes 1982; Brown et al.1980; Lauterwein et al. 1980). In solutions of lower dielectric constant, however, the secondary structure is retained (Drake and Hider 1979; Lauterwein et al.1979; Bony et al. 1980; Brown and Wüthrich 1981).

In view of its pronounced amphiphilic structure (Terwilleger et al. 1982), it is considered that melittin's potency as a cell lytic agent depends on it being able to accumulate at the interface between the cell membrane and the extracellular phase in its monomeric form (Drake and Hider 1979; Lauterwein et al. 1979; Habermann 1980). Lysis may then be a consequence of melittin deforming the bilayer packing and destroying the integrity of the membrane as a whole. Indeed, much detailed work has been reported on the interaction of melittin with lipid systems and various mechanisms have been proposed. (Hegner et al. 1973; Verma et al. 1974; Lavaille et al. 1982; Terwilleger et al. 1982; Dawson et al. 1978; Jähnig et al. 1982).

It should be pointed out, however, that the role of membrane proteins in the action of melittin has

hitherto been largely neglected. Such proteins are fundamental constituents of biological membranes and it might be anticipated that if substantial bilayer disruption were the result of melittin action, then these proteins might themselves register aspects of the process. Alternatively, or in addition, a direct interaction between melittin and the membrane proteins is also conceivable.

To enable a direct comparison with one of the standard assays of melittin efficacy, namely the lysis of erythrocytes, this study of the rotational mobility of a major protein constituent of the erythrocyte membrane (band 3) (Fairbanks et al. 1971) in response to melittin was undertaken. Since the rotational motion of integral membrane proteins like band 3 is particularly sensitive to protein-protein interactions (Cherry 1979), its measurement provides a convenient method of following structural changes in the membrane in which proteins are involved. In order to extend the study, modified derivatives of melittin were tested and the known synergism between melittin and another important bee venom protein, phospholipase A_2 (Yunes et al. 1977), was examined.

Methods

a. Purification of bee venom melittin and phospholipase A_2

Native melittin and phospholipase A_2 were purified in this laboratory from whole bee venom (R. C. Hider and E. Dotimas, unpublished). The purity of the melittin was assessed by reverse-phase HPLC on Synchropak RP-8 using triethylammonium phosphate (25 mM, pH 3.2) and 27.5% n-propanol. It was contaminated with two minor components (probably isotoxins) which together comprised less than 10% of the mixture. The preparation was judged to be free of phospholipase A_2 contamination on the basis of standard assays utilising ^{14}C-labelled phosphatidylcholine. Throughout the study, melittin concentration was determined according to a molar extinction coefficient of 5,600 cm^{-1} at 280 nm (Tatham 1983).

b. Preparation of melittin derivatives

Succinyl and acetyl derivatives were prepared by methods based on those of Habermann and Kowallek (1970). For the succinyl derivative, melittin (16 mg) was dissolved in water (2 ml) and the pH was adjusted to 8.0 with sodium hydroxide (0.1 M). Solid succinic anhydride (28 mg) was then added and the pH was maintained at 8.0 by further addition of sodium hydroxide (0.1 M). Upon completion of the reaction, the pH was increased to 10.0 and the solution allowed to stand for 1 h. The derivative was desalted on a Sephadex G-10 column equilibrated with 2% acetic acid and subsequently lyophilised.

To prepare the acetyl derivative, melittin (16 mg) was dissolved in sodium phosphate buffer (3 ml, 0.5 M, pH 7.4) and mixed with acetic anhydride (100 µl) in methanol (400 µl). After stirring for 15 min, the product was desalted as before and lyophilised.

HPLC analysis showed the succinyl melittin to be essentially homogeneous with less than 5% contamination of the native form. The acetyl melittin contained three fractions. As judged by their retention times, the dominant product (70%) was tetra-acetyl melittin, with 25% triacetylmelittin and 1% unmodified melittin.

The indole absorption spectrum was virtually the same for native melittin and its two derivatives, so throughout, concentrations of the derivatives were determined according to the extinction coefficient known for melittin.

c. Measurement of band 3 rotation

The flash photolysis technique used to measure band 3 rotation has been described in detail elsewhere (Cherry 1978, 1979; Nigg and Cherry 1979). Briefly, band 3 was selectively labelled by incubating intact human erythrocytes for 30 min at room temperature with the triplet probe eosin-5-maleimide (Molecular Probes, Inc.). The eosin-band 3 monomer stoichiometry in the labelled membranes was about 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 polarised light pulse from a Nd-YAG laser (JK Lasers, Ltd.). Excitation was at 532 nm and absorbance changes were recorded at 515 nm for light polarised parallel and perpendicular with respect to the polarisation 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

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

where $A\parallel(t)$ and $A\perp(t)$ are the absorbance changes at time t after the flash for light polarised parallel and

perpendicular, respectively, relative to the polarisation of the exciting flash.

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

$$r(t) = r_1 \exp(-t/T_1) + r_2 \exp(-t/T_2) + r_3 . \quad (2)$$

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 characterising the rotation of rapidly and slowly rotating species of band 3 (Nigg and Cherry 1979). The coefficients r_1 and r_2 are related to the fractional contribution of rapidly and slowly rotating populations. A second equation

$$r(t) = r_1 \exp(t/T_1) + r_3 \quad (3)$$

was used when the experimental decay became minimal at high melittin concentrations and it proved impossible to fit Eq. (2).

d. Hemolysis assay

In order to determine the relative efficacy of melittin, its derivatives and phospholipase A_2 in lysing human erythrocytes, the following procedure was adopted. Recently outdated human blood was washed and centrifuged three times in isotonic buffer (150 mM NaCl, 5 mM Na_2HPO_4, pH 7.4). Packed erythrocytes 1 ml) were diluted to 100 ml in isotonic buffer and allowed to equilibrate at 37° C. For the assay, 2 ml of the blood suspension and 2 ml of the melittin/phospholipase A_2 solution (isotonic buffer, 37° C) were combined and incubated for 5 min. Aliquots were then removed and centrifuged in a Beckman Microfuge for 30 s. The supernatant was separated and its optical density at 578 nm recorded relative to suitable controls.

Results and data analysis

a. Rotational mobility of band 3

The decay of absorption anisotropy arising from eosin-labelled band 3 in untreated and native melittin treated erythrocyte membranes 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 protein which probably occurs only about an axis perpendicular to the plane of the membrane. The curves obtained upon successively increasing melittin concentration demonstrate successive retardations in the rate of the decay, thereby implying a decrease in the rotational motion (curves (ii)-(v), Fig. 1). When the anisotropy decays were fitted to Eq. (2), it was apparent that no clear information was obtainable from the variation of the parameters r_1, r_2, T_1, and T_2 with melittin concentration. This is in part because the curves at higher melittin concentrations can no longer be fitted by Eq. (2) and in part because of the well known complications of fitting multiple exponential decays. The most useful parameter is in fact r_3 which is related to the fraction of band 3 molecules which is immobile over the experimental time range of 2 ms. Figure 2 shows a plot of r_3 (%) as a function of concentration for native melittin.

The effects of the acetylated and succinylated melittin derivatives on band 3 rotation are also shown in Figs. 3 and 4. Whereas the acetyl melittin retains the ability to immobilise, albeit reduced in comparison with the native form, it is apparent that succinylation virtually abolishes the property altogether.

b. Hemolysis

The relative hemolysing powers of native melittin and its two derivatives are shown in Fig. 5. The succinyl melittin is clearly devoid of hemolytic potency whereas the acetyl melittin does retain the property, but in a reduced form. These results are consistent with those obtained by Habermann and Kowallek (1970) save that the samples of native and acetyl melittin used herein appear to be more and less effective respectively.

The abilities of native, acetyl, and succinyl melittin to synergise with the venom phospholipase A_2 in hemolysis were ascertained by varying melittin concentration in the presence of a fixed concentration of the enzyme (0.125 μg/ml). This level of enzyme was chosen so that melittin concentrations of around 0.5 μg/ml (i.e., 6.2×10^{-9} mol/equivalent mg of ghost protein) would reflect the natural ratio of phospholipase A_2/melittin by weight in the whole bee venom (Habermann 1972).

The observed percentages of synergised hemolysis were found to correlate closely with the percentage of hemolysis caused by the melittin derivatives alone. Maximal synergism (achieving 100% hemolysis) occurred when melittin concentrations alone were sufficient to produce about 30% lysis (Fig. 6). This was the case with both the native and acetyl melittins, despite the requirement for the latter to be present in higher concentrations. The succinyl melittin, which does not lyse in its own right, produced no hemolysis in the presence of the enzyme. On an

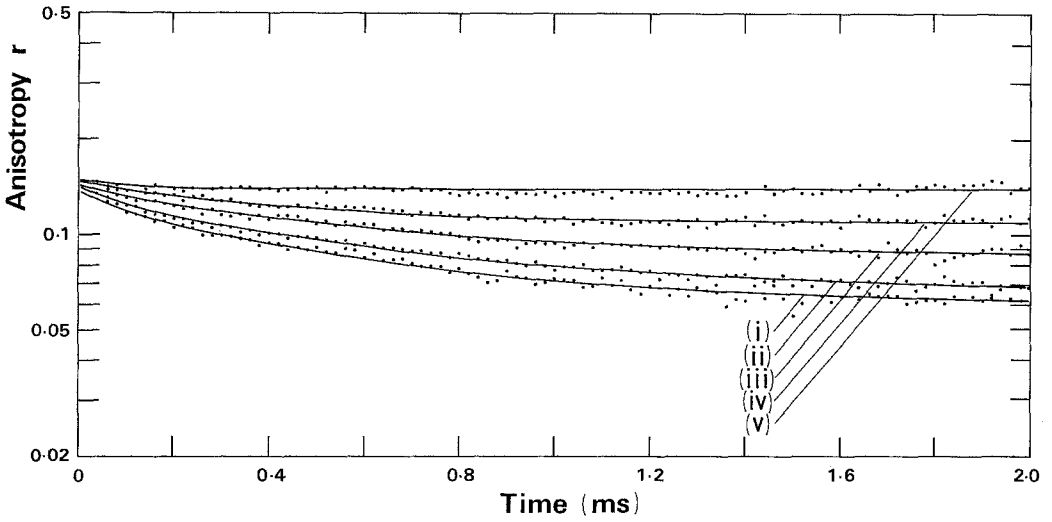

Fig. 1. Anisotropy decay curves for erythrocyte membranes at different concentrations of native melittin. $r(t)$ was measured at 37° C with membranes suspended in 5 mM sodium phosphate buffer, pH 7.4. Concentrations of melittin are expressed as moles per milligram of ghost protein. (*i*) Without melittin. (*ii*) 1.8×10^{-8} mol/mg. (*iii*) 3.5×10^{-8} mol/mg. (*iv*) 4.9×10^{-8} mol/mg. (*v*) 1.1×10^{-7} mol/mg. Curve (*i*) was fitted by Eq. (2), curves (*ii*)–(*v*) by Eq. (3). Ghost protein concentration was 0.6 mg/ml

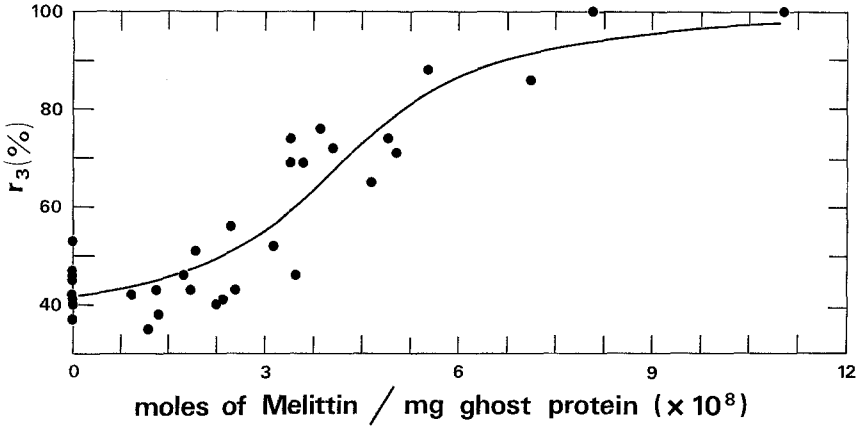

Fig. 2. The dependence of $r_3(\%)$ on the concentration of native melittin. The values of $r_3(\%)$ were obtained after fitting either Eqs. (2) or (3) to the experimentally obtained anisotropy decay curves. The graph was fitted using a non-linear least-squares program with a sigmoidal function. i.e.) $r_3 = k \tan^{-1} f(M - M_0)$ where k is a scaling factor, M_0 is the centre of the sigmoid on the concentration scale and f is a curvature factor. Typically, ghost protein concentrations were 0.6–0.8 mg/ml

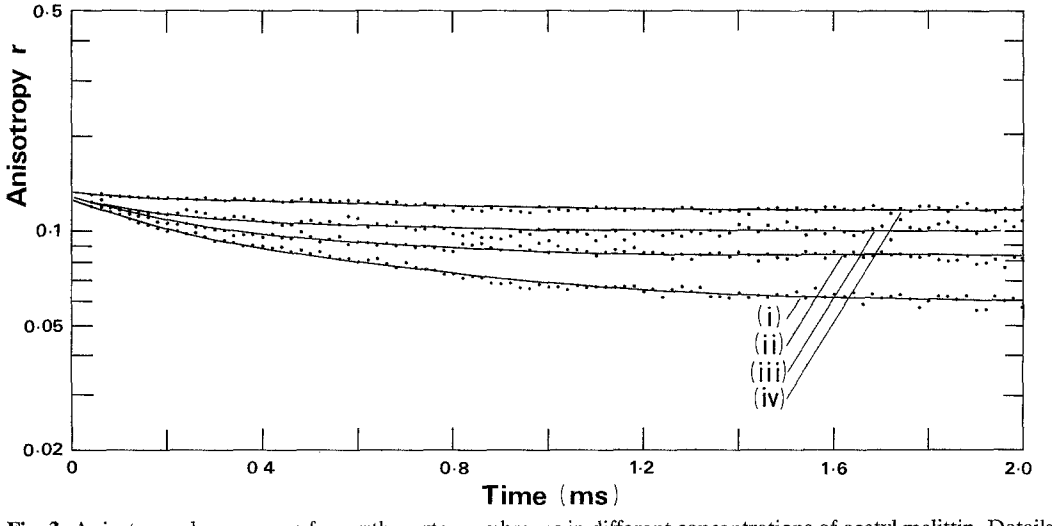

Fig. 3. Anisotropy decay curves for erythrocyte membranes in different concentrations of acetyl melittin. Details as for Fig. 1. (*i*) Without acetyl melittin. (*ii*) 5.5×10^{-8} mol/mg. (*iii*) 5.9×10^{-8} mol/mg. (*iv*) 6.8×10^{-8} mol/mg. Curve (*i*) was fitted by Eq. (2), curves (*ii*)–(*iv*) by Eq. (3)

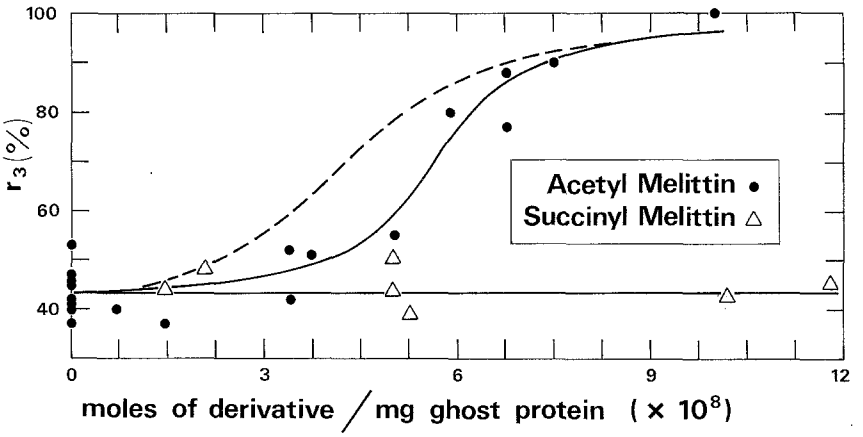

Fig. 4. The dependence of $r_3(\%)$ on the concentration of acetyl and succinyl melittin. ● Acetyl melittin; △ succinyl melittin. The dependence of $r_3(\%)$ on the concentration of native melittin (Fig. 2) is shown by the dashed line

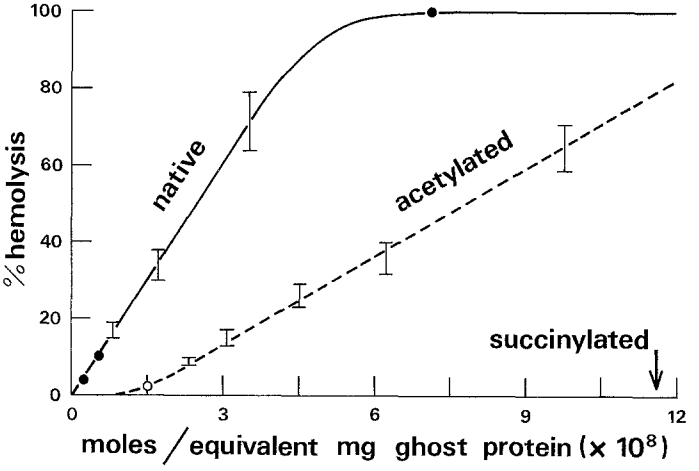

Fig. 5. Percentage of hemolysis of intact erythrocytes as a function of melittin concentration. *Solid line:* Native melittin. *Broken line:* Acetyl melittin. The succinyl melittin produced no hemolysis. Melittin concentration is expressed as moles of melittin/equivalent mg of ghost protein. The conversion was achieved by transforming a known volume of packed erythrocytes into ghosts and determining the protein concentration

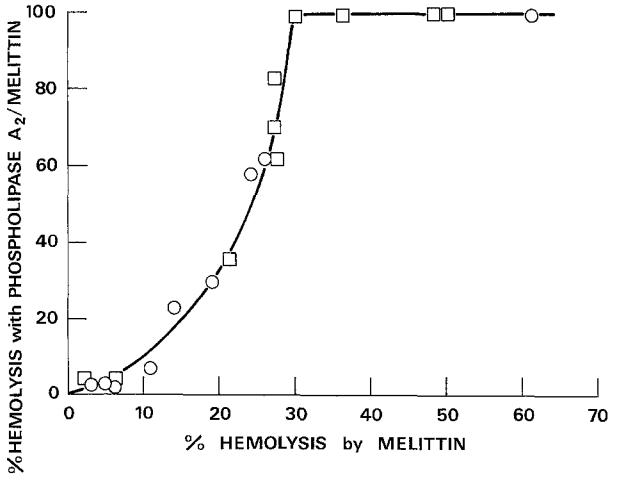

Fig. 6. Percentage hemolysis of intact erythrocytes as induced by melittin/phospholipase synergism compared with the percentage hemolysis induced by the melittin alone. The data were obtained with various melittin concentrations and a fixed concentration of phospholipase A_2 (4.0×10^{-10} mol/equivalent mg ghost protein). □ Native melittin; ○ Acetyl melittin

equimolar basis, therefore, acetyl melittin has a reduced ability to synergise with the enzyme and succinyl melittin has no ability whatsoever.

The phospholipase A_2 alone was notably unable to cause lysis, even after prolonged incubation at higher concentrations.

Discussion

a. Melittin and band 3 mobility

It is clear from the results that the rotational mobility of band 3 is sensitive to the presence of melittin. If the melittin is acetylated, reducing its overall charge from 6+ to 2+, it becomes less effective. Should it be succinylated, converting the overall charge to 2−, there is no detectable effect on mobility.

A possible explanation of the results is that derivitisation stabilises the tetrameric form of melittin in solution, hence reducing the amount of

monomer (which is thought to be the lytic form, Hider et al. 1983) able to reach the membrane. Indeed, it is the case that the order of increasing tetramer stability (native < acetyl < succinyl) (Tatham 1983; Bello et al. 1982) is also the order of decreasing ability to influence band 3. However, in solutions of low dielectric constant there is evidence to show that, like native melittin, these derivatives will adopt a predominantly α-helical and monomeric form (Tatham 1983; Hider et al. 1983). Furthermore, acetyl and succinyl melittin are both surface active, the former more so than native melittin (Habermann and Kowallek 1970). It appears, therefore, that tetramer stabilisation is unlikely to account for the reduced band 3 immobilising powers of the derivatives.

When melittin enters a bilayer, it is envisaged as adopting a conformation that proves destabilising, bringing about thinning and eventual rupture, possibly by a "wedge effect" (Dawson et al. 1978). The sensitivity of band 3 to the presence of melittin can be thought of as arising from the melittin-lipid interactions changing the normal physical state of the bilayer (Posch et al. 1983) or instigating changes in protein-protein interactions within it. An effect transmitted to band 3 solely via the constituent lipids seems unlikely in view of the fact that the surfactant properties of melittin and its derivatives do not correspond with either the ability to influence band 3 or the ability to hemolyse (Habermann and Kowallek 1970; Habermann 1972). Indeed, the discrepancy between surface activity and hemolytic potency is one of the main reasons why a particular "mechanism" is suspected in melittin action (Habermann 1972).

Previously, immobilisation effects on band 3 have been attributed to changes in aggregation equilibria, with tendencies towards slower rotating species showing formation of higher oligomers (Nigg and Cherry 1980). Melittin could influence these equilibria either by directly acting on band 3 or indirectly via the other membrane proteins with which band 3 is intimately associated (i.e., glycophorin and the membrane cytoskeleton) (Nigg and Cherry 1980). That intrinsic membrane proteins can be readily affected by cationic molecules has been shown by experiments in which calcium, magnesium, cationic dyes, polylysine, and basic proteins have produced intramembrane particle aggregation in erythrocyte membranes (Elgsaeter et al. 1976; Lelkes et al. 1983). Clearly, since melittin possesses an extremely cationic C-terminal region and loses potency as the cationic nature is reduced, it is probable that similar modes of action are involved.

Within an erythrocyte membrane, there are moieties which bear marked anionic character, notably the intracellular fragment of band 3 and the extracellular portions of glycophorin. Glycophorin has acidic residues clustered along its polypeptide chain immediately after it emerges on to the extracellular surface and is also rich in sialic acid residues. Significantly, it has been suggested that the anionic character of glycophorin is responsible for maintaining its even distribution over the surface, through mutual electrostatic repulsion (Gahmberg et al. 1978). Furthermore, band 3 and glycophorin are thought to form a complex in the membrane (Nigg et al. 1980). The ghosts used in this study are not resealed, so melittin has access to both faces of the membrane, and therefore both of the major anionic moieties.

A tentative model, therefore, for the melittin-induced immobilisation of band 3 in erythrocyte ghosts is that the melittin is absorbed onto the membrane interface and simultaneously its cationic C-terminal segment comes into proximity with predominantly anionic surface moieties while its membrane penetrating N-terminal region comes proximate to very similar membrane spanning protein segments. As a consequence of both hydrophobic and ionic attraction, aggregation may then be produced. Band 3 could be influenced directly, or indirectly via glycophorin or by its attachment to elements of the spectrin/actin meshwork. It is important to note that melittin can aggregate the membrane spanning protein bacteriorhodopsin when it is reconstituted in lipid vesicles (M. J. Dufton and R. J. Cherry, unpublished). Thus, the aggregating properties of melittin may apply to a range of membrane proteins, and not specifically to band 3 of the erythrocyte.

b. Band 3 immobilisation, hemolysis and synergism

There are two major aspects of the band 3 immobilisation results which imply that the ability to affect intrinsic membrane proteins is relevant to melittin action. Firstly, when the dose-response curves of melittin-induced hemolysis and melittin-induced band 3 immobilisation are compared, the similar concentration dependences demonstrate that the hemolysing events are accompanied by a change in the rotational properties of band 3 (See Figs. 2 and 5). Secondly, when comparing the relative abilities of acetyl and succinyl melittin to immobilise band 3, hemolyse intact erythrocytes and synergise with phospholipase A_2, the order is consistently native melittin > acetyl melittin > succinyl melittin = 0. In contrast, the order of decreasing surface activity is acetyl melittin> native melittin > succinyl melittin > 0 (Habermann and Kowallek 1970). Thus, the former three properties all require the melittin to have an overall positive charge and are sensitive to the degree of this positive charge.

Although it appears that acetylation of melittin reduces the hemolytic powers to a greater extent than the immobilising effect on band 3, it should be emphasised here that there is no reason to expect them to be identical because of the difficulty in quantifying how the rotational diffusion properties have changed.

If the properties of hemolysis, band 3 immobilisation and synergism are examined for possible interdependences, it is noteworthy that the synergism does not become fully effective until native melittin alone is at a sufficient concentration to produce about 30% hemolysis (Fig. 6). It is at this concentration (1.5×10^{-8} mol/equivalent mg of ghost protein) that the first measurable effects on band 3 mobility are seen (Fig. 2). The implication, therefore, is that the aggregation of band 3 may be more pertinent to the ability of phospholipase A_2 to attack melittin-treated cells than the events leading to direct lysis.

c. The possible role of membrane proteins in melittin action

Directing attention to the manner in which whole bee venom brings about cell lysis, it is evident that the presence of phospholipase A_2 renders consideration of how melittin alone induces lysis somewhat of a secondary problem. Thus, as far as the role of melittin as a venom constituent is concerned, it is necessary only to reach a certain sub-lytic concentration in a cell membrane in order to facilitate phospholipase A_2 activity. When venoms from other animals are surveyed, it will be realised that whereas melittin is so far unique to the bee, phospholipase A_2 is a major common component. As a toxin, this enzyme is clearly most suitable for causing widespread cell damage, because not only is its activity continuous, but the products of its catalysis are lysolipids which themselves will destabilise membrane.

In venoms generally, a central problem to the use of this enzyme could be its ability to actually reach the membrane interface and commence catalysis. The normal erythrocyte, in common with other cells, has various proteins disposed over its extracellular surface, several of which are conjugated to large carbohydrate moieties. Since these appear to be uniformly dispersed over the survace (e.g., by charge repulsion as in the glycophorins) they could present an effective physical barrier to an incoming phospholipase, thereby providing the cell with an important line of defence. Melittin, however, being relatively small, amphiphilic and conformationally flexible, may be particularly able to intercalate between the glycoproteins and so gain access to the bilayer. If, as the results suggest, it can then penetrate the bilayer and aggregate the intrinsic proteins/cytoskeleton, it would create patches of protein denuded membrane which could then become accessible to the phospholipase A_2 and allow the catalysis : lysolipid cascade to become fully operational. The observation that "bleb" formation can occur in the presence of basic proteins and cationic dyes, possibly due to contraction of the spectrin-actin meshwork, may be relevant to the envisaged mechanism (Elgsaeter et al. 1976).

In support of the above hypothesis, it is noteworthy that the synergism with phospholipase A_2 achieved by succinyl melittin is still 50% of that achieved by native melittin when hydrolysis of protein-free liposomes is assayed (Yunes et al. 1977). This contrasts with the total lack of such synergism by succinyl melittin in erythrocyte lysis and suggests that the mode of action may be different in the two systems. A role for proteins in erythrocyte lysis is strongly indicated by the fact that immobilisation of band 3 does correlate with lytic activity whereas hydrolysis of phospholipids in a pure lipid system does not.

Conclusion

Subject to the necessary proviso that results obtained with erythrocyte ghosts may not reflect entirely the properties of the intact cells, it seems nevertheless significant that melittin can so readily immobilise the intrinsic membrane protein band 3. The sensitivity of the property to the derivitisation of melittin and its reflection in the reduced hemolytic and synergistic properties strongly suggests an involvement in the melittin mechanism. Therefore, while much valuable information has been obtained about melittin from studies on pure lipid systems, it is clear that intrinsic membrane proteins must not be neglected when mode of action is considered.

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