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Lytic effects of melittin and δ-haemolysin from *Staphylococcus aureus* on vesicles of dipalmitoylphosphatidylcholine

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The effects of the lytic peptides, melittin and δ-haemolysin, are compared in vesicles of gel-phase dipalmitoylphosphatidylcholine (DPPC), using calcein as trapped marker. At low concentration, both toxins cause vesicles to lose contents in 5 mM phosphate buffer near neutral pH, with melittin being the more active. As phosphate concentration is increased, the kinetics of melittin-induced leakage change from a slow, sustained loss to a rapid 'burst' of leakage when melittin is present mainly as tetramer in solution, under conditions where it is reported to lose haemolytic activity towards erythrocytes. At low phosphate concentration, the leakage induced by δ-haemolysin is preceded by a lag phase, though fluorescence measurements show that binding of toxin is rapid. At higher phosphate concentration, the toxin binds rapidly to vesicles, but causes no leakage of entrapped calcein. Steady-state fluorescence spectra show no obvious differences in tryptophan emission for δ-haemolysin bound to lipid in high- or low-phosphate buffer. Spin-label fluorescence-quenching studies show that the single tryptophan residue of δ-haemolysin is buried within the lipid bilayer at all phosphate concentrations used. In gel-phase DPPC, δ-haemolysin shows no tendency to cause vesicle aggregation over several hours, as judged by light scattering, though a slow non-linear effect is seen above the lipid phase transition temperature. These effects are contrasted with those of melittin under similar conditions.

Introduction

The interaction of lytic toxins with membranes has been widely studied by a variety of physical methods [1–5]. The interest arises in part because toxin-lipid interactions might give insight into processes such as membrane protein incorporation, cell fusion and viral interactions with cells.

One of the most widely studied toxins is melittin from bee venom. This is convenient for study, since it is available in reasonable quantities, stable to organic solvents and therefore easily purified by HPLC, and has a single tryptophan residue which can be used as an intrinsic fluorescence probe [3]. Melittin is a positively charged peptide of 26 residues, having clearly defined regions of a hydrophobic nature [6]. The toxin exists in solution in equilibrium between monomeric- and tetrameric forms, with the equilibrium position strongly dependent on the nature and concentration of anion present [7]. In buffers of low ionic strength, and with monovalent anions such as chloride, melittin is present predominantly as monomer at micromolar concentrations. In the presence of multiply

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s).

charged anions such as phosphate at higher ionic strength, the tetrameric form predominates [8].

Melittin causes a variety of effects in cells, but the primary interaction is with the plasma membrane, leading to altered permeability and ultimately cell lysis. The erythrocyte has been used as a model system to study such membrane interactions, and it has been found that melittin causes enhanced permeability to haemoglobin in a complex manner. In particular, it was shown that the tetrameric form of melittin was not lytic towards erythrocytes, while the effect of monomer was consistent with an initial membrane perturbation followed by a slower process, interpreted as a consequence of possible translocation of peptide across the bilayer [9]. The interaction of melittin with phospholipid bilayers has also been studied, and has been shown to result in enhanced permeability to small molecules [10], lipid vesicle aggregation and vesicle fusion on passing through the phase trasition of the host bilayer [11,12]. Vesicle fusion was shown to occur especially readily with vesicles bearing an overall negative charge, and the toxin was shown to be a much more potent fusogen than calcium ion in these circumstances [10].

Another toxin, δ-haemolysin from *Staphylococcus aureus*, has been studied recently, and similarities to melittin have been pointed out [13]. Like melittin, δ-haemolysin is 26 residues in length with a single tryptophan, no tyrosine and with recognisable regions of hydrophobic and hydrophilic nature. In this case, however, the overall charge balance is maintained largely by positively charged residues, unlike melittin which relies on counterions in solution. The aggregation state of δ-haemolysin in solution is strongly influenced by ionic strength but, unlike melittin, aggregates larger than the tetramer are formed.

In this paper, we compare the lytic effects of melittin and δ-haemolysin in vesicles of gel-phase dipalmitoylphosphatidylcholine, using calcein as a trapped fluorescent marker. The toxins are compared in buffers of varying phosphate concentration, and it is shown that there are significant differences in the effects on bilayer vesicles, despite the superficial similarities of the peptides. In particular, it is shown that melittin is able to alter bilayer permeability under conditions of high ionic strength where it is tetrameric. δ-Haemolysin is shown to increase bilayer permeability in media of low ionic strength, but is inactivated by high phosphate concentrations. The inability of δ-haemolysin to increase vesicle permeability in the latter case is shown not to be due to inhibition of peptide binding.

Materials and Methods

DPPC was obtained from Sigma, and was used without further purification. δ-Haemolysin was prepared as previously described [14]. Melittin was purchased from Sigma, and purified from phospholipase A_2 contamination by extraction with aqueous propan-1-ol, followed by treatment with Dowex 1-X8 resin [15] and gel filtration on medium porosity Sephadex G-25 (Pharmacia), eluting with distilled water. Melittin samples were evaporated in vacuum, and stored in solution in 50% aqueous ethanol at 4°C. Control experiments showed that EDTA was effective in inhibiting the phospholipase activity of unpurified melittin, and results obtained in the presence of EDTA were similar for both purified and commercially available melittin.

Unless otherwise indicated, buffers contained EDTA in all cases. Calcein solutions contained 5 mM EDTA, 25 mM calcein, while eluting buffers contained 30 mM EDTA in order to compensate for the osmotic effect of calcein within vesicles. The presence of EDTA also prevented possible quenching of calcein fluorescence by metal ion complexation.

Large unilamellar vesicles were prepared by the deoxycholate-dilution method [16]. Calcein as sodium salt was co-sonicated with the phospholipid/deoxycholate mixture at a concentration of 25 mM before application to a medium porosity Sephadex G-50 column to form vesicles. It was found necessary to maintain both the gel-filtration column and lipid sample above the phase-transition temperature of DPPC, or vesicles would not form. Alternatively, a column could be used at room temperature if the concentration of deoxycholate was reduced 10-fold. In both cases, extensive trapping of calcein was seen, and results obtained on samples prepared by both methods were similar. The calcein used was found to be contaminated with a red material of high molecular weight, which could be removed by gel filtra-

tion or, with difficulty, by repeated recrystallisation from aqueous ethanol. The G-50 column effectively separated this impurity from the vesicle fraction. Experiments with purified calcein showed that the impurity had no discernable effects on leakage from vesicles.

Small unilamellar DPPC vesicles were prepared by the ethanol-injection method [17].

Fluorescence and light-scattering experiments were made using a Schoeffel RRS-1000 spectrofluorimeter interfaced to a microcomputer as previously described [11]. Kinetic measurements used a simple stopped-flow apparatus built in the Department.

The release of calcein from vesicles was monitored fluorimetrically at 520 nm, exciting at 470 nm. The calcein which is trapped in the vesicles is at high concentration and the fluorescence is self-quenched [18]. Leakage is monitored as a relief of quenching. Addition of Triton X-100 to the sample causes total relief of quenching as all trapped calcein is released into the solution.

Electron microscopy was performed using a Corinth 500 transmission electron microscope. Samples were negatively stained using 1% uranyl acetate on Formvar-carbon coated grids. Electron micrographs of large unilamellar DPPC vesicles made by the modified deoxycholate-dilution procedures have shown that vesicles eluted on a column which was preequilibrated above the phase-transition temperature of the lipid had an average diameter over 100 nm. Vesicles made at a molar ratio of phospholipid to deoxycholate of 20 to 1 at room temperature had an average diameter of approx. 80 nm [19].

Fatty acids labelled with the paramagnetic nitroxide spin label (5-, 7-, 9-, 12- and 16-doxylstearic acids) were obtained from Aldrich and used without further purification. Fluorescence-quenching experiments of the δ-haemolysin bound to small unilamellar DPPC vesicles (prepared by the ethanol-injection method [17]) were performed for each spin label molecule at high (500 mM) and low (5 mM) phosphate buffer concentrations in order to study the penetration of the tryptophan residue of the δ-toxin within the bilayer. δ-Haemolysin was added to a dispersion of DPPC vesicles (at a mole ratio of δ-toxin to DPPC of 1 : 99). The samples were left at room temperature for 20 min to allow complete binding of δ-toxin to the vesicles. Aliquots of ethanolic spin label were successively added to samples of bound δ-toxin and the fluorescence intensity after each addition was measured. Fluorescence quenching was analysed according to the Stern-Volmer equation [20]:

$$(I_0/I)-1 = K_{SV}Q \qquad (1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively, Q is the bound quencher concentration and K_{SV} is the Stern-Volmer quenching constant. Plots of $(I_0/I)-1$ vs. Q are presented. Under the experimental conditions used, spin-label binding was substantially complete, as judged from control experiments where lipid vesicle concentration was varied [21].

Several attempts were made to label melittin using fluorescamine and Lucifer Yellow VS (both from Aldrich). Samples of melittin (approx. 0.1 mg/ml) in 20 mM phosphate buffer (pH 8.0) were incubated either with equimolar concentrations of Lucifer yellow VS, or aliquots of fluorescamine (8 mg/ml in acetone) were added while vortexing at room temperature. In both cases, an insoluble fluorescent precipitate formed rapidly. Similar precipitates resulted from attempts to dansylate the peptide in 0.1 M bicarbonate solution. Melittin bound to phospholipid vesicles was labelled with fluorescamine and with Lucifer yellow VS, and fluorescent dispersions resulted. Melittin was added to a sonicated dispersion of DPPC (0.7 mg/ml) in 20 mM phosphate (pH 8.0) to a ratio of 1 : 20 (w/w). Lucifer yellow VS (equimolar to melittin) was added, or an aliquot (10 μl/ml) of fluorescamine in acetone rapidly injected while vortexing the dispersion. Attempts to extract labelled melittin from freeze-dried lipid dispersions using chloroform/methanol mixtures gave insoluble products. Interestingly, melittin-containing vesicles labelled with Lucifer yellow VS showed a reversible increase in turbidity on heating above 42°C, the phase-transition temperature of DPPC. This is in marked contrast to the sharp decrease in turbidity seen when unlabelled vesicles containing no melittin are similarly heated.

Melittin was also fluorescently labelled in dispersions of egg-yolk lysophosphatidylcholine.

However, on dialysis to remove the lysolipid, the products precipitated.

Results

Large unilamellar vesicles (LUV) of DPPC prepared in the presence of sodium deoxycholate retained entrapped calcein for long periods, and could be kept for weeks at 4°C. For the experiments described, only freshly prepared vesicles were used. Vesicles prepared by the ethanol-injection method [17] also retained trapped calcein and behaved similarly to LUV, showing that the effects of peptides were not due to residual deoxycholate

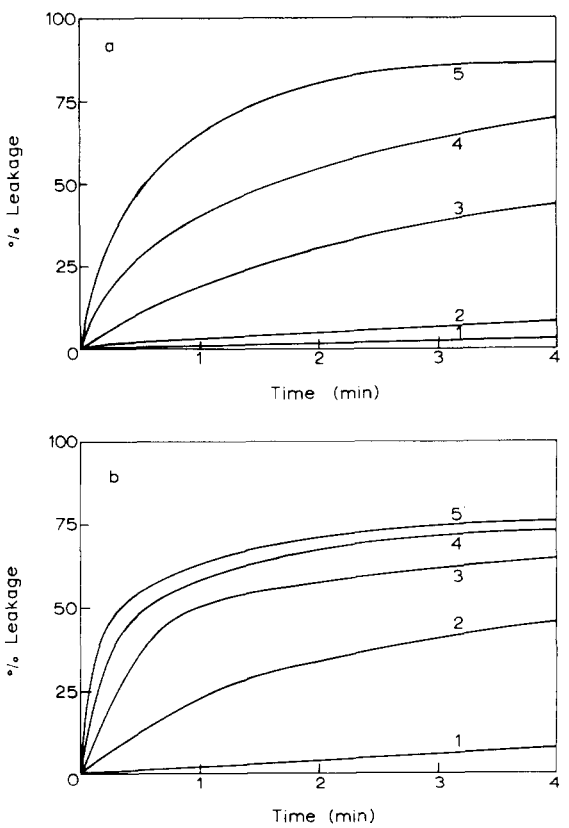

Fig. 1. Effect of increasing melittin concentrations upon the leakage rate of calcein from DPPC LUV. (a) in 5 mM phosphate/35 mM EDTA/0.02% NaN_3 (pH 7.4) and (b) in 500 mM phosphate/35 mM EDTA/0.02% NaN_3 (pH 7.4). DPPC concentration was 136 μM for all experiments. Melittin concentrations were (in both a and b): trace (1), 0.47 μM; (2), 0.93 μM; (3), 1.87 μM; (4), 3.73 μM; (5), 7.46 μM. Experiments were performed at 25°C.

in the LUV bilayer. LUV were used in experiments described because of their higher calcein-trapping efficiency for a given amount of lipid, and because the spontaneous leakage rate was lower above room temperature. On heating calcein-containing LUV to 60°C for 10 min, approx. 50% of trapped marker was released.

The influence of melittin on leakage rates for trapped calcein is shown in Fig. 1. Data are presented for both 5 mM (Fig. 1a) and 500 mM phosphate buffer (Fig. 1b) at pH 7.4, and in the presence of EDTA to osmotically balance-entrapped calcein.

The kinetics of calcein release depend both on ionic strength of buffer and on melittin concentration. At low phosphate concentrations, increase in melittin concentration increases initial leakage rate. In 500 mM phosphate buffer, leakage kinetics at low melittin concentration are broadly similar to those in 5 mM buffer at the same pH, in that a relatively slow linear release of calcein is seen. At higher melittin concentrations a more rapid 'burst' of leakage results. The effects seen with both buffers depend on the lipid-to-melittin ratio rather than on the molarity of melittin in solution at the peptide concentrations studied. This can be seen by comparison of Figs. 1a and 2a (for 5 mM buffer) and of Figs. 1b and 2b (for 500 mM buffer). Data for Fig. 2 were obtained using similar melittin-to-phospholipid mole ratios as Fig. 1, but with half the phospholipid present, with measurement of 100% leakage using Triton X-100 in both cases.

Differences in leakage kinetics are not a consequence of osmotic effects, since vesicles are prepared equilibrated with the buffer to be used in the measurements. Differences in osmotic pressure between entrapped vesicle contents and external buffer do influence leakage rates as shown in Fig. 3. In this case, osmotic balance with EDTA was not attempted, and leakage was measured in the presence and absence of external sucrose.

Fluorescence spectroscopy can be used to monitor melittin aggregation [3], since the tetramer emission is blue-shifted relative to that of the monomer. Such measurements (not shown) confirmed that melittin is solely monomeric in the concentration range used in leakage experiments with 5 mM phosphate buffer (pH 7.4). In the 500

mM phosphate buffer, however, melittin was partially monomeric at the lower melittin concentrations used (up to approx. 3 μM), and completely tetrameric thereafter.

The effects seen with melittin are in clear contrast to those seen with δ-haemolysin. Haemolysin effects on permeability of large unilamellar DPPC vesicles are shown in Fig. 4 for buffers of 5, 100 and 500 mM phosphate at the same pH and temperature. The lytic activity of the peptide is depressed in the 100 mM phosphate buffer relative to that in 5 mM buffer, and a lag phase in leakage is seen in both cases. In 500 mM phosphate buffer, there is no evidence of leakage on the time scale of the experiment. In Fig. 4, trace 5 is included for comparison and shows the effect of melittin on a similar batch of vesicles in 500 mM phosphate (pH 8.0).

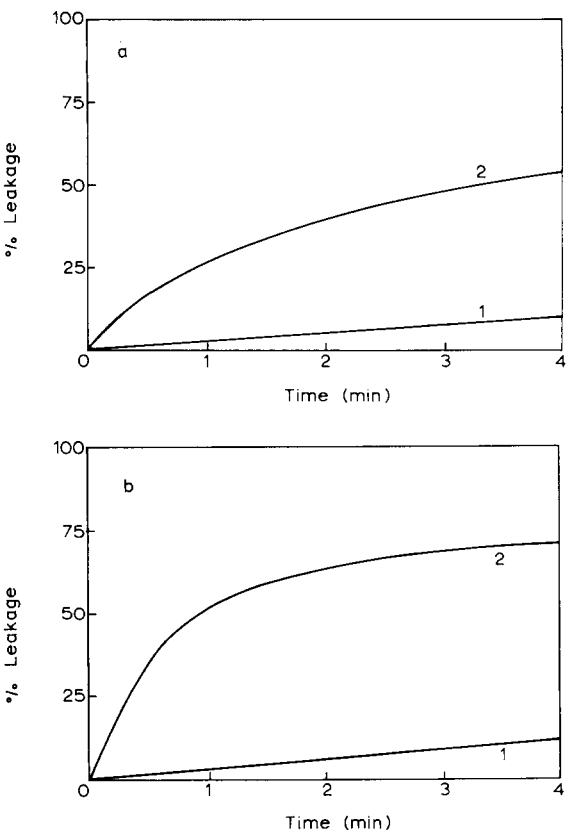

Fig. 2. Effect of increasing melittin concentrations upon the leakage rate of calcein from DPPC LUV. (a) in 5 mM phosphate/35 mM EDTA/0.02% NaN_3 (pH 7.4) and (b) in 500 mM phosphate/35 mM EDTA/0.02% NaN_3 (pH 7.4). DPPC concentration was 68 μM for all experiments. Melittin concentrations were (in both a and b): trace (1), 0.23 μM; (2), 0.93 μM. Experiments were performed at 25°C.

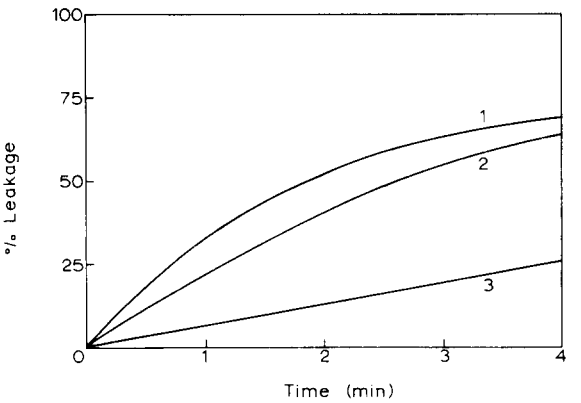

Fig. 3. Effect of sucrose upon leakage of calcein from DPPC LUV. DPPC concentration was 136 μM in all cases; buffer was 5 mM phosphate/5 mM EDTA/0.02% NaN_3 (pH 7.4) containing: trace (1), no sucrose; (2), 150 mM sucrose; (3), 300 mM sucrose. Melittin concentration was 3.73 μM in all cases. Experiments were performed at 25°C.

A comparison of the results presented in Figs. 1, 2 and 4 shows that the lytic potency of δ-

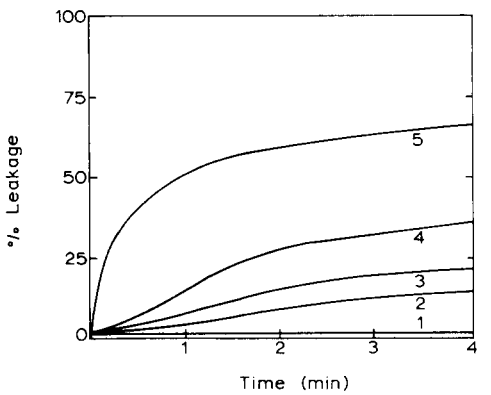

Fig. 4. Leakage of DPPC LUV-entrapped calcein induced by δ-haemolysin. Trace (1), δ-toxin concentration 4.6 μM, in 500 mM phosphate (pH 8.0); (2), δ-toxin concentration 0.66 μM, in 5 mM phosphate (pH 8.0); (3), δ-toxin concentration 4.6 μM, in 100 mM phosphate (pH 8.0); (4), δ-toxin concentration 2.3 μM, in 5 mM phosphate (pH 8.0). DPPC concentration was 136 μM in all cases. Trace (5) is included for comparison and shows the effect of melittin (1.3 μM, in 500 mM phosphate/5 mM EDTA (pH 8.0)) on a dispersion of DPPC LUV containing entrapped calcein as in the δ-toxin experiments. At this phosphate concentration, δ-haemolysin caused no leakage of calcein at concentrations even higher than that used in trace (1). Experiments were performed at 25°C.

haemolysin in buffer of low phosphate concentration is considerably less than that of melittin on a weight basis, as judged by calcein release from DPPC LUV. In buffer of high phosphate concentration, δ-haemolysin has no effect upon the leakage of calcein from DPPC LUV (Fig. 4, trace 1). The δ-toxin is similarly less efficient in causing haemolysis of washed bovine erythrocytes in isotonic phosphate-buffered saline (our observation).

The lack of activity of δ-haemolysin in 500 mM phosphate buffer is not a consequence of inhibition of binding, nor is the lag phase in calcein release at intermediate phosphate concentration reflected in binding kinetics. This can be shown in several ways. Fig. 5 shows the fluorescence-emission spectra of toxin in 5 mM phosphate buffer in the presence and absence of phospholipid vesicles. In this case, small unilamellar DPPC vesicles are prepared in the absence of calcein to avoid quenching of tryptophan fluorescence. The red shift and enhancement of tryptophan fluorescence shows that the peptide binds to lipid vesicles in this buffer. The fluorescence spectra in 500 mM phosphate buffer are very similar to those in 5 mM phosphate at the same pH (not shown, since they are not easily distinguished). Such steady-state spectra suggest that the environment around the tryptophan residue does not change markedly as phosphate concentration is increased. If a suitable wavelength is chosen, it is possible to fluorimetrically monitor the rate of toxin binding to lipid, and Fig. 6 shows such data. As a matter of convenience in this case, fluorescence changes on binding have been enhanced by inclusion in the bilayer of a lipid-soluble fluorescence quencher (an azobenzene-containing phospholipid analogue, the synthesis and properties of which are described elsewhere [22]). However, similar effects are seen without this addition. Although there is a lag phase in leakage of calcein in the presence of δ-haemolysin, no lag phase in binding is seen by this means.

We have also observed fluorimetrically that binding of the toxin is sensed by molecules in the hydrophobic region of the bilayer, even in 500 mM phosphate buffer. This was seen from the emission-intensity changes of a hydrophobic fluorescent probe embedded in the DPPC bilayer at low concentration. The probe used was N-DPHPC

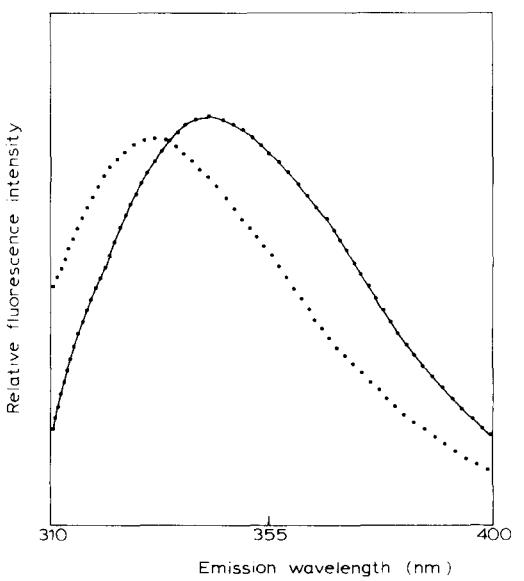

Fig. 5. Fluorescence emission spectra (uncorrected) of δ-haemolysin (2.3 μM) in 5 mM phosphate buffer (pH 8.0) (broken line), and in a dispersion of DPPC SUV (0.68 mM) in the same buffer at 25°C. The excitation wavelength was 280 nm. The spectra in 500 mM phosphate buffer cannot easily be distinguished from these and are therefore not shown.

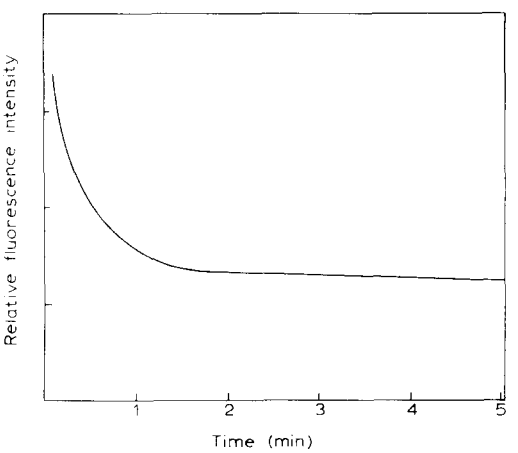

Fig. 6. Trace showing the rate of binding of δ-haemolysin to DPPC SUV. An azobenzene-containing phospholipid (absorption maximum at 332 nm) was incorporated within the SUV at 1 mol%. Total lipid concentration was 0.23 mM. δ-Haemolysin was added at time zero. δ-Toxin concentration in solution was 2.2 μM; buffer used was 20 mM phosphate (pH 8.0). The sample was excited at 280 nm, with detection at 330 nm, at 25°C.

(the synthesis and properties of which have been described previously [23]), a phospholipid analogue the chromophore of which is constrained to lie in the acyl chain region of the bilayer. Binding of δ-haemolysin causes a significant enhancement of N-DPHPC fluorescence emission, an effect which the δ-toxin shares with melittin (results are not shown). The origin of this fluorescence enhancement is at present unclear.

That the single tryptophan residue of δ-haemolysin is buried within the bilayer of DPPC vesicles at both high and low phosphate buffer concentrations is shown by spin-label fluorescence-quenching studies (Fig. 7). Binding of all spin labels used is substantially complete at the spin label-to-DPPC molar ratios used. Binding of spin labels to DPPC vesicles in 5 mM phosphate appeared to be less efficient than in 500 mM buffer at low lipid concentrations, and therefore in order to achieve complete binding of all spin labels, the DPPC concentration used in Fig. 7A was double that used in Fig. 7B. Fluorescence-quenching results are therefore compared as spin label (mol%) relative to DPPC.

The results show that the tryptophan fluorescence of the δ-toxin can be quenched to a certain degree at all depths within the bilayer. The *n*-doxylstearic acids are progressively more efficient at quenching the tryptophan of δ-haemolysin as the nitroxide is moved towards the carboxyl headgroup of the fatty acid, thus suggesting the predominant location of the fluorophore.

Binding of δ-haemolysin to small unilamellar DPPC vesicles below the phase-transition temperature does not appear to induce vesicle aggregation, even at a molar peptide-to-lipid ratio of 1 : 100. This is in marked contrast to the effect of melittin, which causes a rapid aggregation of vesicles, easily monitored by turbidity changes. Above the phase-transition temperature, light-scattering measurements (Fig. 8) with δ-haemolysin show a slow, highly non-linear increase in vesicle turbidity with time. This effect is seen both in 5, and in 500 mM phosphate buffer at the same

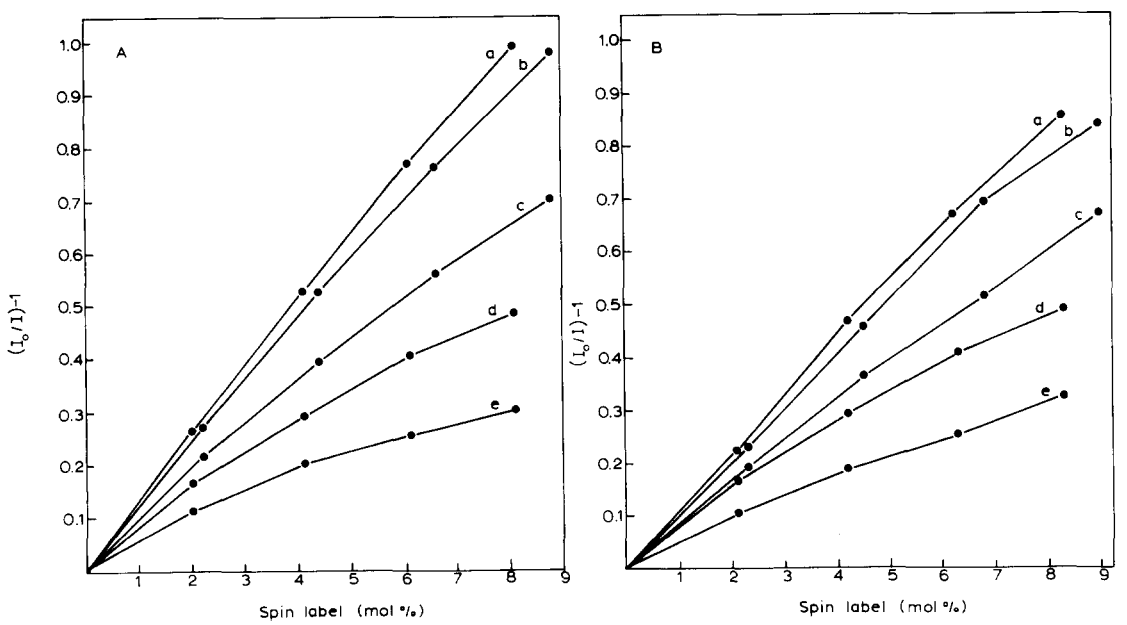

Fig. 7. Stern-Volmer plots showing quenching of fluorescence of DPPC-bound δ-haemolysin by *n*-doxylstearic acid spin labels in: (A) 5 mM phosphate buffer/0.02% NaN_3 (pH 8.0), and (B) 500 mM phosphate buffer/0.02% NaN_3 (pH 8.0). In both graphs, spin labels were: (a) $n = 5$; (b) $n = 7$; (c) $n = 9$; (d) $n = 12$; (e) $n = 16$. DPPC concentrations were 0.92 mM (A), and 0.46 mM (B). δ-Haemolysin concentration was approx. 1 mol% relative to DPPC in (A) and (B). Spin label concentrations (mol% relative to DPPC) are indicated on the horizontal axes. Experiments were performed at 25°C.

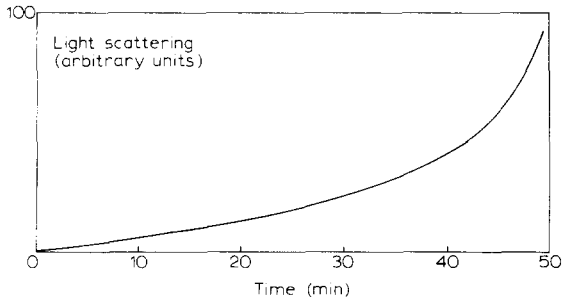

Fig. 8. Time-course of change of light scattering at 540 nm for a dispersion of DPPC SUV at 51°C. δ-Haemolysin concentration was 2.3 μM and DPPC was 0.68 mM in 20 mM phosphate buffer (pH 8.0). Similar results were seen in 500 mM phosphate (pH 8.0). No change in light scattering is seen on the time scale of this experiment for vesicles below the phase-transition temperature for DPPC in the presence of δ-haemolysin, nor are changes seen at 51°C in the absence of δ-toxin.

pH. Large lipid aggregates, visible by light microscopy, eventually form. The time scale of the effect is in marked contrast to the very rapid fusion of similar vesicles by melittin on heating through the phase-transition temperature [11,12].

Discussion

Melittin and δ-haemolysin are superficially similar, in that both are single tryptophan-containing peptides of similar molecular weight, and each contains recognisable sequences of hydrophobic and hydrophilic character. Each peptide is heat-stable, and each can be extracted into organic solvents such as chloroform/methanol without denaturation. Both peptides cause membrane lysis, and in this paper we explore conditions under which this membrane damage occurs for both peptides.

Both melittin and δ-haemolysin are capable of causing loss of trapped fluorescent marker from vesicles of gel-phase DPPC. However, the kinetics of leakage, measured under osmotically neutral conditions, differs for each peptide, and depends strongly on the ionic strength of phosphate buffer used. As shown in Results, a rapid 'burst' phase of leakage is seen under conditions where melittin is known to be tetrameric in solution. It seems reasonable to suggest that this rapid process reflects transient disorder induced by melittin binding to the bilayer, followed by a rearrangement, disaggregation of tetramer or translocation across the bilayer. Previous workers have put forward a model for melittin interaction with erythrocyte membranes, based on studies of haemoglobin leakage [8]. These workers have shown that tetrameric melittin is ineffective in causing haemolysis of erythrocytes, although it has been shown to bind. It has also been suggested that haemolysis induced by monomeric melittin proceeds by transient formation of pores after rapid initial binding, followed by translocation across the bilayer where dimeric species are responsible for a slower phase of haemoglobin loss [9]. The results presented here show that the melittin tetramer is clearly able to interact with lipid bilayer membranes with a concomitant perturbation of structure. The erythrocyte results suggest that other factors such as surface charge, or the presence of surface glycoproteins are involved in melittin interaction. Our own observations (not shown) suggest that the susceptibility of erythrocytes to melittin haemolysis depends on age and source of erythrocytes, and on the conditions used in experiments. The aggregation state of melittin within a lipid bilayer is not known with certainty under any condition, either in lipid vesicles or in erythrocytes, and in any case steady-state measurements would not reveal details of time-dependent processes such as those discussed in this paper .One approach to this problem might be the use of fluorescence resonance energy transfer with labelled melittin derivatives [24]. Such fluorescence measurements are capable of providing time-resolved information of proximity relationships of labelled molecules. However, although it was possible to fluorescently label melittin after binding to phospholipid vesicles, attempts to label the peptide in solution resulted in formation of insoluble products. Similar results have been reported previously by other workers [25]. Melittin labelled after binding to vesicles or lysophosphatidylcholine micelles was insoluble after removal of host lipid. It is known that the lytic potency of melittin is markedly affected by chemical modification [8], and, consequently, energy transfer results might be difficult to extrapolate to the effects of the native molecule.

In 5 mM phosphate buffer δ-haemolysin, like melittin, causes calcein loss from DPPC vesicles. δ-Haemolysin is less potent than melittin in caus-

ing calcein leakage, and kinetics are quite different showing a lag phase of several seconds before leakage ensues. This lag phase is not reflected in binding kinetics. At extremes of pH the lowest aggregate present in solution is known to be tetramer. At neutral pH and low phosphate concentrations much larger aggregates are present [26]. The aggregation state of the δ-toxin at high phosphate concentration is not known. In 500 mM phosphate we have shown that the toxin binds to DPPC vesicles but does not cause leakage of contents. Irrespective of phosphate buffer concentration, binding of toxin is sensed deep within the acyl chain region of the lipid bilayer. This is supported both by the enhancement of fluorescence of a membrane-bound phospholipid probe on peptide addition, and also by the ability of paramagnetic spin-labelled fatty acids to quench the peptide tryptophan fluorescence. These experiments suggest that the tryptophan residue of δ-haemolysin is predominantly located in the acyl chain region close to the lipid headgroup. Qualitatively similar results are seen under conditions where the peptide causes leakage of contents, and where it is inactive. In some cases, Stern-Volmer plots of fluorescence data show downward curvature. There are various possible causes of this. Linear Stern-Volmer plots are expected only in the case of a fluorophore having a single environment, decaying by a first order process. Quenching by 'static' processes gives rise to upward curvature of plots [21], while incomplete binding of quencher, multiple binding sites with differing quenching coefficients or multi-exponential decay characteristics can produce downward curvature. It is likely that more than one of these processes operates in the case of the data presented. Time resolved measurements using synchrotron radiation have shown the fluorescence decay characteristics of the δ-toxin to be multi-exponential (our observation). Quenching with spin-labelled fatty acids must be conducted under conditions where binding of quencher is complete, but at high concentrations of such acids a concentration-independent partition coefficient cannot be assumed. However, direct comparison of the initial slopes of the Stern-Volmer plots is reasonable, since the mole ratio of spin label to phospholipid is low and binding is substantially complete at lipid concentrations used in this study.

δ-Haemolysin does not appear to cause vesicle aggregation for gel-phase DPPC vesicles, unlike melittin. Above the phase-transition temperature, where melittin produces a very rapid vesicle fusion, light scattering shows a slow non-linear effect for δ-haemolysin. The authors have been granted beamtime at the Daresbury Laboratory Synchrotron Radiation Source to further study peptide and vesicle aggregation phenomena using small-angle X-ray scattering and time-resolved fluorescence spectroscopy.

Phospholipid vesicles are currently of interest as potential vehicles for drug-delivery purposes. In this context, selective modification of vesicle permeability, using peptides such as those described above, might have uses. In particular, low concentrations of melittin sensitise thermally-induced loss of contents of DPPC vesicles on heating through the phase-transition temperature [19], and also membrane fusion [10–12]. Any such applications would need to take account of possible allergenic effects of peptides and possible exchange with plasma components. Melittin itself has been reported to be a weak allergen [27], though effects have been reported in susceptible individuals sensitised to bee venom [28]. Allergenic properties of peptides bound to lipid vesicles are largely unknown, and these aspects are worthy of further investigation.

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