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MEMBRANE MATRIX DISRUPTION BY MELITTIN

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SUMMARY

Electron spin resonance spectroscopy was used to examine the interaction of the lytic polypeptide, melittin, with model membrane systems in order to determine the physical basis for its alterations of membrane properties. Sonically dispersed egg lecithin (phosphatidylcholine), phosphatidylserine, extracted *Escherichia coli* phospholipids, and isolated *E. coli* membranes were spin-labeled with fatty acid probes and treated with melittin solutions. Spectral analysis indicates that melittin induces a disruption of the phospholipid matrix without a significant change in the mobility of extensive regions of the acyl chains. This perturbation causes a melittin concentration dependent alteration in the capacity of the lipid matrix for spin label such that more of the probe is taken up. The effects are independent of phospholipid composition and the presence of membrane proteins. It is suggested that melittin participates in a hydrophobic interaction with the hydrocarbon region of the bilayer and acts as a disrupter of matrix ordering.

INTRODUCTION

Antibiotics interact with membranes in numerous ways. Determination of the molecular basis of membrane and antibiotic interaction is important for investigations using these compounds. Several antibiotics possess bactericidal properties due to lysis of the cell membrane and their effects on membrane structure have been examined by physical techniques such as magnetic resonance spectroscopy, differential scanning calorimetry, and monolayer studies. Melittin, a small polypeptide from bee venom, has also been shown to have lytic properties[1].

Model lipid membranes have been used to demonstrate that the protein toxins streptolysin S (refs 2 and 3) and staphylococcal α-toxin[4,5] disrupt the lipid matrix of membranes. Hauser *et al.*[6] used high resolution nuclear magnetic resonance spectroscopy to demonstrate that alamethicin, a cyclic polypeptide which induces ion transport in artificial and natural membranes, disorders the phospholipid lamellar structure in liposomes and immobilizes the hydrophobic regions. The presence of alamethicin molecules in the hydrocarbon region with its polar side chains at the lipid–water interface was demonstrated by ESR and electrophoretic techniques. The authors concluded that alamethicin induced a co-operative disordering of large regions of the phospholipid bilayer through hydrophobic inter-

actions. Pache *et al.*[7] studied the interaction of the peptide antibiotics polymyxin B and gramacidin S with lecithin liposomes by several physical techniques. It was suggested that the polymyxin B amino groups interact with the phospholipid polar head groups and the fatty acid tail inserts into the lipid bilayer resulting in a slight mobilization of the acyl chains. The cyclic peptide region apparently does not penetrate the hydrocarbon zone. Gramacidin S solubilizes the phospholipid vesicles and its interaction was presumed to be entirely electrostatic. After solubilization, the acyl chain mobility in the new aggregate was the same as in the liposome. Pache and Chapman[8] also examined the effects of chlorothicin; a molecule composed of 5-chloro-6-sialic acid, deoxysugars, and an aglycone; on lecithin liposomes. They concluded that this compound formed a strongly bound complex in the bilayer hydrophobic region with a concomitant immobilization of the lipid matrix.

The effect of melittin, a polypeptide of 26 amino acid residues in which the first 20 are hydrophobic and the last six hydrophilic, on liposomes was examined by Sessa and co-workers[9,10]. In an elegant study, the lytic action of melittin released ions or glucose from the interior of liposomes at melittin concentrations greater than 10^{-6} M regardless of the liposome phospholipid composition. Lipid monolayer techniques demonstrated melittin's marked affinity for lipid-water interfaces irrespective of the surface charge of the monolayer. These authors suggested that melittin disrupts liposomes by hydrophobic interactions. We wish to report an electron spin resonance (ESR) spectroscopic examination of the molecular interaction of melittin with model membrane systems.

MATERIALS AND METHODS

Melittin was obtained from Sigma Chemical Co., St. Louis, Mo. and dissolved in 0.1 M Tris–HCl buffer (pH 7.2). Egg lecithin ($>99\%$ pure) was the generous gift of Dr Craig Jackson and phosphatidylserine (92% pure, 0.1% cholesterol) was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. The spin label 12-nitroxide stearic acid was synthesized according to Keana *et al.*[11] and 5-nitroxide stearic acid was purchased from Synvar Assoc., Palo Alto, Calif. *Escherichia coli* phospholipids were extracted from isolated membranes by the method of Bligh and Dyer[12].

Sonically dispersed lipids were prepared in 0.1 M Tris–HCl buffer (pH 7.2) by dissolving the phospholipids in chloroform, evaporation of the solvent under nitrogen, addition of buffer, and sonication in an ice bath in 30-S bursts for a total of 5 min. *E. coli* H139 was grown, membranes prepared according to Mavis *et al.*[13], and suspended in 0.1 M Tris–HCl buffer (pH 7.2). All sonically dispersed lipids and membrane preparations were 10 mg/ml in phospholipid.

The spin labels 12-nitroxide stearic acid and 5-nitroxide stearic acid were added to the liposome and membrane preparations to a final concentration of 50 μM which gave maximal signal strength and negligible exchange broadening. Spectra were obtained on a Varian E-3 spectrometer at 23 °C before and after addition of melittin of varying concentrations (final melittin concentration in sample was 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6} M).

RESULTS

The spin-labeled acids employed in these studies are rigidly anchored at the carboxyl end to the phospholipid–water interface, displaying anisotropy due to incomplete averaging of the nitroxide axes in the slow tumbling range[14]. This permitted the observation of changes in partitioning of the spin label between the aqueous environment and the lipid matrix. Pache and Chapman[8] used the methyl ester derivatives, which give isotropic spectra, in order to calculate mobility changes. Although changes in mobility of spin labels displaying anisotropic motion in the slow tumbling mode may be more difficult to observe, the degree of immobilization in the controls was such that enhancement of immobilization upon addition of melittin was detectable.

Fig. 1A illustrates the spectrum obtained from egg lecithin (phosphatidylcholine) liposomes and 12-nitroxide stearic acid. The high and low field lines are heterogeneous, the arrows indicating aqueous signal components as determined by the hyperfine coupling. The spectrum is the result of the instrument summing an isotropic aqueous signal and an immobilized anisotropic signal from the lipid matrix (Fig. 2). Addition of 10^{-3} M melittin caused a disappearance of the mobile aqueous component and full visualization of the anisotropic immobilized signal (Fig. 1B). Melittin at 10^{-4} M induced a similar change to a lesser extent and lower concentrations gave spectra identical with the controls (Fig. 1C). Calculations of $T_{\parallel}/T_{\perp}$ (Fig. 2) showed a slight increase in the degree of immobilization of the

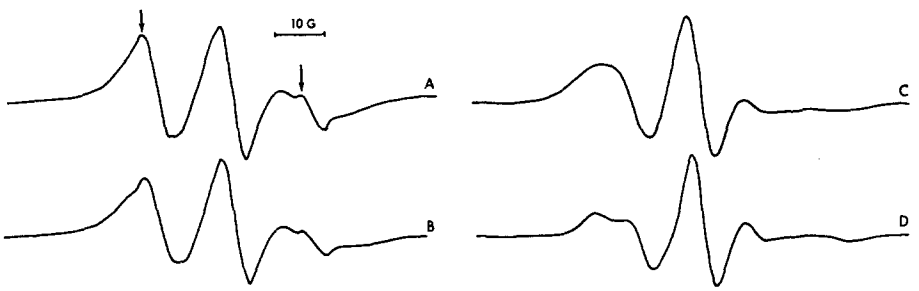

Fig. 1. ESR spectra of spin-labeled sonically dispersed phosphatidylcholine. A. Phosphatidylcholine *plus* 12-nitroxide stearic acid with arrows indicating aqueous, isotropic components as determined by the hyperfine coupling. B. Phosphatidylcholine–12-nitroxide stearic acid treated with 10^{-3} M melittin. C. Phosphatidylcholine–12-nitroxide stearic acid treated with 10^{-4} M melittin. D. Phosphatidylcholine *plus* 5-nitroxide stearic acid.

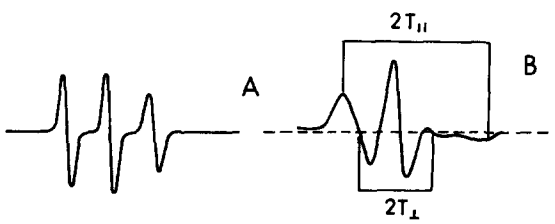

Fig. 2. ESR spectra of an isotropic, freely tumbling spin label (A) and of an immobilized signal demonstrating anisotropy in the slow tumbling range (B). Points of measurement of $T_{\parallel}$ and $T_{\perp}$ are shown.

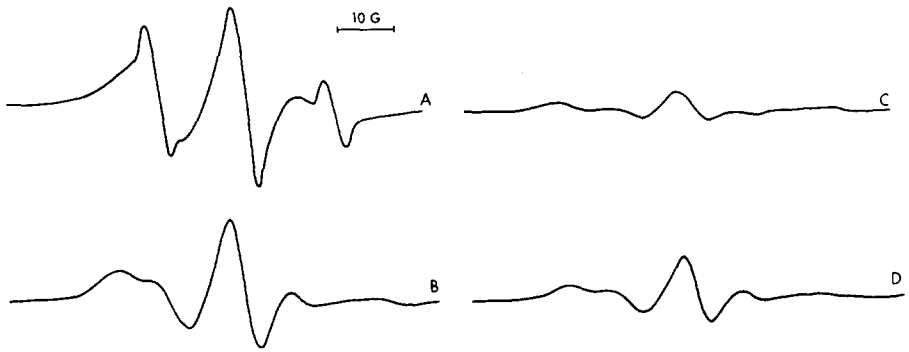

Fig. 3. ESR spectra of spin-labeled sonically dispersed phosphatidylserine. A. Phosphatidylserine *plus* 12-nitroxide stearic acid. B. Phosphatidylserine *plus* 5-nitroxide stearic acid. C. Phosphatidylserine–5-nitroxide stearic acid treated with 10^{-3} M melittin. D. Phosphatidylserine–5-nitroxide stearic acid treated with 10^{-4} M melittin.

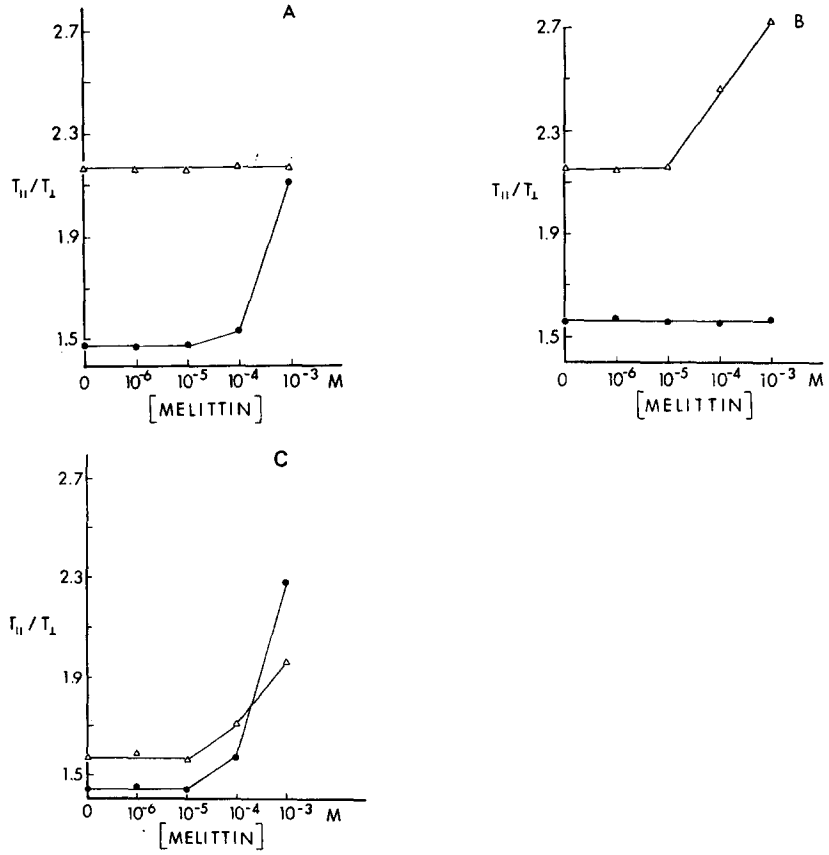

Fig. 4. $T_{II}/T_{\perp}$ values *vs* melittin concentration. A. Phosphatidylcholine–12-nitroxide stearic acid (●) and phosphatidylcholine–5-nitroxide stearic acid (△). B. Phosphatidylserine–12-nitroxide stearic acid (●) and phosphatidylserine–5-nitroxide stearic acid (△). C. Extracted *E. coli* phospholipids *plus* 12-nitroxide stearic acid (●) and isolated *E. coli* membranes *plus* 12-nitroxide stearic acid (△).

hydrocarbon component dependent on melittin concentration (Fig. 4A). Sonically dispersed phosphatidylcholine spin-labeled with 5-nitroxide stearic acid (Fig. 1D) gave spectra identical with phosphatidylcholine–12-nitroxide stearic acid *plus* 10^{-3} M melittin (Fig. 1B) and addition of melittin caused no changes in $T_{\parallel}/T_{\perp}$ values (Fig. 4A). The close proximity of the nitroxide moiety to the anchored carboxyl group in 5-nitroxide stearic acid causes the signal to be strongly immobilized and thus prevents increases in the degree of immobilization of the hydrocarbon region from being observed even if melittin enters the bilayer.

Sonically dispersed phosphatidylserine doped with 12-nitroxide stearic acid gave spectra (Fig. 3A) similar to phosphatidylcholine–12-nitroxide stearic acid (Fig. 1A) in that a mobile aqueous signal is superimposed over an anisotropic immobilized component. Addition of melittin had no effect on the spectra or the $T_{\parallel}/T_{\perp}$ values (Fig. 4B). The charge repulsion of the polar head groups may prevent penetration of the hydrophobic residues of melittin deep enough into the hydrocarbon matrix to effect the nitroxide moiety at C-12 or to locally disturb the lipid matrix sufficiently to allow more spin label to enter. Fig. 3B illustrates that the spectrum from sonically dispersed phosphatidylserine and 5-nitroxide stearic acid are similar to those from phosphatidylcholine–5-nitroxide stearic acid (Fig. 1D) due to the positional effect of the nitroxide group. The absence of an aqueous signal component when 5-nitroxide stearic acid is the spin label with phosphatidylcholine or phosphatidylserine may reflect a perturbation of the phospholipid packing by the bulky nitroxide near the anchoring polar head groups. A slight increase in immobilization proportional to the melittin concentration was observed (Figs 3E, 3D, and 4B). Although no such immobilization was seen with phosphatidylcholine–5-nitroxide stearic acid *plus* melittin because of the close proximity of the nitroxide moiety to the anchoring carboxyl, charge repulsion between the net anionic phosphatidylserine head groups and the polar nitroxide may distort the polar head group–water interface sufficiently to allow some melittin entry and subsequent effect on hydrocarbon chain mobility. The possibility of electrostatic interaction between the anionic phosphatidylserine molecules and the polar residues of melittin affecting the nitroxide of 5-nitroxide stearic acid near the interface cannot be excluded. In either case, melittin induced immobilization would occur in the phosphatidylserine–5-nitroxide stearic acid system and not in the phosphatidylcholine–5-nitroxide stearic acid system. The interpretation of molecular events affecting the 5-nitroxide stearic acid spin label is difficult due to its structure, especially with the added complication of a net charged polar head group interface.

Sonically dispersed *E. coli* phospholipids were spin-labeled with 12-nitroxide stearic acid and gave spectra (Fig. 5A) similar to phosphatidylcholine–12-nitroxide stearic acid (Fig. 1A). Melittin addition at 10^{-3} M and 10^{-4} M also gave results identical to sonically dispersed phosphatidylcholine in that the aqueous signal component disappeared (Figs 5B and 5C), and slight immobilization occurred (Fig. 4C). Spin-labeling of isolated *E. coli* membranes with 12-nitroxide stearic acid (Figs 5 D–5F) gave spectra identical to sonically dispersed *E. coli* phospholipids and to sonically dispersed phosphatidylcholine before and after melittin addition (Fig. 4C).

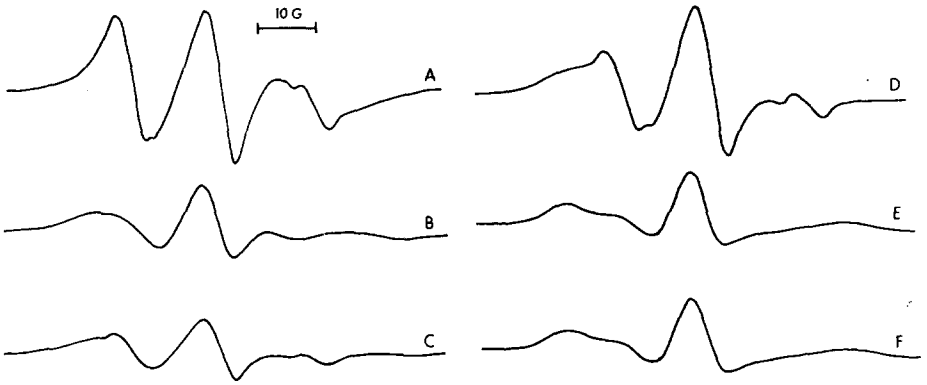

Fig. 5. ESR spectra of spin-labeled *E. coli* phospholipids and membranes. A. Phospholipids *plus* 12-nitroxide stearic acid. B. Phospholipids–12-nitroxide stearic acid treated with 10^{-3} M melittin. C. Phospholipids–12-nitroxide stearic acid treated with 10^{-4} M melittin. D. Membranes *plus* 12-nitroxide stearic acid. E. Membranes–12-nitroxide stearic acid treated with 10^{-3} M melittin. F. Membranes–12-nitroxide stearic acid treated with 10^{-4} M melittin.

DISCUSSION

Melittin is a small polypeptide (mol. wt. 3400) which associates in a tetramer in solution as the individual polypeptide chains are too small to assume a globular configuration with the hydrophobic residues buried[10]. The amino acid sequence indicates that the polypeptide chain is strongly amphiphilic. The ESR spectra show that melittin causes only a slight increase in immobilization of the spin label. The predominant ESR effect of melittin on sonically dispersed phosphatidylcholine, *E. coli* phospholipids, and *E. coli* membranes is to increase the lipid matrix's capacity to absorb spin label from the aqueous environment. The identical nature of this effect in sonically dispersed phosphatidylcholine and *E. coli* phospholipids, which have different polar head group compositions, and in *E. coli* membranes which contain bound protein and lipopolysaccharide, indicates the relative independence of this phenomenon upon surface charge. Therefore, the major interaction of melittin with phospholipid bilayers appears to be hydrophobic. That only minor changes in mobility occur upon melittin interaction with sonically dispersed lipids and *E. coli* membranes is of interest as other antibiotics such as chlorothicin and polymyxin B, which interact with bilayers hydrophobically, induce co-operative immobilization over large regions of the lipid matrix[7,8]. Similarly, intercalation of cholesterol and subsequent hydrophobic interaction produces significant immobilization[15].

Melittin affects the permeability properties of liposomes at concentrations as low as 10^{-6} M (ref. 10). In the present study, no changes in the ESR spectra were observed at 10^{-6} M, while changes were clearly seen at 10^{-4} M. Ion leakage could result from a few molecules of melittin interacting with a liposome. In such an instance, the percent of the liposome disturbed by melittin would be too small to be measured by ESR spectroscopy. Changes in the ESR spectra would only be seen when a larger number of melittin molecules interacting with a liposome cause

disturbances of a large percentage of the spin-labelled membrane matrix. This is due to instrument summing of signals from the entire sample. Sessa *et al.*[10] reported that liposomes treated with melittin at 10^{-4} M were observed by electron microscopy to be disrupted into lamellar aggregates. Thus at high melittin concentrations, total disruption of a fraction of the liposomes, dependent on the amount of melittin present, and reaggregation into lamellar arrays with bilayer regions would account for the melittin concentration dependent increased uptake of spin label without concomitant major changes in probe immobilization. Also, our studies didn't show a time-dependent change in the ESR spectra and permeability effects were time dependent at 10^{-6} to 10^{-4} M melittin. Possibly, therefore, at low melittin concentrations partial disturbance of each liposome occurs allowing ion leakage and at higher concentrations there is total disruption of the liposomes permitting greater incorporation of spin labels.

Melittin's ability at low concentrations to alter the lipid matrix allowing ion leakage, yet causing little change in the ESR spectra, suggests the following mechanism. Melittin contacts the polar head group–water interface and may initially undergo electrostatic interaction, permitting a possible dissociation of the tetramer, followed by conformation changes. The hydrophobic residues of the polypeptide chain could enter the hydrocarbon region of the lipid matrix and interact with the phospholipid acyl chains. The interaction is sufficient to stabilize the melittin's position, but not great enough to induce cooperative immobilization of large regions of the matrix. Thus at low concentrations of melittin little or no immobilization effects will be observed, but ion leakage through localized matrix disruptions could be measured. It seems reasonable that the energy of interaction between a hydrophobic polypeptide chain (with its dipoles) and fatty acid chains would be less than acyl chains with the hydrophobic chlorothicin, cholesterol, or the fatty acid chain of polymyxin B. Similarly, the insertion of a linear polypeptide into the bilayer would be expected to be less disruptive than a bulky, cyclic antibiotic such as alamethicin. At higher concentrations of melittin, intercalation of this lytic polypeptide disorders the lipid matrix sufficiently to totally disrupt the liposomes.

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