Lipopolysaccharides in Bacterial Membranes Act like Cholesterol in Eukaryotic Plasma Membranes in Providing Protection against Melittin-Induced Bilayer Lysis†

Daniel Allende and Thomas J. McIntosh*

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: Melittin is a small, cationic peptide that, like many other antimicrobial peptides, lyses cell membranes by acting on their lipid bilayers. However, the sensitivity to antimicrobial peptides varies among cell types. We have performed direct binding and vesicle leakage experiments to determine the sensitivity to melittin of bilayers composed of various physiologically relevant lipids, in particular, key components of eukaryotic membranes (cholesterol) and bacterial outer membranes (lipopolysaccharide or LPS). Melittin binds to bilayers composed of both zwitterionic and negatively charged phospholipids, as well as to the highly charged LPS bilayers. The magnitude of the free energy of binding ($\Delta G°$) increases with increasing bilayer charge density; $\Delta G° = -7.6$ kcal/mol for phosphatidylcholine (PC) bilayers and -8.9 to -11.0 kcal/mol for negatively charged bilayers containing phosphatidylserine (PS), phospholipids with covalently attached polyethylene glycol (PEG-lipids), or LPS. Comparisons of these data show that binding is not markedly affected by the steric barrier produced by the PEG in PEG-lipids or by the polysaccharide core of LPS. The addition of equimolar cholesterol to PC bilayers reduces the level of binding ($\Delta G° = -6.4$ kcal/mol) and reduces the extent of melittin-induced leakage by 20-fold. LPS and 1:1 PC/cholesterol bilayers have similar high resistance to melittin-induced leakage, indicating that cholesterol in eukaryotic plasma membranes and LPS in Gram-negative bacteria provide strong protection against the lytic effects of melittin. We argue that this resistance is due at least in part to the similar tight packing of the lipid acyl chains in PC/cholesterol and LPS bilayers. The addition of bacterial phospholipids to LPS bilayers increases their sensitivity to melittin, helping to explain the higher sensitivity of deep rough bacteria compared to smooth phenotypes.

A variety of organisms, from insects to humans, deploy a chemical barrier at the first possible point of contact with microbes. This line of defense depends largely on small, secreted peptides that are effective against a wide range of microbial invaders (*1*). Many of these peptides do not act primarily on specific processes or proteins in the invading microbe, but rather begin killing the bacterium by disrupting its cell membrane (*2*). Specifically, the main target of these peptides is the lipid bilayer of the microbe's membrane (*3*, *4*).

The action of these antimicrobial peptides can be quite rapid. For example, at a concentration 4-fold higher than the minimum inhibitory concentration (MIC), which is often a few micromolar, some antimicrobial peptides can kill up to 99.9% of bacteria within 5 min (*5*). Moreover, because these peptides work on basic physical properties of the membrane bilayer to initiate cell death, microbes have had difficulty in developing resistance to such peptides (*1*). As noted by Zasloff (*6*), "Despite their ancient lineage, antimicrobial peptides have remained effective defensive weapons, confounding the general belief that bacteria, fungi and viruses can and will develop resistance to any conceivable substance." Thus, one reason for the recent interest in antimicrobial peptides is the rapid emergence of bacterial strains that are resistant to many conventional antibiotics (*1*, *6*, *7*).

One of the best-characterized antimicrobial peptides is melittin, which is the focus of this study. Melittin is a linear 26-amino acid amphipathic peptide derived from honeybee venom that is a potent antibiotic (minimum inhibitory concentration of 5 μM for *Escherichia coli*) (*8*). Melittin is surface active, binds to both electrically neutral and negatively charged lipid bilayers (*9*−*12*), forms an α-helix upon binding (*13*), and induces channel formation in bilayers (*14*).

The interaction of antimicrobial peptides such as melittin with lipid bilayers can be modulated by the lipid composition (*3*, *6*, *11*, *15*, *16*), which is perhaps a reason that some peptides are lytic to bacteria, but not to the host cell or to eukaryotic cell membranes (*1*, *17*, *18*). The key factors that provide the specificity of these peptides to microbial membranes, rather than to the membranes of the host cells, are not well understood (*19*). However, one possible reason for the relative insensitivity of eukaryotic plasma membranes to antimicrobial peptides is the presence of cholesterol in these membranes (*6*). Cholesterol modifies the physical properties of membranes by decreasing the area per phospholipid molecule and increasing the bilayer compressibility modulus (*20*). Indeed, experiments have shown that the presence of cholesterol reduces the bilayer binding efficiency and the level of leakage caused by the antimicrobial peptides melittin (*21*) and magainin 2 (*22*), although melittin is more

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* To whom correspondence should be addressed. Phone: (919) 684-8950. Fax: (919) 681-9929. E-mail: T.McIntosh@cellbio.duke.edu.

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hemolytic to human red blood cells than is magainin (*7*, *23*).

In Gram-negative bacteria, an outer membrane, surrounding the cytoplasmic membrane, provides an additional barrier that plays an important role in bacterial antibiotic resistance and protects strains of these bacteria against many antimicrobial peptides, including melittin (*8*, *24*). In particular, Gram-negative bacteria are more resistant to melittin than Gram-positive bacteria (that lack this outer membrane) (*8*). This outer membrane has an unusual lipid composition, as its outer monolayer contains high concentrations of lipopolysaccharide (LPS),[1] a complex molecule composed of polysaccharides covalently linked to a lipid moiety, lipid A (*25*). Although some Gram-negative bacteria are not very susceptible to antimicrobial peptides, presumably because of this protective LPS coating, the susceptibility can vary among mutant strains. In particular, "smooth" and "rough" mutants are less susceptible than "deep rough" mutants to antimicrobial peptides such as melittin (*24*, *26*−*29*). There are at least two major differences in the lipids in the outer monolayers of the outer membranes of these different mutants. The first difference is in the structure of the LPS molecule, in particular, the length and complexity of the hydrophilic polysaccharide region. In smooth bacteria, the LPS molecule contains an O-antigen tetrasaccharide repeating unit attached to a complete polysaccharide core region; in rough bacteria (for instance, the Ra chemotype), the O-antigen repeat is not present, and in deep rough mutants (for instance, the Rd chemotype), both the O-antigen repeating unit and the outer polysaccharide core region are absent (*30*). A second difference between these mutants is that the phospholipid:LPS ratio in the outer monolayer of the outer membrane is larger for deep rough bacteria than for smooth or rough mutants (*31*). Therefore, the different susceptibilities of these mutants could be due to either differences in the structure of their LPS molecules or differences in the LPS:phospholipid composition in their outer monolayers.

Another factor to consider for both eukaryotes and Gram-negative bacteria is the presence of negatively charged lipids in the bilayer, as melittin is a basic peptide with six positive residues. It is known that positively charged peptides bind more strongly to negatively charged lipids than to neutral lipids (*10*, *32*−*37*), and previous studies (*15*, *38*−*40*) have shown that negatively charged phospholipids can strongly influence melittin-induced leakage from vesicles. Both eukaryotic plasma membranes and bacterial outer membranes contain negatively charged phospholipids, and LPS molecules contain multiple negative charges.

There are three major goals of this paper. The first is to determine how specific lipid components of both the eukaryotic plasma membrane (phospholipids and cholesterol) and the outer membrane of Gram-negative bacteria (bacterial phospholipids and LPS) modulate the interactions of melittin with the membrane bilayer. As noted above, of these two types of membranes, only the eukaryotic membrane contains cholesterol and only the outer bacterial membrane contains LPS. Moreover, the phospholipid compositions of the two membranes are quite different, as a major component of eukaryotic plasma membranes is phosphatidylcholine (PC), whereas the bacterial membrane contains a mixture of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin. A second goal is test the hypothesis that the LPS in bacterial outer membranes performs a role similar to that of cholesterol in eukaryotic plasma membranes by stabilizing the membrane against peptide-induced leakage. Third, we test the hypothesis that deep rough mutants are susceptible to antimicrobial peptides because of the presence of phospholipids in the outer monolayer of their outer membrane.

To address these goals, we measure both the extent of binding and the effects on bilayer integrity (vesicle leakage) of melittin for a variety of bilayer compositions. To determine the role of cholesterol in the plasma membrane of eukaryotic cells, we compare the binding and melittin-induced leakage of bilayers containing phospholipids with and without cholesterol. To test the effects of LPS structure and LPS−phospholipid interactions, we compare the melittin-induced leakage of vesicles composed of deep rough (Rd) LPS in the presence and absence of bacterial phospholipids. Finally, to determine the possible steric effects of the hydrophilic polysaccharide core of LPS, we perform binding and leakage experiments on lipids containing covalently attached polyethylene glycol chains (PEG-lipids) with molecular weights similar to those of the LPS polysaccharides.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (EPC), brain phosphatidylserine (PS) (sodium salt), cholesterol, total bacterial phospholipid extract (BPL), and PEG-lipid (PEG-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). The BPL, isolated from *E. coli*, consists of 67% phosphatidylethanolamine, 23% phosphatidylglycerol, and 10% cardiolipin (Avanti Polar Lipids). The PEG-lipid consists of polyethylene glycol with a molecular weight of 2000 covalently attached to the amine group of 1-palmitoyl-2-oleoylphosphatidylethanolamine. LPS from the rough phenotype *E. coli* EH100 (Ra mutant) and LPS from deep rough phenotype *Salmonella minnesota* R7 (Rd mutant) were purchased from Sigma Chemical Co. (St. Louis, MO). The peptide melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) was purchased from Sigma Chemical Co. and used without further purification. Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] and the dye sulforhodamine B (SRB) were purchased from Sigma Chemical Co.

Preparation of Liposomes. Multilamellar vesicles (MLVs) were made using the following procedure. For lipid mixtures containing phospholipids, cholesterol, or PEG-lipid, the lipids were codissolved in chloroform and the solvent was removed by rotary evaporation. The lipids were then hydrated in 5 mM Hepes and 25 mM KCl buffer (pH 7.4) and extensively vortexed. In the case of LPS, the dry lipid was suspended in buffer, heated to 60 °C, and vortexed. The LPS/bacterial phospholipid mixture (1:1) was dissolved in a petroleum ether/chloroform/phenol mixture (8:5:2) and dried in a vacuum overnight. The lipid film was hydrated at 60 °C with the same buffer and vortexed.

For peptide binding experiments, large unilamellar vesicles (LUVs) were prepared from MLVs by the following

[1] Abbreviations: LPS, lipopolysaccharide; PC, phosphatidylcholine; EPC, egg yolk phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; BPL, bacterial phospholipids; PEG, polyethylene glycol; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles; SRB, sulforhodamine B.

procedures. MLVs, at concentrations of 5−15 mg/mL, were frozen and thawed five times and extruded with a thermobarrel extruder (Northern Lipids, Vancouver, BC) 10 times through two stacked 0.1 μm polycarbonate filters. To ensure proper encapsulation, the extrusions were performed at temperatures above the main phase transition temperature of the lipid (*41*). For vesicles composed of EPC, PS, cholesterol, or PEG-lipids, the extrusions were performed at 20 °C, whereas for LPS and LPS/BPL mixtures, the extrusions were performed at 50 °C. Phospholipid concentrations in the LUVs were measured by phosphate analysis (*41*).

For leakage experiments, LUVs encapsulating sulforhodamine B (SRB) were produced by the procedure by Rex et al. (*37*). Briefly, lipids were hydrated in 70 mM SRB, 5 mM Hepes, and 25 mM KCl (pH 7.4) to give a 10 mM liposomal suspension. [For some experiments, 5 mM EDTA was added to the buffer to inactivate possible phospholipase A_2 contamination (*38*); however, no difference was observed in leakage results with or without EDTA.] LUVs were produced by extrusion as described above. To prevent leakage of SRB from the vesicles that might occur at the lipid phase transition, LUVs were maintained at a temperature above the lipid's melting temperature during and after extrusion. The SRB-containing vesicles were separated from free SRB by size-exclusion chromatography using a column filled with Sephadex G-50 fine gel swollen in an isosmotic buffer [5 mM Hepes and 130 mM KCl (pH 7.4)]. The osmotic contribution of the fluorophore was balanced by increasing the salt concentration of the buffer to avoid potential artifacts (*43*). Phospholipid concentrations were measured by phosphate analysis (*42*).

X-ray Diffraction Experiments. X-ray diffraction experiments were used to characterize the structure and phase of the bacterial lipids (BPL, Rd LPS, and 1:1 Rd LPS/BPL mixture) used in our binding and leakage studies. For each lipid composition, MLVs were prepared as described above, except that they were hydrated in water rather than buffer. Oriented lipid multilayers were made by drying a drop of the lipid-water MLV suspension onto a curved glass substrate (*44*−*46*). The oriented multilayers were then mounted in a temperature-controlled chamber in which the relative humidity was controlled by saturated salt solutions (*45*−*47*). X-ray patterns were recorded on Kodak DEF X-ray film contained in flat-plate cassettes.

Peptide Binding Measurements. For peptide binding experiments, melittin was used at a concentration of 10 μM in 5 mM Hepes and 25 mM KCl (pH 7.4). At this concentration, melittin is monomeric (*48*, *49*). The binding of melittin to LUVs was assessed with an ultrafiltration assay (*50*) that separated lipid and lipid−peptide complexes from free peptide with Centricon-10 filters (Millipore Inc.). Melittin was added to LUVs and incubated for 30 min before a 1 h centrifugation at 6000*g* through the filter was carried out (*51*, *52*). For all lipid preparations, the peptide incubation and centrifugation procedures were performed at a temperature above the lipid's phase transition temperature. The free melittin concentration in the eluate was determined by measuring the tryptophan fluorescence at an emission wavelength of 356 nm in a Jobin Yvon SPEX fluorometer (DM-3000) and comparing the measured fluorescence to the fluorescence intensity of melittin standards. A correction was made for the small amount (less than 10% of the peptide) that was found to bind to the filters in control experiments in the absence of lipid. The amount of melittin bound to the lipid was determined by subtracting the free peptide concentration from the total peptide concentration. Control experiments showed that no detectable lipid was found in the eluate by phosphate analysis (*42*), either in the presence or in the absence of melittin.

The molar partition coefficient K_p was calculated from binding measurements as described by Ladokhin (*53*). Under conditions where the molar concentration of peptide in the bilayer is much smaller than the molar concentration of lipid, the mole fraction partition coefficient (K_p) can be written as

$$K_p = (P_{bil}W)/(P_{wat}L) \quad (1)$$

where P_{bil} and P_{wat} are the bulk molar concentrations of peptide in the bilayer and water phases, respectively, and L and W are the molar concentrations of lipid and water, respectively. For these LUVs, L was taken to be 50% of the total lipid concentration. The free energy of transfer was calculated from

$$\Delta G^\circ = -RT \ln(K_p) \quad (2)$$

where R is the molar gas constant and T is the temperature in kelvin.

Leakage Experiments. The leakage induced by melittin was assessed by recording the release of vesicle-encapsulated SRB following published procedures (*21*, *37*). Experiments were performed at 20 °C for most lipids and at 50 °C for vesicles containing LPS, temperatures above each lipid's phase transition temperature. The fraction loaded with SRB that eluted from the column was diluted with isosmotic buffer [5 mM Hepes and 130 mM KCl (pH 7.4)] to obtain a range of lipid concentrations (1−3 μM) in the cuvette. The high concentration of SRB (70 mM) leads to self-quenching of its fluorescence, resulting in a low background fluorescence intensity of the liposome dispersion (I_B). The addition of melittin caused the release of SRB to the medium. SRB leakage was monitored by measuring the increased fluorescence intensity (I_F) of the dye into the medium. After 5 min, Triton X-100 (0.1 vol %) was added to obtain a 100% SRB leakage value (I_T). The percentage of SRB release was calculated according to

$$\% \text{ release} = (I_F - I_B)/(I_T - I_B) \times 100\% \quad (3)$$

The excitation and emission wavelengths of SRB were 560 and 590 nm, respectively. For all fluorescence experiments, the temperature was controlled with a temperature cell and the sample was continually stirred with a small stirring bar.

RESULTS

X-ray Diffraction. X-ray patterns were recorded at 98% relative humidity from bacterial phospholipid (BPL) at 20 °C and from Rd LPS or the 1:1 Rd LPS/BPL mixture at 45 °C, a temperature above the melting transition of Rd LPS (*54*). Each of these specimens produced a diffraction pattern consisting of two to seven sharp orders of a single lamellar repeat period and a single broad wide-angle band centered at 4.5 Å. Such patterns are consistent with the presence of liquid-crystalline phase bilayers (*55*). The repeat periods were 49.5, 65.2, and 62.2 Å for BPL, Rd LPS, and the 1:1 Rd

Table 1: Binding and Leakage Data for Melittin with LUVs

lipid	$\Delta G°$ (kcal/mol)	% leakage[a]
EPC	-7.6 ± 0.1	72.9 ± 1.9
EPC/cholesterol (1:1)	-6.4 ± 0.2	2.7 ± 0.9
EPC/PS (85:15)	-8.9 ± 0.3	53.9 ± 2.0
BPL	-8.7 ± 0.1	11.4 ± 0.3
EPC/PEG-2000 (85:15)	-9.2 ± 0.4	10.4 ± 1.6
Rd LPS	-10.0 ± 0.3	4.1 ± 0.5
Ra LPS	-11.0 ± 0.7	−
Rd LPS/BPL (1:1)	-9.0 ± 0.5	12.3 ± 0.3

[a] Represents % SRB released after 5 min for a 200:1 lipid:peptide ratio.

LPS/BPL mixture, respectively. The relatively large repeat periods for the LPS-containing bilayers are due in part to the width of the polysaccharide headgroup (*41*). Thus, at the temperatures that were used for the binding and leakage studies, the bacterial lipid systems (BPL, Rd LPS, and 1:1 Rd LPS/BPL mixture) formed single lamellar phase liquid-crystalline bilayers. That is, there was no three-dimensional phase separation in any of these samples. Previous X-ray studies have shown that the other bilayer systems used in our binding and leakage studies also consist of single-phase liquid-crystalline bilayers (*56*−*59*).

Binding of Melittin to Lipid Bilayers. Table 1 presents the free energy of transfer ($\Delta G°$) of melittin from the aqueous phase to LUV bilayers. These data show that the partitioning of melittin depended strongly on the bilayer composition. In terms of electrically neutral bilayers, melittin partitioned into egg phosphatidylcholine (EPC) bilayers with a free energy of transfer ($\Delta G°$) of -7.6 ± 0.1 kcal/mol (mean $\pm$ standard deviation, $n = 4$ experiments). The incorporation of equimolar cholesterol into EPC bilayers caused a decrease in magnitude in $\Delta G°$ from -7.6 to -6.4 kcal/mol.

The free energy of transfer of melittin into several types of negatively charged bilayers was greater (Table 1). The inclusion of 15% of the negatively charged lipid phosphatidylserine (PS) or PEG-lipids into EPC increased $\Delta G°$ to -8.9 and -9.2 kcal/mol, respectively. The molar free energy for transfer of melittin to bacterial phospholipids (BPL), which contain the negatively charged lipids PG and cardiolipin, was -8.7 kcal/mol. There was a large free energy of transfer to bilayers of both rough Ra LPS and deep rough Rd LPS; $\Delta G°$ was -11.0 kcal/mol for Ra LPS and -10.0 kcal/mol for Rd LPS. The molar free energy for transfer of melittin into bilayers composed of the 1:1 Rd LPS/BPL mixture was -9.0 kcal/mol, which was between the $\Delta G°$ values obtained for Rd LPS and BPL bilayers.

Leakage Studies from Electrically Neutral Vesicles. The ability of melittin to induce leakage from LUVs was studied using the self-quenching properties of sulforhodamine B (SRB). A typical curve of SRB leakage as a function of time is shown in Figure 1 for a 200:1 EPC:melittin ratio. Although a complete kinetic study was not performed, it is clear the curve can be subdivided into three sections. The first region is the flat background intensity (I_B) of entrapped SRB in self-quenching concentrations before the addition of melittin. In the second region of the curve, the addition of melittin caused the leakage of SRB, leading to an increase in fluorescence intensity (I_F). The fluorescence increased rapidly in the first minute or two after melittin addition, and then leveled off to a nearly constant value. The third region of

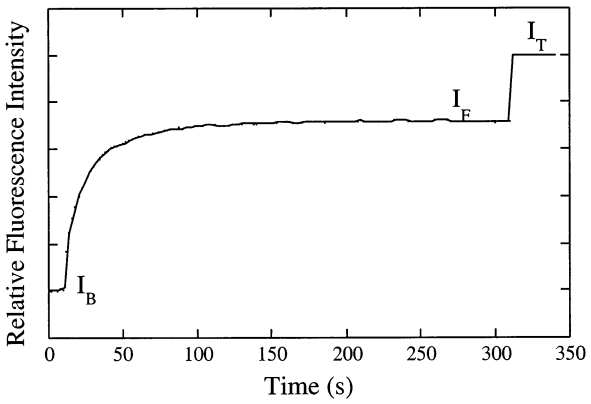

FIGURE 1: Typical SRB leakage curve for EPC LUVs. The baseline intensity is noted I_B. At 10 s, melittin at a 200:1 EPC:melittin ratio was added and the fluorescence intensity rapidly increased until it reached a steady state value, I_F. At 310 s, detergent was added and the total fluorescence intensity reading (I_T) was obtained.

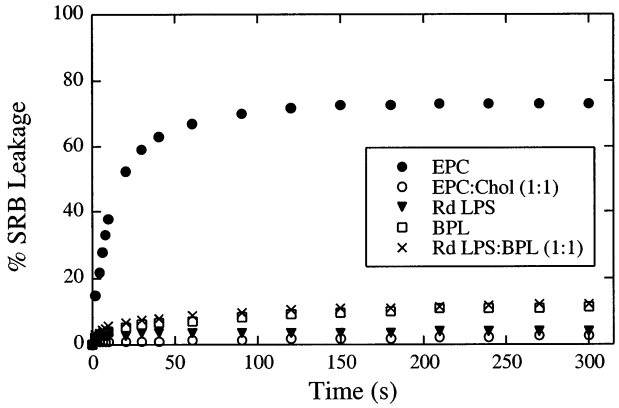

FIGURE 2: Plots of SRB leakage from LUVs composed of EPC, a 1:1 EPC/cholesterol mixture, Rd LPS, BPL, and a 1:1 Rd LPS/BPL mixture for lipid:melittin ratios of 200:1. The melittin was added at time zero. Note the similarity of the leakage profiles for 1:1 EPC/cholesterol and LPS bilayers. Values are the means of three or four measurements.

the plot shows the total fluorescence intensity (I_T) or 100% release caused by the complete disruption of all vesicles by the addition of Triton X-100. Similar profiles have been observed for melittin-induced leakage of SRB from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) LUVs (*60*), carboxyfluorescein from EPC LUVs (*15*), or calcein from POPC LUVs *(40)*.

The percentage of SRB released from the LUVs depended on the lipid:peptide ratio. For a 200:1 EPC:melittin ratio, $\sim 72.9 \pm 1.9\%$ ($n = 4$) of SRB was released after 5 min (Table 1). This is in quantitative agreement with previous studies (*39*, *40*). For a 400:1 EPC:melittin ratio, $\sim 52.0 \pm 0.6\%$ ($n = 3$) of the marker was released after 5 min. A similar release from small unilamellar vesicles (SUVs) was reported by Ohki (*38*) with carboxyfluorescein. For all further experiments, a 200:1 lipid:peptide ratio was used.

The addition of equimolar cholesterol into EPC bilayers decreased the lytic ability of melittin to 2.7% (Figure 2 and Table 1). This is in agreement with previous results in which the lytic power of melittin is inhibited and dependent on the concentration of cholesterol in the membrane (*21*).

Effect of Negative Charge on Melittin-Induced Leakage. Next we investigated the membrane leakage induced by melittin in the presence of negatively charged lipids.

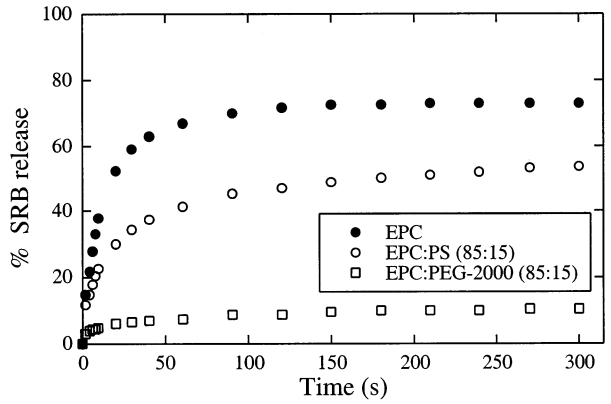

FIGURE 3: Plots of SRB leakage from EPC, 85:15 EPC/PS, and 85:15 EPC/PEG-2000 LUVs at lipid:melittin ratios of 200:1. The melittin was added at time zero. Values are the means of three to six measurements.

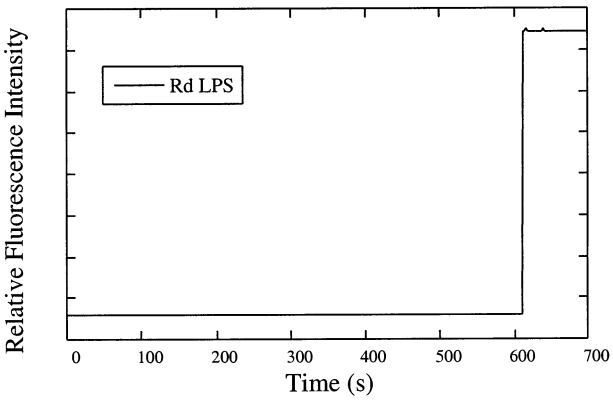

FIGURE 4: Demonstration of SRB encapsulation for LUVs composed of Rd LPS. The detergent was added at 610 s.

Comparison of melittin-induced leakage between neutral and negatively charged lipids is presented in Figure 3 and Table 1. Despite the favorable electrostatic interaction between melittin and bilayers containing 15% PS and 15% PEG-2000 (Table 1), the presence of these charged lipids decreased the extent of release of SRB. The inclusion of 15% PS in EPC vesicles reduced the lytic activity of melittin from 72.9 to 53.9%. A similar protective effect has been previously observed for the negatively charged lipids PS and PG (*15*, *38*−*40*). The inhibition of melittin-induced leakage was even more pronounced with the inclusion of 15% PEG-2000, as the extent of release of SRB was reduced to 10.4% (Table 1). This result is in agreement with previous studies of melittin-induced leakage of SRB from POPC liposomes in which the lytic activity of melittin decreased with the addition of PEG-2000 and PEG-5000 (*37*).

Effect of LPS and E. coli Phospholipids on Melittin-Induced Leakage. A requisite for the testing of bilayer leakiness is the ability to form sealed liposomes. At 45 °C, a temperature above its main phase transition temperature (32−33 °C), Rd LPS formed very stable, sealed vesicles (Figure 4). In the absence of melittin, there was no appreciable leakage for 10 min, but there was an abrupt increase in fluorescence after treatment with detergent.

Figure 2 compares the leakiness caused by melittin to those of LUVs composed of Rd LPS, BPL, and a 1:1 Rd LPS/BPL mixture. For Rd LPS bilayers, there was only 4.1% leakage of SRB, compared to 72.9% with EPC bilayers. Significantly, the leakage from Rd LPS bilayers was similar to that from EPC/cholesterol bilayers. The bacterial phospholipids (BPL) showed a leakiness 11.4% greater than that of Rd LPS, but considerably lower than that of EPC vesicles (Table 1). The incorporation of equimolar BPL into the Rd LPS vesicle increased the leakiness of vesicles to a value of 12.3%, similar to the leakage from bilayers composed of pure BPL (Figure 2).

DISCUSSION

The data presented in this paper show that both melittin binding and melittin-induced bilayer leakage depend strongly on the bilayer lipid composition. The data demonstrate that specific lipid components of eukaryotic plasma membranes (cholesterol) and the outer membranes of Gram-negative bacteria (LPS) provide protection against melittin-induced bilayer disruption and leakage.

For baseline measurements, we studied the interactions of melittin with egg phosphatidylcholine (EPC), since phosphatidylcholine is the most common phospholipid in many eukaryotic plasma membranes and EPC has a hydrocarbon chain composition that is typical of these membrane lipids. Our $\Delta G°$ value of −7.6 kcal/mol is in close agreement with previous measurements of −7 to −8 kcal/mol for melittin partitioning into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) vesicles (*9*, *13*, *61*−*64*). At a 200:1 lipid:peptide ratio, melittin caused 72.9% leakage of SRB from sealed EPC vesicles.

The addition of equimolar cholesterol to EPC bilayers caused a decrease in both the free energy of melittin binding (Table 1) and the extent of melittin-induced leakage. Benachir et al. (*21*) also found an inhibition of the lytic power of melittin by the addition of 30 mol % cholesterol to PC bilayers, which they attributed to a cholesterol-induced reduction in the level of melittin binding. Since there is no net charge on either EPC or 1:1 EPC/cholesterol vesicles, the observed changes in binding and leakage cannot be attributed to electrostatics. Instead, it is likely that both the decreased binding and decreased leakage levels are due to the related effects cholesterol has on bilayer structure and mechanical properties. The introduction of cholesterol reduces the area per phospholipid molecule or area per hydrocarbon chain (A_{lc}) (*57*, *65*) and also increases the isothermal area compressibility modulus (K_A) (*20*). Both A_{lc} and K_A would be expected to influence the partitioning of amphipathic peptides into the interfacial region of the bilayer. As A_{lc} decreases, less of the bilayer hydrocarbon surface is exposed to the peptide, which decreases the hydrophobic attraction of the peptide to the bilayer. K_A is related to the energy needed to change the area per lipid molecule. Since the bilayer surface must expand to incorporate the amphipathic peptide melittin, the larger the value of K_A, the more energy is required to partition melittin into the bilayer. Indeed, we have shown for melittin that the free energy of partitioning is small for bilayers with high values of K_A (*66*), and $\Delta G°$ decreases linearly with an increasing area compressibility modulus (*67*). Moreover, melittin-induced lysis is thought to be due to the penetration of melittin into the bilayer hydrocarbon region and the subsequent formation of

transmembrane pores (*68*, *69*). The penetration of melittin deep into the bilayer would be expected to be reduced by a decrease in A_{lc} or an increase in K_A. Thus, the reduction in both $\Delta G°$ and the extent of leakage with the introduction of cholesterol can be explained in terms of cholesterol reducing A_{lc} and increasing K_A.

The addition of several types of charged lipids to EPC liposomes, including PS and PEG-lipids, increased the level of melittin binding (Table 1), but decreased the level of melittin-induced leakage (Figure 3). Previous workers have noted similar phenomena with negatively charged phospholipids (*15*, *38*−*40*) or PEG-lipids (*37*). In terms of binding, the observation that $\Delta G°$ is similar for EPC bilayers containing either 15 mol % PS or 15 mol % PEG-lipid indicates that (1) the improved binding is due to electrostatic attraction due to the presence of the negatively charged phosphate group in both PS and PEG-lipid and (2) the PEG chains do not provide an effective steric barrier for inhibiting melittin binding to the bilayer surface. These conclusions are consistent with the work of Rex et al. (*37*), who found that the addition of electrically neutral PEG-lipids (PEG-ceramides) did not appreciably modify melittin binding to POPC vesicles. At 15 mol % PEG-lipid, the PEG chains form a crowded "brush" conformation (*70*, *71*) that has been shown to form an effective steric barrier to the close approach of apposing PEG-lipid bilayers (*58*, *72*, *73*). Moreover, this concentration of PEG-2000 increases the blood circulation time of PEG-liposomes (*74*−*76*), presumably due to the PEG barrier preventing opsonization of the liposomes by serum proteins (*77*, *78*). Grafted PEG layers have been shown to inhibit the absorption to bilayers of proteins such as albumin and fibronectin (*78*). However, relatively small molecules such as lysolipids are able to partition into PEG-liposomes (*79*). Thus, like lysolipid, melittin is apparently small enough to pass through the PEG barrier.

Compared with EPC bilayers, bilayers composed of bacterial outer membrane lipid (LPS, BPL, or LPS and BPL) gave a much larger free energy of transfer ($\Delta G°$) for melittin (Table 1). This is at least partly due to the negative charge on these bilayers. Rd LPS and Ra LPS molecules typically contain four and six negative charges, respectively, and BPL contains 23% phosphatidylglycerol (one negative charge per molecule) and 10% cardiolipin (two negative charges per molecule) along with 67% zwitterionic phosphatidylethanolamine. Because LPS molecules contain six or seven hydrocarbon chains, PG contains two acyl chains, and cardiolipin contains four acyl chains, the number of negative charges per unit area varies as follows: Ra LPS > Rd LPS > BPL. Thus, for these bacterial lipids, the negative charge per unit area is a factor in melittin partitioning. The observation that $\Delta G°$ is larger for Ra LPS than for Rd LPS is an indication that lipid charge, and not the length (steric barrier) of the polysaccharide chain, is more critical for peptide binding. It has been shown that the range of the polysaccharide steric barrier is considerably larger for Ra LPS than for Rd LPS (*46*).

Although electrostatic attraction is a major factor in enhanced binding of melittin to the negatively charged bilayer systems compared to EPC (Table 1), electrostatics alone cannot explain why $\Delta G°$ is comparable for 85:15 EPC/PS and BPL bilayers because EPC/PS bilayers have a smaller charge:unit area ratio than the bacterial systems. As noted above, other possible factors in $\Delta G°$ are the area compressibility modulus (K_A) and the area per lipid hydrocarbon chain (A_{lc}). K_A has not been determined for either LPS or BPL. However, the area per hydrocarbon chain has been measured to be 26 Å^2 for LPS (*46*), which can be compared to an A_{lc} of 32 Å^2 for EPC (*44*, *65*) and an A_{lc} of ≈26 Å^2 for a fully hydrated 1:1 EPC/cholesterol mixture (*65*). Compared to PCs with similar hydrocarbon chain compositions, charged lipids such as PS, PG, and cardiolipin would be expected to have larger values of A_{lc} due to electrostatic repulsion, whereas zwitterionic phosphatidylethanolamines (PEs) have a smaller A_{lc} than PCs (*80*, *81*). The major fatty acid chains in each of the phospholipids in BPL (PE, PG, and cardiolipin) are 16:0 and 16:1 (*82*), similar to the major fatty acid chains (16:0 and 18:1) in EPC. Therefore, considering this hydrocarbon composition and the fact that BPL contains 67% PE, we assume that BPL has a smaller A_{lc} than EPC. Thus, to a first approximation, for the lipid systems in Table 1, values of A_{lc} should vary as follows: EPC/PS ≥ EPC > BPL > LPS ≈ EPC/cholesterol. Therefore, we argue that the comparable values of $\Delta G°$ for 85:15 EPC/PS and BPL bilayers reflect balancing differences in charge per unit area and area per hydrocarbon chain.

In terms of vesicle leakage, previous experiments have also shown that the presence of negatively charged lipids decreases melittin-induced permeability (*15*, *37*−*40*). Several authors have argued that this is due to the charged lipids anchoring melittin to the bilayer surface, thereby preventing melittin from penetrating deeply into the hydrocarbon region of the bilayer or forming defects (*15*, *38*−*40*). In the case of PEG-lipids, Rex et al. (*37*) argue that the PEG chain itself is involved, perhaps by inhibiting lateral diffusion in the plane of the bilayer. Although all of these factors may be involved, we argue that in the case of LPS another important factor is the small area per hydrocarbon chain with this lipid. In support of this, we note that (1) melittin-induced leakage rates for LPS bilayers are similar to those of EPC/cholesterol bilayers, a lipid system with a similar A_{lc} value, and (2) the extent of leakage of Rd LPS bilayers is less than that of EPC/PEG-2000 bilayers (Table 1).

The data in Figure 2 and Table 1 indicate that both cholesterol, a component of eukaryotic plasma membranes, and LPS, a major component of the outer membrane of Gram-negative bacteria, inhibit melittin-induced bilayer leakage. In addition, the addition of phospholipid to Rd LPS bilayers makes the membranes more sensitive to melittin (Table 1). These data help to explain the increased sensitivity to antimicrobial peptides such as melittin of deep rough bacteria compared to rough or smooth bacteria (*24*, *26*−*28*). The outer monolayers of the outer membranes of all of these bacteria contain large amounts of LPS. However, deep rough bacteria have a larger phospholipid:LPS ratio on their surface than do smooth or rough phenotypes (*31*). Therefore, we argue that it is the presence of phospholipids in the outer monolayer, rather than differences in structure of the individual LPS molecules, that causes the peptide sensitivity of deep rough bacteria. Previously, Snyder and McIntosh (*41*) have shown that the sensitivity of deep rough bacteria to small, hydrophobic antibiotics (such as cephaloridine) is due to the presence of phospholipids in the outer monolayers of their outer membranes.

SUMMARY

Thus, compared to phospholipid bilayers, both phospholipid/cholesterol and LPS bilayers are insensitive to melittin-induced leakage. We argue that this insensitivity is due at least in part to the tight packing of the hydrocarbon chains in the plane of both bilayers. For LPS bilayers, the steric barrier provided by the polysaccharide chain does not appear be a major factor influencing melittin binding.

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