Interaction of Bee Venom Melittin with Zwitterionic and Negatively Charged Phospholipid Bilayers: A Spin-Label Electron Spin Resonance Study

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ABSTRACT   Electron spin resonance (ESR) spectroscopy was used to study the penetration and interaction of bee venom melittin with dimyristoylphosphatidylcholine (DMPC) and ditetradecylphosphatidylglycerol (DTPG) bilayer membranes. Melittin is a surface-active, amphipathic peptide and serves as a useful model for a variety of membrane interactions, including those of presequences and signal peptides, as well as the charged subdomain of the cardiac regulatory protein phospholamban. Derivatives of phosphatidylcholine and phosphatidylglycerol spin-labeled at various positions along the sn-2 acyl chain were used to establish the chain flexibility gradient for the two membranes in the presence and absence of melittin. Negatively charged DTPG bilayer membranes showed a higher capacity for binding melittin without bilayer disruption than did membranes formed by the zwitterionic DMPC, demonstrating the electrostatic neutralization of bound melittin by DTPG. The temperature dependence of the ESR spectra showed that the gel-to-liquid crystalline phase transition is eliminated by binding melittin to DTPG bilayers, whereas a very broad transition remains in the case of DMPC bilayers. None of the spin labels used showed a two-component spectrum characteristic of a specific restriction of their chain motion by melittin, but the outer hyperfine splittings and effective chain order parameters were increased for all labels upon binding melittin. This indicates a reduced flexibility of the lipid chains induced by a surface orientation of the bound melittin. Whereas the characteristic shape of the chain flexibility gradient was maintained upon melittin addition to DMPC bilayers, the chain flexibility profile in DTPG bilayers was much more strongly perturbed. It was found that the steepest change in segmental flexibility was shifted toward the bilayer interior when melittin was bound to DTPG membranes, indicating a greater depth of penetration than in DMPC membranes. pH titration of stearic acid labeled at the C-5 position, used as a probe of interfacial interactions, showed net downward shifts in interfacial pK of 0.8 and 1.2 pH units contributed from the positive charge of melittin, outweighing upward shifts from interfacial dehydration, when melittin was bound to DTPG and DMPC, respectively. The perturbation of the outer hyperfine splitting was used to determine the interactions of melittin with spin-labeled lipids of different polar headgroups in DTPG and DMPC. Anionic lipids (phosphatidylserine, phosphatidylglycerol, and stearic acid) and zwitterionic lipids (phosphatidylethanolamine and phosphatidylcholine) had the largest outer splittings in the presence of melittin. Neutral lipids (protonated stearic acid and diacylglycerol) displayed the largest increase in outer splitting on binding melittin, which was attributed to a change in the vertical location of these lipids in the bilayer. Both effects were more pronounced in DTPG than in DMPC.

INTRODUCTION

Melittin, isolated from honey bee venom (for a review, see Habermann, 1972; Dempsey, 1990), is a small, surface-active peptide that consists of 26 amino acids; 10 of these are strongly hydrophobic, and six are positively charged at neutral pH. It has a strong hemolytic activity (Sessa et al., 1969; Habermann, 1972) and belongs to the class of amphiphilic basic peptides that bind to neutral membranes as well as to membranes with a negative electrostatic surface potential. In addition to the surface activity, melittin has been found to elicit single-channel conductances in planar bilayers and to induce pore formation in lipid vesicles (Tosteson and Tosteson, 1981; Stankowski et al., 1991). The amino acid sequence of the peptide possesses a basic center composed of two arginine and two lysine residues that is located at the C-terminal end of the peptide (Habermann and Jentsch, 1967):

Gly(+)-Ile-Gly-Ala-Val^5-Leu-Lys(+)-Val-Leu-Thr^{10}-

Thr-Gly-Leu-Pro-Ala^{15}-Leu-Ile-Ser-Trp-Ile^{20}-

Lys(+)-Arg(+)-Lys(+)-Arg(+)-Gln^{25}-Gln-CONH$_2$

Because signal peptides and the presequences of mitochondria-destined proteins are also characterized by an amphiphilic amino acid composition that consists of both basic and hydrophobic residues, it is of special interest to study the interaction of peptides such as melittin with model membrane systems and with biological membranes (Engelman and Steitz, 1981). Furthermore, because of its similarity to a subsequence of phospholamban, the melittin peptide has been used to study the regulation of Ca^{2+}-ATPase activity in sarcoplasmic reticulum membranes (Chiesi et al., 1991; Mahaney and Thomas, 1991; Mahaney et al., 1992; Voss et al., 1995).

In aqueous solution, melittin exists either as a monomer or a tetramer, depending on concentration, ionic strength, and pH (Faucon et al., 1979). Although the monomer does not show any secondary structure in water (Talbot et al., 1979; Lauterwein et al., 1980), melittin exhibits an α-helical structure, both when in the tetrameric form and when bound to membranes or micelles (Dawson et al., 1978; Vogel and Jähnig, 1986; Ikura et al., 1991). The crystal structure of α-helical melittin shows that the molecule is highly amphipathic, with most of the hydrophobic residues located on one side and most of the hydrophilic residues on the other side of the helix long axis (Terwilliger and Eisenberg, 1982; Terwilliger et al., 1982). The net hydrophobic moment of the entire peptide is $\langle \mu_\alpha \rangle = 0.35$, whereas the mean hydrophobicity is only $\langle H \rangle = 0.10$ (cf. Eisenberg et al., 1984). It has been shown that melittin causes micellization of saturated phosphatidylcholine bilayers (Dufourcq et al., 1986), and of egg phosphatidylglycerol, dioleoylphosphatidic acid, phosphatidylserine, and cardiolipin vesicles (Batenburg et al., 1987a,b). The orientation of melittin and the depth of its insertion into the membrane have been the subject of some controversy. Accessibility measurements of spin-labeled melittin with chromium oxalate (Altenbach and Hubbell, 1988; Altenbach et al., 1989) suggest that melittin is located on the membrane surface, with only the hydrophobic residues inserted into the lipid bilayer. ^{13}C-NMR measurements carried out in the presence of aqueous shift reagents (Stanislawski and Rüterjans, 1987) support this result. In contrast, polarized transmission IR spectroscopy (Vogel et al., 1983) and polarized attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) (Brauner et al., 1987) used to determine the orientational order parameter of melittin in dry multibilayers showed that the α-helical part of melittin was preferentially oriented parallel to the lipid acyl chains. This result was confirmed on partially hydrated oriented multibilayers (Vogel, 1987). More recent studies have revealed the dependence of the melittin orientation on the degree of hydration of the host membrane (Frey and Tamm, 1991). Such considerations are essential, for instance, to establishing the mechanism by which melittin forms ion channels and pores in lipid bilayers (Tosteson and Tosteson, 1981; Stankowski et al., 1991).

In the present work we have studied the lipid interaction with melittin by using electron spin resonance (ESR) spectroscopy of spin-labeled lipids in fully hydrated lipid vesicles. By this means, detailed information on the local motions of phospholipids in the presence of melittin and their selectivity of interaction with melittin could be obtained. In zwitterionic phosphatidylcholine as well as in anionic phosphatidylglycerol host membranes, lipid probes were used that are spin-labeled at different carbon atom positions in the sn-2 acyl chains, ranging from carbon atom 4 to carbon atom 14. The effect of melittin on the ESR spectra of these spin labels was investigated. First, the dependence of the maximum outer hyperfine splitting on the melittin-to-lipid ratio was determined for both host lipid bilayers by using spin labels with the nitroxyl group at carbon atom 5 of the

sn-2 acyl chain. Experiments with different spin label positional isomers were then carried out using melittin/lipid ratios at which the outer hyperfine splittings for the 5-position labels reached their maximum values. The effect of melittin on the bilayer chain-melting phase transition of both lipid host matrices was monitored by ESR spectroscopy. Furthermore, we investigated the effect of melittin binding on the interfacial titration behavior of stearic acid spin-labeled at carbon atom 5, incorporated in phosphatidylglycerol as well as in phosphatidylcholine vesicles. These experiments give information regarding the influence of melittin on the bilayer surface electrostatics and interfacial polarity. In a final set of experiments, we focused on the lipid specificity of melittin by measuring the increase in the maximum outer hyperfine splitting for spin-labeled lipids with various headgroups upon addition of the peptide. These experiments were also carried out in zwitterionic as well as in negatively charged host lipid membranes.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Fluka, Switzerland) was checked for purity by thin-layer chromatography (single spot). 1,2-Ditetradecyl-sn-glycero-3-phosphoglycerol (DTPG) was synthesized as described by Harlos and Eibl (1980). 3-(N-morpholino)propanesulfonic acid (MOPS) and melittin were obtained from Sigma (St. Louis, MO). The melittin was subsequently purified according to the method of Wille (1989), with modifications described by Voss et al. (1991). Phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylserine spin labels with the nitroxyl group on the C-n atom of the sn-2 chain (n-PCSL,[1] n-PGSL, n-PESL, and n-PSSL, respectively) were synthesized from the corresponding spin-labeled stearic acids (n-SASL), as described by Marsh and Watts (1982). Phosphatidylglycerol spin labels with $n = 4$–10, 12, and 14 as well as phosphatidylcholine spin labels with $n = 4$–14 were used. Spin-labeled diacylglycerol, 5-DGSL (1-acyl-2-[5-(4,4-dimethyloxazolidine-N-oxyl)]stearoyl-sn-glycerol), was synthesized from 5-PCSL, as described by Heimburg et al. (1992).

Sample preparation

Lipid dispersions were prepared by first codissolving the lipids (0.5 mg) with 1 mol% spin label in chloroform/methanol (ratio 2:1 v/v), then evaporating the solvent using a nitrogen gas flow and drying the sample under vacuum overnight. The dried lipid films were dispersed in buffer (10 mM buffer with 5 mM EDTA: MOPS for pH range 6.5–7.5, citric acid for pH range 3–6, sodium borate for pH range 7.6–9.2, and sodium borate/ NaOH for pH above 9.2). Melittin dissolved in the same buffer at a concentration of 0.7 mM (determined by UV absorbance at 280 nm using the extinction coefficient $\epsilon_{280} = 5400$ M^{-1} cm^{-1}) was then added to give the desired lipid/peptide ratio. The final total volume was 0.2 ml. The samples were vortexed well and, after incubation at 35°C for 1 h with intermittent vortexing, they were centrifuged using a low-speed tabletop centrifuge and then transferred into ESR capillaries. Control samples without melittin were treated identically, except that the melittin-contain-

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Abbreviations: n-PCSL and n-PGSL, 1-acyl-2-[n-(4,4-dimethyloxazolidine-N-oxyl)]stearoyl-sn-glycero-3-phosphocholine, and -phosphoglycerol; 5-PESL and 5-PSSL, 1-acyl-2-[5-(4,4-dimethyloxazolidine-N-oxyl)]stearoyl-sn-glycero-3-phosphoethanolamine, and -phosphoserine; 5-SASL, 5-(4,4-dimethyloxazolidine-N-oxyl)stearic acid.

ing buffer solution was replaced by the same buffer without the peptide. Essentially identical effects of melittin were observed on the ESR spectra of the spin-labeled lipids, if the melittin-containing samples were prepared alternatively by hydrating the dry lipid with solutions of melittin in buffer (final volume 0.2 ml). No time-dependent changes were observed in the ESR spectra of lipid spin labels in the melittin-containing samples after the incubation procedure used in the sample preparation, either while recording the spectra or after a period of several hours. After measurement, the samples were tested for possible degradation by thin-layer chromatography using solvent systems of $CHCl_3/CH_3OH/ammonia$ (65/30/3 v/v/v) and hexane/ether (1/1 v/v). No detectable free fatty acid or lyso compounds were found. Lipid/peptide ratios were determined from lipid phosphate (Rouser et al., 1970) and protein (Lowry et al., 1951) assays, where the latter was standardized against melittin solutions.

ESR spectroscopy

ESR spectra were recorded with a 9-GHz spectrometer (model E-12, Century Line; Varian, Palo Alto, CA) equipped with a TE_{102} rectangular cavity (Varian). Samples were contained in 1-mm-ID sealed glass capillaries placed within a standard 4-mm quartz ESR tube that contained light silicone oil for thermal stability. Temperature was controlled to $\pm 0.3°C$ or better by using nitrogen gas flow temperature regulation, and the sample temperature was measured with a fine-wire thermocouple situated at the top of the microwave cavity. The spectrometer was interfaced to a personal computer for digitizing and analyzing the measured ESR data. Spectral hyperfine splittings were determined by fitting the maxima and minima in the outer wings of the spectrum empirically to a Gaussian curve and calculating the field difference between the two extrema. Apparent order parameters were calculated from the expression

$$S^{eff} = \frac{A_\parallel - A_\perp}{A_{zz} - 1/2(A_{xx} + A_{yy})} \frac{a_0'}{a_0}, \qquad (1)$$

where $2A_\parallel$ is given by the maximum outer ^{14}N-hyperfine splitting, $2A_{max}$, and $A_\perp$ is given by (Gaffney, 1976)

$$A_\perp = A_{min} + 1.4\left(1 - \frac{A_\parallel - A_{min}}{A_{zz} - 1/2(A_{xx} + A_{yy})}\right), \qquad (2)$$

where $2A_{min}$ is the inner ^{14}N-hyperfine splitting. a_0 is the effective isotropic ^{14}N-hyperfine coupling constant and is given by

$$a_0 = 1/3 (A_\parallel + 2A_\perp) \qquad (3)$$

and a_0' is the isotropic ^{14}N-hyperfine coupling constant in the single crystal environment in which the ^{14}N-hyperfine coupling tensor was determined and is given by

$$a_0' = 1/3 (A_{xx} + A_{yy} + A_{zz}), \qquad (4)$$

where $A_{xx} = 5.9$ G, $A_{yy} = 5.4$ G, and $A_{zz} = 32.9$ G are the principal values of the ^{14}N-hyperfine coupling tensor of doxylpropane (Jost et al., 1971). Detailed simulations of the lineshapes of the ESR spectra from chain-labeled lipids have demonstrated the presence of long-axis motions in the slow motional regime for fluid-phase bilayers (Lange et al., 1985; Moser et al., 1989). Therefore the order parameters calculated from Eq. 1, using motional narrowing theory, are only effective values, which will approximate the ordering associated with fast chain motions, viz. *trans-gauche* isomerism. In the fluid phase, the maximum outer ^{14}N-hyperfine splitting, A_{max}, is related directly to S^{eff} by Eqs. 1 and 3. In the gel phase, values of A_{max} will be dominated by the slower rate of *trans-gauche* isomerism, however. In the fluid phase, either parameter may be used to characterize the chain dynamics at different positions of chain labeling, or for comparison between systems in the presence and absence of melittin, but A_{max} is the more useful parameter for the gel phase. In general, the two parameters are sensitive both to lipid chain mobility and to order, to a greater or lesser extent, depending on conditions, as indicated above.

RESULTS

Membrane binding of melittin

The effect of binding melittin on the lipid chain mobility in bilayer membranes was determined from the dependence of the outer hyperfine splittings in the ESR spectra of 5-C atom spin-labeled lipids on the melittin/lipid ratio used for membrane sample preparation. The ESR spectra of the 5-C atom spin labels in pure lipid dispersions correspond to an axially symmetric, partially motionally averaged, anisotropic system. Binding of the peptide has the effect of decreasing the degree of motional averaging of the anisotropy in the spectra from the lipid chains. Fig. 1 shows the behavior in a zwitterionic and in an anionic host lipid bilayer: 5-SASL in phosphatidylcholine/melittin complexes (Fig. 1 *a*) and 5-PCSL in phosphatidylglycerol/melittin complexes
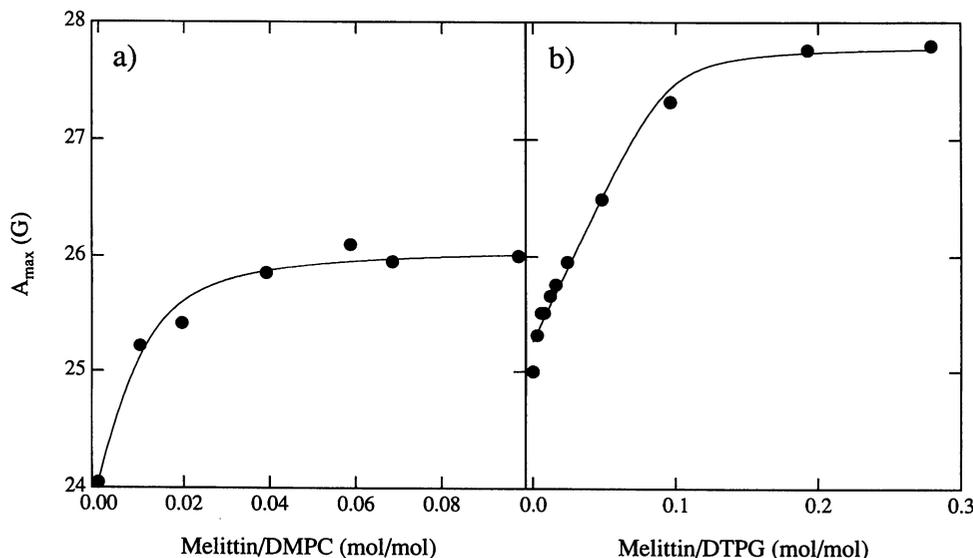
FIGURE 1 Dependence of the spin label outer hyperfine splitting constants, A_{max}, of (*a*) 5-SASL in DMPC membranes and (*b*) 5-PCSL in DTPG membranes on the mole ratio of total melittin added per lipid. Buffer: 10 mM MOPS, 5 mM EDTA, and 10 mM NaCl, pH 7.2. Temperature: 30°C.

(Fig. 1 *b*). The outer hyperfine splitting constant, A_{max}, of the spin labels is plotted as a function of the total melittin/ lipid ratio in the samples. The figure shows that the degree of motional averaging is decreased progressively in the ESR spectra of the lipid-melittin complexes as melittin binds to the membrane. This is reflected by an increase in the outer hyperfine splitting, $2A_{max}$, and an accompanying decrease in the inner hyperfine splitting, $2A_{min}$ (data not shown). The 5-SASL label was used to define the melittin-induced perturbations with DMPC because the effects registered by 5-PCSL were much smaller (see later section on lipid selectivity). Interestingly, the melittin-induced change in A_{max} of 5-PCSL in DMPC was increased at high ionic strength.

A plateau for the increase in A_{max} is reached at a lower ratio of added melittin to lipid in the case of the DMPC complexes (0.2–0.25 mg melittin/mg lipid) as compared to the DTPG complexes (0.7–0.9 mg melittin/mg lipid). Estimation of the lipid/peptide binding stoichiometry in the complexes from the intercept of the linear increase in A_{max} on initial binding with the plateau value of A_{max} yields lipid/peptide molar ratios of approximately 60 DMPC/ melittin and 10 DTPG/melittin, respectively. This corresponds to the binding level at which maximum perturbation of the lipid mobility in bilayer membranes is achieved. The ESR spectra of the spin-labeled lipids at melittin concentrations corresponding to the plateau values in A_{max} were all typical of those for a bilayer or other liquid crystalline phase. At higher melittin concentrations than these (not used in this study), the samples became difficult to spin down, and finally a clear solution was achieved with excess melittin (see also Ohki et al., 1994). This resulted in less anisotropic ESR spectra with lower values of A_{max} that are characteristic of micellar phases, at very high melittin concentrations. The plateau values of A_{max} in Fig. 1 therefore

represent the maximum or saturation perturbations of the lipid mobility in the bilayer membranes before their disruption by micellization. In the experiments to be described below, melittin concentrations corresponding to the plateau values of A_{max} were used. This protocol largely corrects for any changes in binding capacity with temperature and pH, etc. (cf. Dufourcq and Faucon, 1977).

Influence on the bilayer phase transition

The effect of melittin on the thermotropic lipid phase behavior was investigated for bilayers formed by DMPC and DTPG, respectively. Melittin was used in amounts corresponding to the plateau levels of A_{max}, as determined in Fig. 1, in both cases. Fig. 2 shows the temperature dependence of the outer hyperfine splittings for the 5-position spin labels in DMPC (Fig. 2 *a*) and in DTPG (Fig. 2 *b*), both in the absence and in the presence of melittin. Also shown are the temperature dependences of the maximum amplitudes in the ESR spectra of the 14-position spin labels in DMPC (Fig. 2 *c*) and in DTPG (Fig. 2 *d*), both of which are normalized to the second integral of the corresponding ESR spectra. Both spectral parameters for the two spin labels register the thermotropic phase transition from the lipid gel state to the liquid crystalline lipid state, which occurs at temperatures of approximately 23°C and 26°C for DMPC and DTPG, respectively, in the absence of melittin. The outer hyperfine splittings decrease, and the spectral lineheights increase, abruptly at the phase transition, both of which correspond to the greater freedom of chain mobility in the fluid, liquid crystalline phase (cf. also Schulze et al., 1987). As can clearly be seen, melittin has a large effect on the spin label outer hyperfine splittings, as well as on the amplitudes of
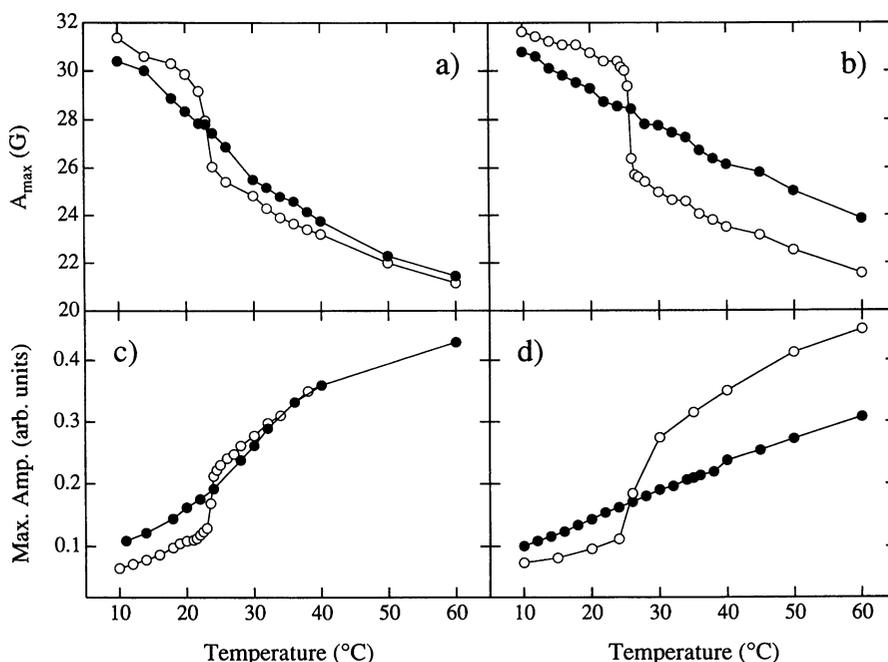
FIGURE 2 Temperature dependence of the outer hyperfine splitting constants, A_{max}, of (*a*) the 5-PESL spin label in DMPC and (*b*) 5-PCSL in DTPG bilayers, and of the maximum spectral lineheights (normalized by the second integral of the ESR spectra) of (*c*) 14-PCSL in DMPC and (*d*) 14-PGSL in DTPG bilayers, in the absence (○) and in the presence (●) of melittin. The melittin concentration was that required to achieve the plateau value of A_{max} in each case. Buffer: 10 mM MOPS and 5 mM EDTA, pH 7.0.

the ESR spectra. In the system containing DTPG, the cooperative lipid chain-melting phase transition is completely eliminated by binding melittin. The lipid chain mobility in DTPG is increased at temperatures corresponding to the lipid gel phase and decreased at those corresponding to the fluid phase on binding of melittin. Fig. 2 c shows that in the DMPC-melittin complexes there is still a residual transition, but this takes place over a rather broad temperature range, as found also by Schulze et al. (1987) with the 5-SASL spin label. The lipid chain mobility in DMPC is increased in the gel phase, but is affected less in the fluid phase by the binding of melittin.

Lipid chain flexibility gradient

The effects of binding melittin on the chain flexibility gradient in lipid membranes at the plateau values for perturbation of the chain mobility were determined for DMPC and DTPG bilayers. Spin probes used were n-PGSL (n = 4–10, 12, and 14) for DTPG systems and n-PCSL (n = 4–14) for samples with DMPC. Fig. 3 shows the ESR spectra of the spin probe positional isomers at 40°C in a DMPC environment (Fig. 3 a) and in a DTPG environment (Fig. 3 b), both in the absence (dotted lines) and in the presence (solid lines) of melittin. This comparison shows that overall, the degree of motional averaging at the plateau values of A_{max} is decreased much more upon the addition of melittin to the DTPG membranes than upon addition to the

DMPC membranes. The latter is reflected by the larger increase in the maximum outer hyperfine splitting of the n-PGSL spin labels in DTPG than of the n-PCSL spin labels, in DMPC, in the presence of the peptide. Although the addition of melittin to the lipid bilayers increases the ESR splittings to a large extent, the spectra of the spin labels close to the terminal methyl group do not exhibit a second component representing a second more motionally restricted lipid population, such as is the case with integral membrane proteins (see, e.g., Marsh, 1985). Fig. 4 shows the effective order parameters determined as a function of the position, n, of the nitroxyl group along the sn-2 acyl chain of the n-PCSL spin labels in DMPC and of the n-PGSL spin labels in DTPG, and how it is influenced by melittin at a temperature in the fluid phase. The apparent order parameter profile reflects quantitatively the qualitative observations made above on the ESR spectra. The complexes of melittin with DTPG possess the largest degree of spectral anisotropy (largest A_{max}) and exhibit higher effective order parameters. The difference in the latter from the DTPG bilayers alone is greater than the difference between the effective order parameters of DMPC/melittin complexes and DMPC bilayers alone, and the effective order parameters remain high over a considerably greater length of the acyl chain. This represents a qualitative difference between the chain flexibility profiles for DTPG bilayers in the presence and absence of melittin: the steepest change in flexibility gradient is shifted toward the bilayer interior on
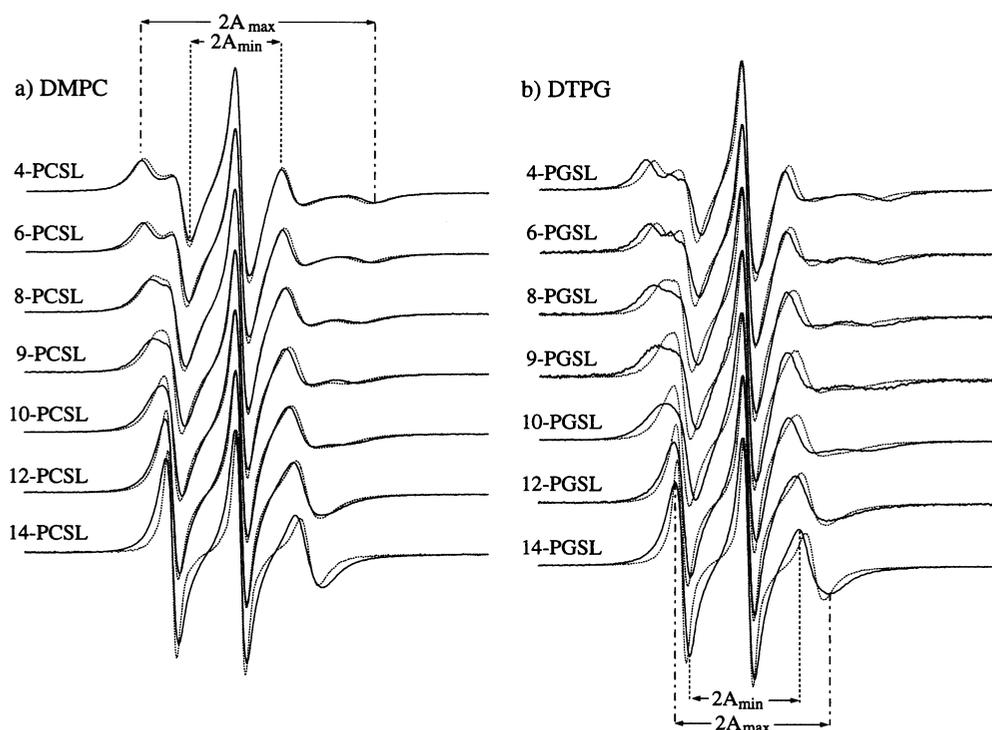
FIGURE 3   ESR spectra of phospholipids spin-labeled at various positions along the sn-2 acyl chain (n-PCSL and n-PGSL) in (a) DMPC and (b) DTPG bilayers in the absence (⋯) and in the presence (——) of melittin. The maximum (outer) and minimum (inner) hyperfine splittings, $2A_{max}$ and $2A_{min}$, respectively, are indicated. The melittin concentration was that required to achieve the plateau value of A_{max} in each case. The spectra were recorded at 40°C with a spectral width of 100 G. Buffer: 10 mM MOPS, 10 mM NaCl, and 5 mM EDTA, pH 7.2.
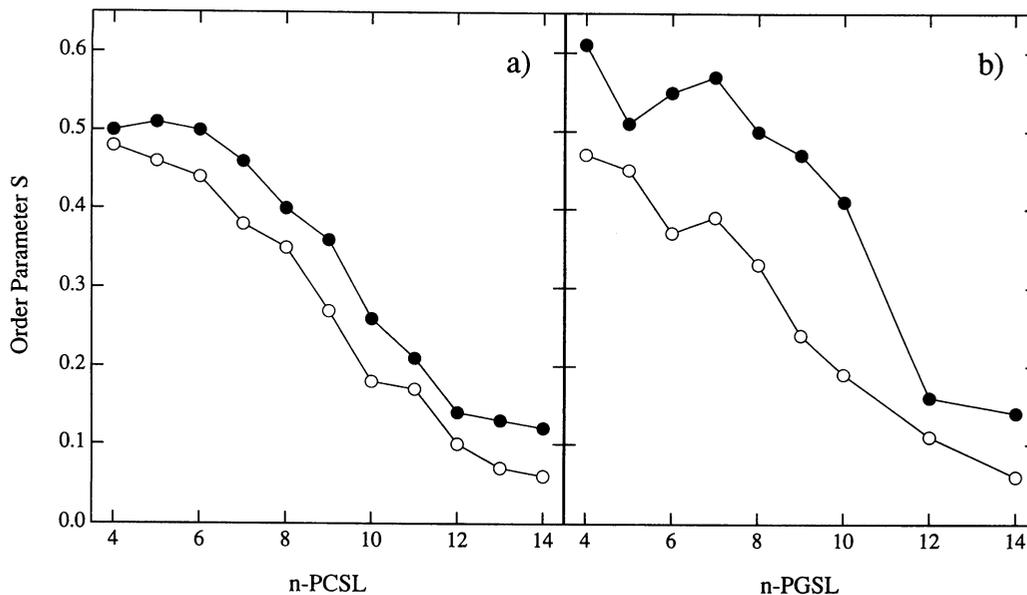
FIGURE 4   Effective order parameters, S, at 40°C as a function of the position, n, of the spin label in the sn-2 acyl chain of (a) the n-PCSL spin labels in DMPC, and (b) the n-PGSL spin labels in DTPG, in the absence (O) and in the presence (●) of melittin. The melittin concentration was that required to achieve the plateau value of A_{max} in each case. Buffer: 10 mM MOPS, 10 mM NaCl, and 5 mM EDTA, pH 7.0.

binding melittin. For DMPC bilayers, however, the overall shapes of the flexibility profiles are qualitatively similar in the presence and absence of melittin. Measurements have been performed over a wide range of temperatures, and qualitatively similar results were obtained at other temperatures in the fluid bilayer phase, for both systems.

Fatty acid pH titration

The ESR spectra of the 5-SASL stearic acid spin label in DMPC-melittin and in DTPG-melittin complexes, as well as in the lipid environments alone, were measured as a function of pH to determine the influence of the peptide association on the pH titration of the fatty acid (cf. Sankaram et al., 1990). The dependences of the outer hyperfine splittings of 5-SASL on the bulk pH are given in Fig. 5. The data were fitted using the equation for a conventional acid-base titration according to

$$A_{max} = \frac{A_{max}^{5\text{-SASLH}} - A_{max}^{5\text{-SASL}^-}}{1 + 10^{pH - pK}} + A_{max}^{5\text{-SASL}^-}, \qquad (5)$$

with $A_{max}^{5\text{-SASLH}}$, $A_{max}^{5\text{-SASL}^-}$, and the interfacial pK of the fatty acid spin label as parameters to be determined. $A_{max}^{5\text{-SASLH}}$ is the maximum outer hyperfine splitting constant of the protonated form of stearic acid and $A_{max}^{5\text{-SASL}^-}$ that of the corresponding anion. As seen from the titrations in Fig. 5, the presence of the peptide shifts the interfacial pK of stearic acid in DMPC (Fig. 5 a) from pK = 7.1 ($\pm$ 0.1) to pK = 5.9 ($\pm$ 0.2). In DTPG (Fig. 5 b) the shift is from pK = 8.1 ($\pm$ 0.2) in the absence of melittin to pK = 7.3 ($\pm$ 0.3) in the presence of melittin. In both cases, binding of the peptide

shifts the effective pK toward a lower value. At very low and at very high pH, the maximum outer hyperfine splitting of stearic acid in DTPG-melittin complexes is roughly the same as in the pure lipid system, indicating that melittin does not bind to the membrane under these conditions because of titration of the charge on the phospholipid and on the peptide, respectively. A similar observation is made for the DMPC-melittin samples at high pH. For these reasons, the points at the extreme pH values were not included in fitting the fatty acid titration.

Interaction with different spin-labeled lipid species

The perturbation by melittin binding of the mobility of spin-labeled lipids with different polar headgroups was determined for lipid probes labeled at the 5 C-atom position of the chain (cf. Sankaram et al., 1989). Measurements were made on DMPC host bilayers (at 30°C) as well as on DTPG host matrices (at 40°C) with a concentration of 1 mol% of the various spin-labeled lipid species. ESR spectra for the various spin labels differing in their lipid headgroup, both in the lipid matrices alone and in the lipid-peptide complexes at amounts of melittin corresponding to the plateau values of A_{max}, are given for the two lipid systems in Fig. 6. A larger increase in the outer hyperfine splitting for a given lipid species upon the addition of melittin indicates a stronger perturbation in the mobility of that particular lipid by the peptide binding with the bilayer host. As can be seen from Fig. 6 and Table 1, the overall increase in spectral anisotropy is larger in the case of DTPG as the host bilayer lipid
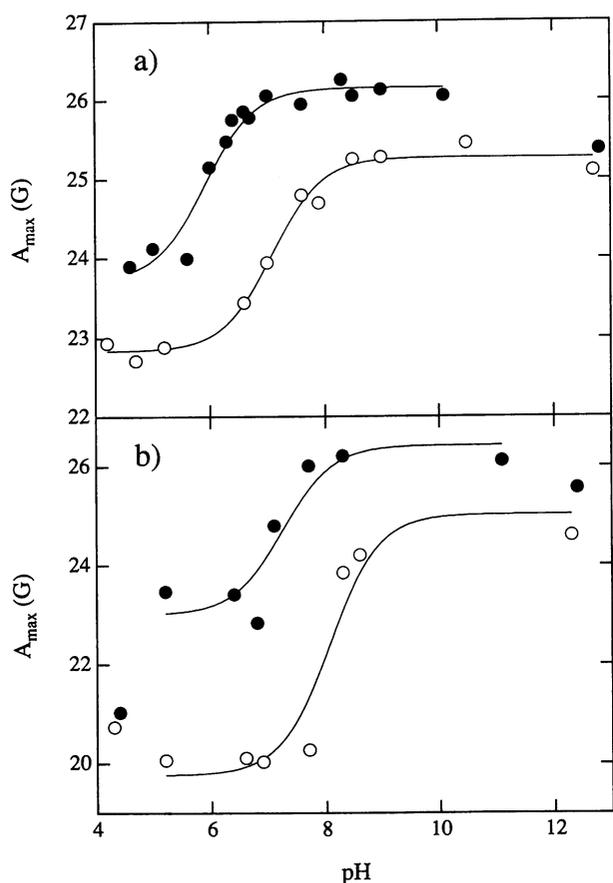
FIGURE 5 pH dependence of the outer hyperfine splitting constants, A_{max}, of the 5-SASL stearic acid spin-label in (a) DMPC host lipid bilayers at 30°C and (b) DTPG host lipid bilayers at 40°C, both in the absence (O) and in the presence (●) of melittin. The melittin concentration was that required to achieve the plateau value of A_{max} in each case. The full lines represent nonlinear least-squares fits to a conventional acid-base titration (Eq. 5). For DTPG, the data points at pH 4.2 were not included in the fit, because of partial protonation of the lipid, nor was that at the very highest pH in the presence of melittin for both systems, because of deprotonation of the peptide.

a) DMPC

$$5\text{-SASLH} \geq 5\text{-DGSL} \approx 5\text{-PESL}^{\pm} \approx 5\text{-PGSL}^{-}$$

$$\geq 5\text{-SASL}^{-} \approx 5\text{-PSSL}^{-} > 5\text{-PCSL}^{\pm}$$

b) DTPG

$$5\text{-SASLH} > 5\text{-PCSL}^{\pm} \approx 5\text{-DGSL} \geq 5\text{-PESL}^{\pm}$$

$$> 5\text{-PGSL}^{-} \geq 5\text{-PSSL}^{-} \approx 5\text{-SASL}^{-}.$$

It should be emphasized that these differences in outer hyperfine splitting are referred to the state of the corresponding lipid spin label in the lipid bilayers alone and therefore include differences between the various labels in the latter environment. Evidently, the rearrangements of the labels on binding of melittin are greatest for the more hydrophobic lipids such as protonated fatty acid and diacylglycerol, even though the values of A_{max} in the presence of melittin are smaller for these lipids than are those for the more polar lipids (cf. Table 1). Measurements have been performed over a wide range of temperatures in the fluid phase, and qualitatively similar results for the selectivity series were obtained at other temperatures, including at the same temperature for both lipids.

DISCUSSION

Membrane binding and bilayer saturation

The binding of melittin to the lipid bilayers initially causes a restriction of lipid acyl chain motion, which is evidenced by the increases in A_{max}, which then reach a plateau value at a melittin content that is characteristic of the particular lipid type (see Fig. 1). At melittin concentrations beyond the plateau value (not studied here), further binding of melittin induces micellization of the lipid, which is accompanied by a decrease in the values of A_{max}. The plateau values of A_{max} arise at lower melittin concentrations for DMPC than for DTPG, as does the point at which micellization occurs. The bilayer form of DTPG therefore has a higher capacity for binding melittin before the onset of bilayer disruption than does DMPC. This is because neutralization of the positive charge of melittin by the negatively charged lipid inhibits its micelle-forming tendency. This represents a significant difference in the interaction of melittin with negatively charged and zwitterionic lipid membranes. For this reason, experiments were carried out at melittin concentrations corresponding to the plateau values of A_{max}, which directly reflect this difference in interaction.

It should be noted that melittin concentrations corresponding to the plateau values for DTPG are inappropriate for DMPC because these already induce micellization of the zwitterionic lipid. An indication of the relative perturbations of the anionic and zwitterionic lipid bilayers at the same melittin concentrations can be deduced from Fig. 1. At a lipid/peptide ratio of 0.1 mol/mol the increase in A_{max} for 5-PCSL in DTPG is greater than that for 5-SASL in DMPC,

when compared to DMPC. Consequently, the increases in hyperfine splittings in DMPC lie in a rather similar range, with the exception of 5-PCSL, which corresponds to the host lipid headgroup and is hardly perturbed at all. In both bulk lipid environments, protonated stearic acid is most strongly perturbed by the binding of melittin, whereas its deprotonated form shows the least significant increase in the hyperfine anisotropy upon melittin binding in DTPG. Overall, the spectra of the neutral or zwitterionic spin-labeled lipid species are most perturbed by the binding of melittin, relative to the spectra in its absence, in both bilayer systems. Table 1 summarizes the hyperfine splittings determined for the different C-5 spin-labeled lipids and gives the differences of the splittings in the presence and in the absence of melittin (ΔA_{max}). The increases in the maximum outer hyperfine splittings, i.e., the values of ΔA_{max}, are found to be in the following order:

a) DMPC   b) DTPG

FIGURE 6   ESR spectra of different lipid species spin-labeled at the fifth position of the sn-2 acyl chain (a) in DMPC bilayers recorded at 30°C in the absence (···) and in the presence (——) of melittin; (b) in DTPG bilayers recorded at 40°C in the absence (···) and in the presence (——) of melittin. The melittin concentration was that required to achieve the plateau value of A_{max} in each case. Buffer: 10 mM MOPS, 10 mM NaCl, and 5 mM EDTA, pH 7.0, in all cases except for the protonated (5-SASLH) and deprotonated (5-SASL⁻) forms of stearic acid, where the buffers were 10 mM citric acid, 10 mM NaCl, 5 mM EDTA, pH 5.2, and 10 mM sodium borate, 10 mM NaCl, 5 mM EDTA, pH 9.5, respectively. Total scan width: 100 G.

even though 5-SASL displays a selectivity of interaction with melittin. At the same mole ratio, the effects on 5-PCSL in DMPC are smaller still, by a considerable amount (data not shown).

It can also be seen from Fig. 1 that the plateau level of the outer hyperfine splitting is reached at a higher molar lipid/melittin input ratio (approx. 16–20) for DMPC than for DTPG (approx. 4–6), showing the electrostatic enhancement of the binding of melittin. This is in good agreement with earlier studies of Dufourcq and Faucon (1977), who found that saturation was achieved at lipid/melittin mole ratios of 3–4 in the case of phosphatidylserine membranes and of 25 in the case of phosphatidylcholine vesicles, by monitoring the intrinsic tryptophan fluorescence of melittin. In the latter case, the experiments were performed by titrating melittin with lipid, rather than studying the binding of melittin to preformed membranes, as in the present study. The lower capacity for uptake of melittin by zwitterionic phosphatidylcholine membranes in comparison to negatively charged bilayers is attributed to the electrostatic repulsion between bound and aqueous melittin (Stankowski and Schwarz, 1990). In agreement with this, the lipid perturbation on saturation binding of melittin to DMPC is greater at high ionic strength than in the absence of salt.

The stoichiometry of melittin binding (as opposed to the input ratio at which the greatest value of A_{max} is reached), which is deduced from the initial slopes and plateau values of A_{max} with increasing binding (cf. Fig. 1), is approxi-

TABLE 1   Outer hyperfine splitting constants, A_{max}, of the ESR spectra from lipids spin-labeled on the 5 C-atom of the chain in DMPC and DTPG host bilayers at 30°C and 40°C, respectively, in the presence and absence of melittin

Spin label	Charge	A_{max} (G)		$\Delta A_{max}(G)$*
T = 30°C		DMPC	DMPC + melittin	
5-SASLH	0#	22.9	24.1	1.2
5-DGSL	0	24.1	25.2	1.0
5-PESL	±	24.8	25.8	1.0
5-PGSL	−	24.9	25.9	1.0
5-PSSL	−	25.3	26.0	0.7
5-SASL	−§	25.3	26.1	0.8
5-PCSL	±	25.2	25.4	0.2
T = 40°C		DTPG	DTPG + melittin	
5-SASLH	0¶	20.1	23.5	3.4
5-DGSL	0	22.3	24.9	2.6
5-PESL	±	23.3	25.7	2.4
5-PGSL	−	23.8	25.9	2.1
5-PSSL	−	24.2	26.2	2.0
5-SASL	−‖	24.2	26.2	2.0
5-PCSL	±	23.7	26.3	2.6

The values in the presence of melittin correspond to the plateau values (cf. Fig. 1).
*ΔA_{max}: increase in the hyperfine splitting induced by binding the peptide.
#pH 5.0.
§pH 8.5.
¶pH 5.2.
‖pH 8.6.
Buffers: 10 mM MOPS, 10 mM NaCl and 5 mM EDTA, pH 7; 10 mM citric acid, 10 mM NaCl and 5 mM EDTA, pH 5.2 or 5.0; 10 mM sodium borate, 10 mM NaCl and 5 mM EDTA, pH 8.5 or 8.6.

mately 60 DMPC/melittin and 10 DTPG/melittin, for the zwitterionic and anionic lipid bilayer systems, respectively. For DMPC membranes, the stoichiometry lies well below that for saturation surface coverage of the bilayer and represents the inhibitory effect of the accumulating positive surface potential arising from the bound melittin and, consequently, the stronger tendency to induce micellization. For DTPG membranes, the surface potential still remains negative at maximum binding of melittin, which has a maximum of six positive charges. At the level of binding to DTPG bilayers that corresponds to the plateau value of A_{max}, the surface area per melittin molecule is approximately 600 Å^2, corresponding to ~10 lipid molecules (cf. Marsh, 1990). The area occupied by a 26-residue α-helix oriented parallel to the bilayer surface is minimally ~400 Å^2. Thus, at the plateau value of A_{max}, the melittin covers most of the surface of DTPG bilayers, although not in a close-packed fashion. Likely reasons for the latter are the distributional entropy of the bound melittin on the surface (essentially a two-dimensional surface pressure) and electrostatic repulsions between the bound melittin molecules (cf. Heimburg and Marsh, 1995). Additionally, the hydrophobic interaction with the lipid chains may limit the binding stoichiometry corresponding to the plateau value of A_{max} at which these latter interactions may be optimized.

Membrane penetration and flexibility gradient

The difference in the amount of melittin bound to neutral and negatively charged membranes before bilayer destabilization, referred to above, could also be the reason for the different effects on the lipid phase transition behavior. The phase transition of the negatively charged lipid membranes is eliminated (see Fig. 2), whereas a very broad transition remains in the case of the neutral DMPC membranes (Fig. 2 c). The elimination, or very pronounced broadening, of the lipid phase transition suggests a direct interaction of melittin with the lipid chains, at least in the case of DTPG. Binding of melittin strongly disrupts the cooperative packing of the lipid chains in the gel-phase bilayers, for both DTPG and DMPC (see Fig. 2). Nevertheless, the melittin-containing samples do not show any second motionally restricted component in the ESR spectra of the spin labeled lipids, such as arises from the direct interaction of the lipid chains with the intramembranous sections of integral membrane proteins (Marsh, 1985). Therefore, melittin does not appear to form transmembrane aggregates with an intramembranous hydrophobic face similar to that presented to the lipid by integral membrane proteins. Interestingly, however, in sarcoplasmic reticulum membranes, the binding of melittin enhances the population of motionally restricted lipids that is associated with the Ca-ATPase (Mahaney et al., 1992). In lipid bilayer membranes, instead, the overall anisotropy of the spectra is increased by melittin in a depth-dependent manner.

In the fluid phase of the negatively charged membrane, the shape of the chain flexibility profile is perturbed mark-

edly by binding of melittin, whereas for DMPC membranes the shape of the profile remains the same, although the effective order parameters are increased by binding. This suggests that melittin penetrates more deeply into the DTPG membranes than into DMPC membranes. The steepest part of the flexibility gradient is shifted from carbon atom 8 to carbon atom 10 of the sn-2 acyl chain on binding melittin to DTPG membranes. This is not consistent with a transmembrane orientation of the 20-residue N-terminal amphipathic helix of melittin, for which a much larger effect would be expected also for the spin label at the carbon 14 position. This argues for an orientation in which the amphipathic melittin molecule lies with its axis more closely parallel to the membrane surface and the hydrophobic side chains penetrate only partially into the hydrophobic interior. The extent of penetration cannot be deduced exactly from the spin-label chain flexibility profile, because the effect of melittin represents perturbation of a chain flexibility gradient that already exists in bilayers of the phospholipid alone.

These conclusions with regard to the surface orientation of melittin are consistent with its amphipathic character and large hydrophobic moment. As already mentioned, this orientation is also that established by other experiments (Altenbach et al., 1989; Stankowski and Rüterjans, 1987; Frey and Tamm, 1991), including more recent high-resolution NMR studies (Dempsey and Butler, 1992). These results are not consistent with models for the membrane pores induced by melittin that consist of transmembrane α-helical bundles (cf., e.g., Sansom, 1991). It cannot be excluded, however, that such structures are present at low concentration and are capable of forming the ionic channels that are observed in electrical measurements (Tosteson and Tosteson, 1981), nor that their population may be enhanced by the application of a transmembrane potential.

Interfacial effects

The pK of the spin-labeled stearic acid in DMPC bilayers is higher than that in bulk solution because of the lower polarity at the bilayer surface, which shifts the acid-base equilibrium (cf. Fernández and Fromherz, 1977). In DTPG bilayers, the apparent pK is even higher because of the electrostatic gathering of protons at the negatively charged bilayer surface (cf. Sankaram et al., 1990). Binding of melittin was found to shift the interfacial pK of the bilayer-incorporated stearic acid spin label toward lower pH values, in both cases. For DMPC vesicles, the shift in interfacial pK is $\Delta pK \approx -1.2$, from $pK \approx 7.1$ in DMPC alone to $pK \approx 5.9$ in DMPC-melittin complexes, whereas in DTPG the observed shift of $\Delta pK \approx -0.8$ is from $pK \approx 8.1$ in the lipid alone to $pK \approx 7.3$ in the complex with melittin. The comparable extent of the shifts for the two cases is surprising at first sight, in view of the considerably greater degree of melittin binding to DTPG than to DMPC (cf. Fig. 1). In fact, the shift is slightly smaller for DTPG than for DMPC. The total observed shift in interfacial pK of the fatty acid on

binding melittin is given generally by (see e.g. Cevc and Marsh, 1987; Sankaram et al., 1990)

$$\Delta pK^{int} = \Delta pK^{pol} + \Delta pK^{el} + \Delta pK^{LP}, \qquad (6)$$

where ΔpK^{pol} is the shift due to the change in interfacial polarity, ΔpK^{el} that due to the change in the surface electrostatic potential, and ΔpK^{LP} that arising from the different strengths of interaction of the two titrating forms of stearic acid with the peptide (cf. Table 1). The basic melittin peptide has a net charge of +5 to +6 (Stanislawski and Rüterjans, 1987); therefore the negative surface potential will be reduced and the anionic form of stearic acid will be preferentially stabilized on binding of melittin. This means that both ΔpK^{el} and ΔpK^{LP} will result in downward shifts in pK, to an extent that depends on the degree of binding. The rather similar values of the experimentally observed shifts for DMPC and DTPG, in spite of the very different extents of binding of the basic peptide, imply that the downward shifts by ΔpK^{el} and ΔpK^{LP} are partially compensated for by an upward shift in ΔpK^{pol} that is greater for DTPG than for DMPC. The latter again correlates with the greater extent of melittin binding to DTPG. A positive value for ΔpK^{pol} results from a destabilization of the ionic form of the fatty acid that must be caused by a decrease in the interfacial polarity on binding of melittin (cf. Fernández and Fromherz, 1977). Effectively, therefore, melittin gives rise to a partial dehydration of the lipid peptide interface. This could be caused by the strong interaction between opposite electrical charges, as in the binding of basic peripheral proteins (Sankaram et al., 1990), that might be augmented also by the presence of hydrophobic groups in the amphipathic peptide.

Lipid headgroup selectivity

The melittin-induced changes in the outer hyperfine splittings of the membrane-incorporated spin-labeled lipids were used to obtain information on interactions of the bound peptide with the different lipid headgroups (see Fig. 6 and Table 1). Because the outer hyperfine splittings of the various spin labels in the host lipids are not identical, two different types of effect may be distinguished (cf. Sankaram et al., 1989). These are the interactions characterized by the absolute values of A_{max} in the presence of melittin, and those characterized by ΔA_{max}, the increase in A_{max} induced by binding melittin, respectively. For the former, larger values are obtained with the polar lipids, either anionic or zwitterionic. In part, this is what is expected for the interaction of anionic lipid headgroups with the basic side chains of the peptide, which may be optimized in DTPG, where melittin penetrates deeper into the membrane and the effect is most pronounced. The selectivity also with zwitterionic lipids may reflect the amphiphilic character of melittin and indicate an optimization, in this case, also in the interaction with the hydrophobic side chains of the peptide. The in-

creases in A_{max} on binding melittin additionally reflect the differences in the outer hyperfine splitting between the various spin-labeled lipids in the absence of the peptide. These reference values of A_{max} are considerably smaller for the neutral lipids, diacylglycerol and protonated fatty acid, than for the polar lipids. This is because these lipids are situated deeper in the hydrophobic core of the lipid membrane (cf. Sankaram et al., 1989, 1990, and Fig. 5). The unusually large values of ΔA_{max} for these neutral lipids indicate that they move vertically upward in the membrane, in the direction of the lipid polar headgroups, on binding of melittin. This marked rearrangement is not found for the polar lipids, which are expected to remain in register with the host phospholipids of the membrane and may be facilitated by optimization of the interactions with the hydrophobic regions of the bound melittin. In spite of this strong interaction, the absolute values of A_{max} for the neutral lipids nonetheless remain smaller than those of the polar lipids, still favoring a more hydrophobic contact.

It is interesting to note that, in sarcoplasmic reticulum membranes, the binding of melittin preferentially increased the outer hyperfine splitting of spin-labeled stearic acid, relative to that of spin-labeled phosphatidylcholine (Mahaney et al., 1992), in agreement with the present results on lipid bilayers. Additionally, in the same work, it was found that the motionally restricted population of spin-labeled stearic acid interacting with the Ca-ATPase was preferentially increased, relative to that of phosphatidylcholine, on binding melittin.

It is likely that the present results will be of general significance for the interaction with lipid bilayers of other amphipathic, membrane-active peptides of biological significance. Among these are the magainins, cecropins, MARCKS protein, annexins, and the pseudo-substrate region of protein kinase C. Not least important, as already noted, is phospholamban, the membrane-bound Ca-ATPase regulator protein of cardiac sarcoplasmic reticulum, and signal peptides, presequences, and leader sequences, which contain both hydrophobic and basic residues, as does melittin.

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