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Melittin Binding to Mixed Phosphatidylglycerol/Phosphatidylcholine Membranes†

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ABSTRACT: The binding of bee venom melittin to negatively charged unilamellar vesicles and planar lipid bilayers composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) was studied with circular dichroism and deuterium NMR spectroscopy. The melittin binding isotherm was measured for small unilamellar vesicles containing 10 or 20 mol % POPG. Due to electrostatic attraction, binding of the positively charged melittin was much enhanced as compared to the binding to neutral lipid vesicles. However, after correction for electrostatic effects by means of the Gouy-Chapman theory, all melittin binding isotherms could be described by a partition equilibrium with the same surface partition constant of $K_P = (4.5 \pm 0.6) \times 10^4$ M^{-1}. It was estimated that about 50% of the total melittin surface was embedded in a hydrophobic environment. The melittin partition constant for small unilamellar vesicles was by a factor of 20 larger than that of planar bilayers and attests to the tighter lipid packing in the nonsonicated bilayers. Deuterium NMR studies were performed with coarse lipid dispersions. Binding of melittin to POPC/POPG (80/20 mol/mol) membranes caused systematic changes in the conformation of the phosphocholine and phosphoglycerol head groups which were ascribed to the influence of electrostatic charge on the choline dipole. While the negative charge of phosphatidylglycerol moved the N^+ end of the choline $^-P-N^+$ dipole toward the bilayer interior, the binding of melittin reversed this effect and rotated the N^+ end toward the aqueous phase. No specific melittin-POPG complexes could be detected. The phosphoglycerol head group was less affected by melittin binding than its choline counterpart.

Melittin, the main component of bee venom, is a cationic, amphiphilic peptide which binds to membranes and, at higher concentrations, disrupts the bilayer structure [cf. Batenburg et al. (1988), Altenbach and Hubbell (1988), and Dufourc et al. (1986a,b) and references cited therein]. Melittin carries a net charge of +5 to +6, and its binding to negatively charged membranes is distinctly enhanced compared to neutral membranes. This has been demonstrated experimentally in a series of binding studies on melittin-phosphatidylserine (Dufourcq & Faucon, 1977), melittin-cardiolipin (Batenburg et al., 1987a,b), melittin-phosphatidylglycerol (Batenburg et al., 1987c), and melittin-phosphatidylcholine/phosphatidylethanolamine membranes (Batenburg et al., 1988). The quantitative analysis of these binding isotherms was based on a chemical model with a defined number of lipids constituting a peptide "binding site". This type of approach corresponds to a Scatchard analysis and, though empirically useful, is inadequate to describe the binding of large ligands to a fluidlike

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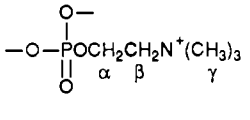
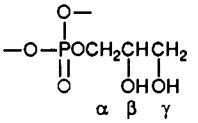
lipid bilayer [cf. Stankowski (1983a,b, 1984)]. Moreover, electrostatic effects such as the attraction of melittin to the negatively charged membrane surface or the repulsion of melittin from membranes with like charge were not taken into account. Since the concentration of free peptide near the lipid–water interface was not evaluated, such an approach yields incorrect chemical binding constants. We have previously measured melittin binding to nonsonicated bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)[1] and have corrected electrostatic effects by means of the Gouy–Chapman theory (Kuchinka & Seelig, 1989). In the present work, we have extended this approach to membranes containing 10 or 20 mol % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and have obtained a consistent description of all binding isotherms with a surface partition equilibrium. Even though the extent of melittin binding was distinctly enhanced in the presence of negatively charged lipid, this effect was due exclusively to an electrostatic attraction; the chemical partition equilibrium was not influenced by the membrane composition, and all binding data could be described by the same partition coefficient.

Deuterium NMR was used to explore the effect of melittin on the choline and glycerol head groups in mixed POPC/POPG membranes. The choline head group has been shown to be sensitive to electric charges at the membrane surface (Seelig et al., 1987, 1988; Scherer & Seelig, 1989), and it was of particular interest to evaluate the simultaneous influence of two oppositely charged molecules (melittin and POPG) on the orientation of the $^{-}P–N^{+}$ dipole of the choline head group.

Materials and Methods

To simplify the discussion, the following notations are introduced for the phosphocholine and phosphoglycerol headgroup segments:





Materials. Melittin was purchased from Sigma (St. Louis, MO) (grade II, phopholipase free) and purified according to Batenburg et al. (1987a) and Kuchinka and Seelig (1989). The lipid samples were stable over extended periods (weeks), and no lipid hydrolysis could be detected. The peptide concentration was determined with UV spectroscopy using an absorption coefficient of 5570 M^{-1} cm^{-1} at 280 nm (Quay & Condie, 1983).

Nondeuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA) were purchased from Avanti Polar Lipids (Birmingham, AL). Head group deuterated POPC and POPG were synthesized as described previously (Tamm & Seelig, 1983; Wohlgemuth et al., 1980).

Preparation of Lipid Samples for CD and NMR Experiments. Small unilamellar vesicles (SUV) were required for the CD experiments and were prepared as follows. A lipid dispersion (approximately 3 mg of lipid/mL) was sonicated under a nitrogen atmosphere for about 35 min (at 10 °C) until an almost clear solution was obtained. Metal debris from the titanium tip was removed by centrifugation for 10 min in an Eppendorf centrifuge. For NMR samples, lipids were mixed in dichloromethane in the appropriate molar ratios, and the solvent was removed in a stream of dry nitrogen and then by high vacuum. The total amount of lipid varied between 10 mg when deuterated POPC was used to 20 mg for samples containing deuterated POPG. Defined quantities of melittin were dissolved in 50 μL (deuterated POPC) or 100 μL (deuterated POPG) of buffer (0.1 M NaCl, 10 mM Tris-HCl, and 0.1 mM Na_2EDTA, pH 7.4), and the solution was added to the dried lipid. The samples were strongly vortexed, followed by several freeze–thaw cycles and further vortexing to ensure a homogeneous distribution of melittin.

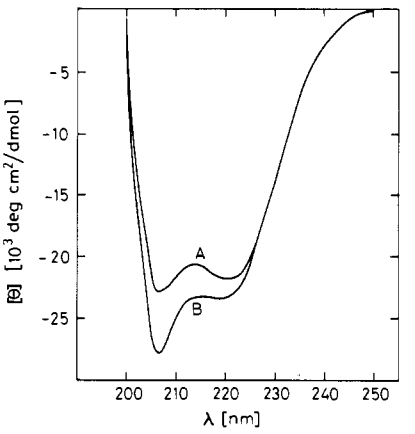


FIGURE 1: CD spectra of melittin in methanol and bound to POPC/POPG vesicles. Curve A, melittin (2×10^{-5} M) and POPC/POPG (80/20 mol/mol) small unilamellar vesicles (2×10^{-4} M) in buffer. Curve B, melittin in methanol ($c = 2 \times 10^{-5}$ M).

CD and NMR Measurements. CD measurements were made with a Cary 61 spectrometer calibrated with *d*(+)-10-camphorsulfonic acid. The optical length of the cuvette was 2 mm. The ^2H NMR spectra were recorded on a Bruker CXP-300 spectrometer operating at 46.1 MHz and, in part, on a Bruker MSL-400 spectrometer at 64.1 MHz. The experimental parameters were essentially the same as detailed previously (Kuchinka & Seelig, 1989). In all experiments, the temperature was maintained at 25 °C.

Results

Helix Content of Melittin. The CD spectrum of melittin in water is typical of a random coil conformation. However, upon binding to lipid vesicles, melittin adopts a more ordered structure, and the CD spectrum indicates a rather high helix content. This change in the CD spectrum has been used to measure the binding isotherm of melittin to phosphatidylcholine bilayers (Vogel, 1981) and has also been used in the present study to monitor the interaction of melittin with negatively charged lipids.

The helix content of membrane-bound melittin was estimated by Vogel (1981) as ~65%. We have reevaluated this question since it bears on the extent of membrane expansion when melittin penetrates into the lipid membrane. Our analysis was guided by the structure of melittin in methanol which has recently been determined by 2D NMR revealing a helix content of melittin close to 90% (Bazzo et al., 1988). Figure 1 then compares CD spectra of melittin in methanol and bound to POPG/POPC (20/80 mol/mol) bilayer vesicles, revealing a very close similarity of the two spectra. At the characteristic wavelength of the α-helix structure (λ = 222 nm), the ellipticity of melittin in methanol is $\theta_{222} = -23\,200$ deg cm^2/dmol (at 222 nm), and since this value, according

[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; SUV, small unilamellar vesicle(s).

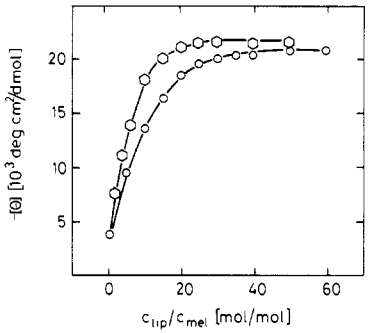


FIGURE 2: Variation of the negative ellipticity per residue of total melittin at 222 nm versus the total lipid-to-peptide ratio. The POPC/POPG composition of the small unilamellar vesicles was either 90/10 (mol/mol) (○) or 80/20 (mol/mol) (○). The initial melittin concentration was 2×10^{-5} M. All measurements made in buffer (0.1 M NaCl, 10 mM Tris-HCl, and 0.1 mM Na_2EDTA, pH 7.4).

to 2D NMR, corresponds to a helix content of 90% the ellipticity of a hypothetical , purely α-helical melittin can be estimated as $\theta_{helix} \simeq -25\,000$ deg cm^2/dmol.

This value is smaller than reference data in the literature of about $-30\,000$ deg cm^2/dmol characteristic of helical stretches with 10–20 amino acids (Chen et al., 1974). A possible explanation for this difference could be the fact that the latter data refer to large globular proteins where part of the α-helices are not accessible to solvent. In contrast, all amino acid residues in melittin are fully exposed to solvent.

The CD spectrum of melittin bound to POPG/POPC vesicles is very similar to that of methanol. With $\theta_{helix} = -25\,000$ deg cm^2/dmol, we estimate a helix content of $f_H = 0.85$ (0.82) for POPC vesicles with 20% (10%) POPG. This is about 20–30% larger than previous calculations (Vogel, 1981).

Melittin Binding to POPC/POPG Vesicles. A centrifugation assay has been used to measure the binding of melittin to phosphatidylethanolamine and phosphatidylcholine membranes (Batenburg et al., 1988; Kuchinka & Seelig, 1989). Unfortunately, this approach was not feasible for negatively charged membranes since under typical experimental conditions almost all of the added melittin was bound to the membrane surface. The concentration of free melittin in solution was then too small to allow an accurate analysis. We have therefore followed the method of Vogel (1981) and have titrated melittin solutions of a given initial concentration with small unilamellar vesicles (SUV), monitoring the binding by the change in the CD spectrum (Vogel, 1981; Schwarz & Beschiaschvili, 1989). This assay is contigent on the use of SUV in order to minimize the detrimental influence of light scattering. However, the lipid packing in SUV is different from that in planar lipid bilayers, and quantitative differences for melittin binding between planar bilayers with densely packed lipids and small vesicles with more loosely packed lipids are to be expected.

Typical titration curves for melittin with sonicated POPC/POPG vesicles, containing 10 or 20 mol % POPG, are shown in Figure 2. Melittin free in solution adopts a disordered conformation, and the molar ellipticity is $\theta_f = -3850$ deg cm^2/dmol at 222 nm. Upon addition of lipid vesicles, melittin binds to the lipid surface, and the ellipticity decreases, reaching a plateau value of $\theta_b = -21\,000$ deg cm^2/dmol (10% POG) or $-21\,500$ deg cm^2/dmol (20% POPG) when all melittin is bound to the membrane surface. Knowing the ellipticities of the free and the bound form, the fraction of membrane-bound melittin, f_b, can be determined according to

$$f_b = (\theta - \theta_f)/(\theta_b - \theta_f) \quad (1)$$

Using this information, it is then possible to calculate the equilibrium concentration of free melittin remaining in the aqueous phase, C_{eq}, as well as the extent of binding, X_b, defined as the molar amount of melittin bound per mole of total lipid.

In the experiments reported below, the concentration of melittin was chosen low enough so that (i) only monomers were in the aqueous phase and (ii) the bilayer structure remained intact. Under these conditions, melittin binds only to the outer half-layer of the unilamellar vesicles and does not permeate into the vesicle interior (Stanislawski & Rüterjans, 1987; Altenbach & Hubbell, 1988). Evidence for asymmetric binding was also obtained by the following experiment: SUV were added to a melittin solution under conditions where some melittin remained in solution. After the CD spectrum was recorded, the suspension was sonicated, leading to a temporary opening of the vesicles and making the vesicle interior accessible to melittin. The subsequent CD measurement demonstrated a distinctly enhanced melittin binding which was quantitatively consistent with the increase in melittin-accessible lipid.

From the size of the POPG/POPC vesicles and from NMR measurements with shift reagents, it has been estimated that about 60% of the total lipid is in the outer half-layer (Horwitz et al., 1974). We have therefore corrected the measured extent of binding, X_b, according to

$$X_b^* \simeq X_b/0.6 \quad (2)$$

Even though the correction factor is only approximate, X_b^* gives a better representation of the melittin density at the vesicle outside.

A plot of X_b^* versus the free melittin concentration, C_{eq}, yields the conventional binding isotherm, and the experimental results for POPC/POPG vesicles (10 and 20% POPG) (this work) as well as those obtained for electrically neutral DOPC vesicles (Schwarz & Beschiaschvili, 1989) and DMPC vesicles (Vogel, 1981) are shown in Figure 3. The latter data have been normalized according to eq 2. Addition of 10 or 20 mol % POPG leads to a dramatically enhanced melittin binding. This is purely an electrostatic effect since all binding isotherms are characterized by the same chemical binding constant as will be discussed below.

^{2}H NMR of Melittin Binding to Deuterated POPC/POPG Membranes. Figure 4 illustrates the effect of melittin on the ^{2}H NMR spectra of POPC/POPG (80/20 mol/mol) membranes in which the choline head group was deuterated at the α or β segment, respectively. The membranes were prepared by addition of buffered melittin solutions to dried lipid mixtures followed by vortexing and freeze–thawing. This led to the formation of coarse liposomes with almost planar bilayer domains. All spectra are characterized by a single quadrupole splitting, indicating a homogeneous environment of all phosphatidylcholine molecules. This is also confirmed by the ^{2}H NMR spectra of Figure 5 which refer to the same membrane system, this time with the deuterium label at the α segment of the glycerol head group. With and without melittin, the spectra show no evidence for a demixing of lipids or of two different lipid populations, i.e., lipids bound to melittin and lipids free in the membrane.

However, quantitative differences in the residual quadrupole splittings are observed upon melittin binding. The quadrupole splittings of the α and β segments of POPC vary in opposite directions upon addition of melittin: the quadrupole splitting decreases for the α segment and increases for the β segment, in agreement with results obtained previously for pure POPC membranes (Kuchinka & Seelig, 1989) and for phosphatidylcholine–phosphatidylserine membranes (Dempsey et al.,

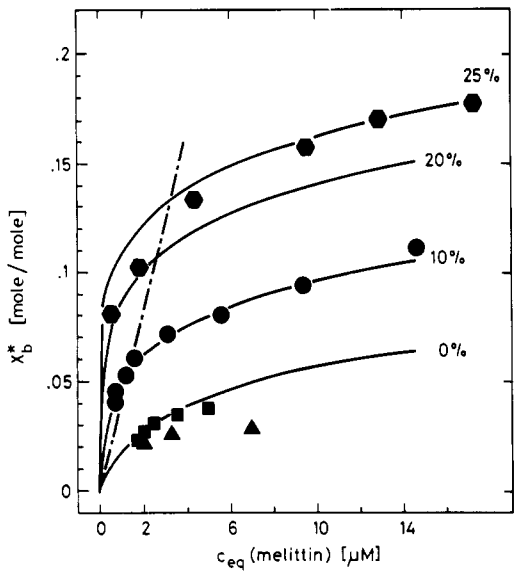


FIGURE 3: Melittin binding to phosphatidylcholine/phosphatidylglycerol membranes. The amount of melittin bound per mole of lipid (considering only the outer vesicle layer) is plotted versus the equilibrium concentration of melittin free in solution. Experimental data: (●) POPG/POPC 10/90 (mol/mol); (●) POPG/POPC 20/80 (mol/mol); (■) DOPC [data calculated from Schwarz and Beschiaschvili (1989), Figure 2; the r values of this figure were divided by 0.6 in order to correct for the asymmetric binding to the outer monolayer]; (▲) DMPC [data calculated from Vogel (1981); same correction as above]. The solid lines are theoretical binding isotherms calculated on the basis of a partition equilibrium with K = 45 600 M^{-1}, but taking into account the different electric surface charge by means of the Gouy–Chapman theory. The dashed line corresponds to a partition equilibrium neglecting electrostatic effects. For details of the calculation, cf. the text.

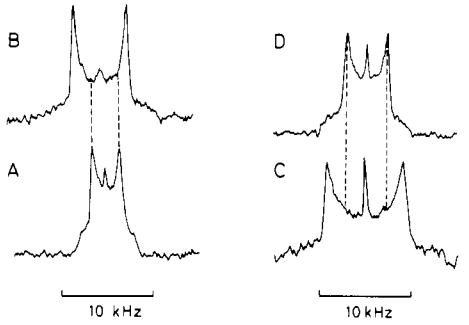


FIGURE 4: Deuterium NMR spectra of multilamellar dispersions of deuterated POPC/nondeuterated POPG (80/20 mol/mol) with and without melittin at 25 °C in buffer. (A) [β-CD_2]POPC without melittin; $\Delta\nu_\beta$ = 2.98 kHz. (B) [β-CD_2]POPC with melittin; X_b = 40.5 (mmol/mol); $\Delta\nu_\beta$ = 5.7 kHz. (C) [α-CD_2]POPC without melittin; $\Delta\nu_\alpha$ = 8.3 kHz. (D) [α-CD_2]POPC with melittin; X_b = 45.6 (mmol/mol); $\Delta\nu_\alpha$ = 4.4 kHz.

1989). The membrane samples used for ^2H NMR were prepared such that virtually all of the added melittin was bound to the membrane surface. This was confirmed experimentally by measuring the supernatant after membrane equilibration and centrifugation. In no case could melittin be detected in the supernatant with UV spectroscopy.

Figure 6 shows the variation of the quadrupole splittings of the α and β segments with the amount of bound melittin. Since the membranes were equilibrated by several freeze–thaw cycles, melittin binds symmetrically to both halves of the bilayer. In Figure 6, the quadrupole splittings, $\Delta\nu_Q$, are plotted against

$$r = X_b/[1 + X_b(A_P/A_L)] \quad (3)$$

r is proportional to the charge density at the membrane surface and takes into account the surface charge dilution due to protein penetration [cf. Seelig et al. (1988)]. Since $A_P/A_L \approx (150/68) = 2.2$ and $X_b \leq 0.05$, this yields a correction by at most 5% at the highest X_b. Figure 6 demonstrates a linear variation of $\Delta\nu_Q$ with r, and linear regression analysis yields the following relationships for the two choline head-group segments:

$$\Delta\nu_\alpha \text{ (kHz)} = 8.3 - 93.9r \text{ (mol/mol)} \quad (4)$$

$$\Delta\nu_\beta \text{ (kHz)} = 3.1 + 72.8r \text{ (mol/mol)} \quad (5)$$

The correlation coefficient is close to 1 in both cases. Eliminating r from the latter two equations yields a linear relation between the two quadrupole splittings [α–β plot; cf. Akutsu and Seelig (1981) and Roux et al. (1989)]:

$$\Delta\nu_\beta = 9.5 - 0.78\Delta\nu_\alpha \quad (6)$$

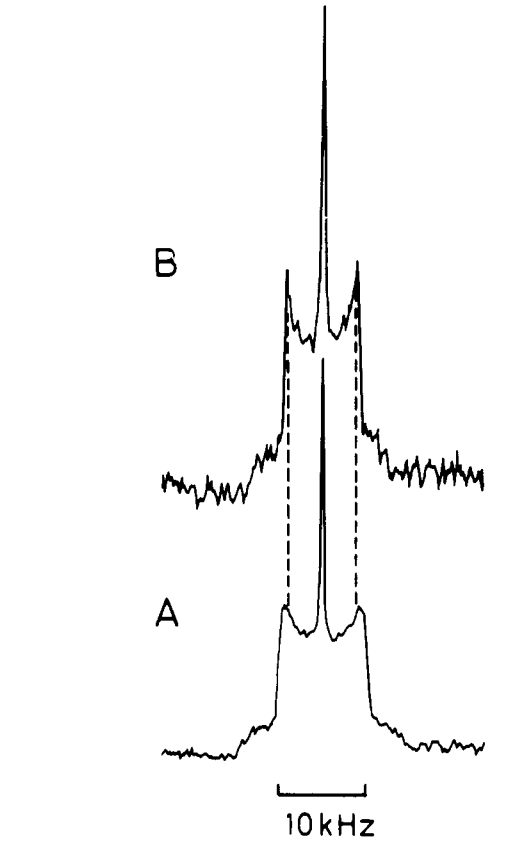


FIGURE 5: Deuterium NMR spectra of nondeuterated POPC/deuterated POPG (80/20 mol/mol) membranes with and without melittin. Measurements made in buffer at 25 °C. (A) [α-CD_2]POPG without melittin; $\Delta\nu_\alpha$ = 9.6 kHz. (B) [α-CD_2]POPG with melittin; X_b = 26.0 (mmol/mol); $\Delta\nu_\alpha$ = 8.5 kHz.

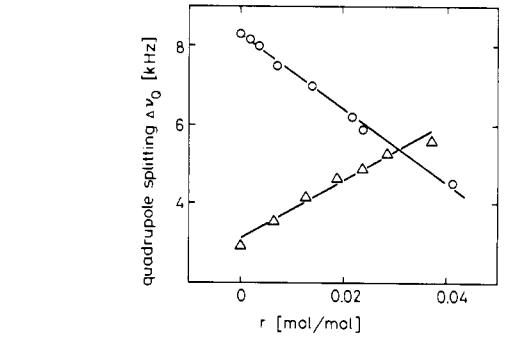


FIGURE 6: Conformational change of the phosphocholine head group in membranes composed of POPC/POPG (80/20 mol/mol) upon binding of melittin. POPC was deuterated at the α segment (○) and at the β segment of the choline moiety. The figure shows the variation of the quadrupole splittings with the mole fraction of bound melittin, r (cf. eq 3).

Table I: Binding of Melittin to POPG/POPC (10/90 mol/mol) Vesicles (25 °C; 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1 mM Na_2EDTA)

C_{eq} (μM)	X_b[a] (mmol/mol)	X_b*[b] (mmol/mol)	σ[c] (mC/m²)	Ψ_0 (mV)	C_M (μM)	X_b^*/c_M (M^{-1})
0	0	0	−23.5	−29.2		
0.77	24.1	40.2	−5.1	−6.7	1.26	31 900
0.74	27.6	46.0	−2.7	−3.5	0.96	47 900
1.19	31.5	52.5	−0.05	−0.01	1.20	43 900
1.78	36.7	61.2	3.4	4.4	1.29	47 600
3.13	42.9	71.5	7.3	9.5	1.55	46 000
5.84	49.0	81.7	11.0	14.2	2.03	40 100
9.44	57.1	95.2	15.7	20.0	2.14	44 500
14.6	67.1	111.8	21.2	26.6	2.05	54 600

[a] Measured by CD spectroscopy. [b] Calculated according to eq 2. [c] Calculated according to eq 3.

The effect of melittin on the quadrupole splitting of the glycerol head group was not investigated in detail. Figure 5 demonstrates that the α splitting of POPG decreases from 9.6 kHz (POPC/POPG, 80/20 mol/mol) in the absence of melittin to 8.5 kHz when $X_b = 0.026$ (mol/mol). Qualitatively, the α segments of POPG and POPC vary in the same direction. Quantitatively, the variation is by a factor of 2 smaller in POPG than in POPC.

Discussion

Analysis of the Binding Isotherm. Melittin will bind to POPG/POPC membranes for two reasons, namely, (i) the hydrophobic interaction between the nonpolar amino acids and the phospholipid hydrocarbon layer and (ii) the electrostatic interaction between the negatively charged membrane surface and the positively charged melittin. The binding step is probably accompanied by membrane expansion since the peptide molecule can intercalate between the lipid molecules as evidenced by monolayer experiments. A second consequence of melittin binding is a partial neutralization of the negative membrane surface charge. Both factors, membrane expansion and electric charge compensation, are taken into account in the following analysis.

Specific details of the model have been discussed in connection with the binding of Ca^{2+} to POPC membranes containing varying amounts of POPG or cardiolipin (Macdonald & Seelig, 1987a,b) and also for the binding and penetration of charged local anesthetics and substance P into POPC membranes (Seelig et al., 1988; Seelig & Macdonald, 1989). The present model is an extension of our previous analysis of melittin binding to electrically neutral POPC membranes (Kuchinka & Seelig, 1989) including here the effect of a negative surface charge.

In brief, the electric surface charge density, σ, at the outer leaflet of a POPC/POPG vesicle is given by

$$\sigma = (e_0/A_L)(-X_{PG} + X_b^* z_P)/[1 + X_b^*(A_P/A_L)] \quad (7)$$

Here X_{PG} denotes the mole fraction of POPG; A_L is the surface area of phospholipid which is assumed to be identical for POPC and POPG; e_0 is the elementary electric charge; z_P and A_P are the effective valency and area, respectively, of melittin at the membrane surface; X_b^* is the mole fraction of melittin bound to the outer monolayer as defined above. In the following analysis, we shall use $A_L = 68$ Å² (Altenbach & Seelig, 1984) and $A_P = 150$ Å². The latter value corresponds to the cross-sectional area of the α-helical region of melittin.[2] The exact value of A_P is not too critical since X_b^* is generally small and the denominator of eq 3 introduces only a small correction. The effective charge was $z_P = 1.9$ and was distinctly smaller than the true electric charge of 5–6. Possible explanations for this discrepancy have been discussed previously [cf. Schwarz and Beschiaschvili (1989) and Kuchinka and Seelig (1989)].

Knowledge of the membrane surface charge density as evaluated from X_b^* allows the calculation of the membrane surface potential, Ψ_0, using the Gouy–Chapman theory [cf. Aveyard and Haydon (1973) and MacLaughlin, 1977)]:

$$\sigma^2 = 2000\epsilon_0\epsilon_R RT\sum_i C_{i,eq}(e^{-z_i F_0\Psi_0/RT} - 1) \quad (8)$$

where $\epsilon = 78$ is the dielectric constant of water (at 25 °C), ϵ_0 the permittivity of free space, R the gas constant, F_0 the Faraday constant, $C_{i,eq}$ the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), and z_i the signed valency of the ith species. A computer program was employed to numerically evaluate the surface potential Ψ_0 from the experimentally known surface charge density σ.

The effect of a negative membrane surface charge is to attract positively charged melittin to the membrane surface. The concentration of melittin, C_M, immediately above the plane of binding is thus distinctly larger than the melittin concentration in the bulk aqueous phase, C_{eq}, and can be calculated according to

$$C_M = C_{eq}\exp(-z_P F_0\Psi_0/RT) \quad (9)$$

Numerical data for POPC/POPG (90/10 mol/mol) membranes are given in Table I.

For the set of parameters chosen, the ratio X_b^*/C_M remains approximately constant over the whole concentration range. After correction for electrostatic effects, the binding of melittin to this particular membrane can thus be described by a simple surface partition equilibrium of the form

$$X_b^* = K_P C_M \quad (10)$$

with binding constant $K_P = (4.5 \pm 0.6) \times 10^4$ M^{-1}.

Using this result, we have included in Figure 3 theoretical binding isotherms (solid lines) for POPC vesicles with varying POPG content. Also shown is the (hypothetical) partitioning of melittin in the absence of any electrostatic repulsion or attraction (dashed line). In the latter case, a strictly linear relationship between the amount of bound melittin and the equilibrium concentration of free melittin is expected. Inspection of Figure 3 reveals that the experimental data are in very good agreement with the theoretical predictions and can indeed be described by the same binding model and the same binding constant. The deviation of the DMPC data (Vogel, 1981) may be explained by the fact that this lipid does not contain cis-double bonds and is more densely packed than POPC (this work) or DOPC (Schwarz & Beschiaschvili, 1989).

[2] The surface area was estimated by two different methods. Computer modeling of the crystal structure showed that the radius of the helical region was about 7 Å. Measurement of the surface activity of pure melittin solutions yielded a surface area of melittin of around 140 Å².

The experimental data for POPC/POPG vesicles with 20% POPG do not exactly fit the corresponding theoretical binding curve but are better described by a binding isotherm with 25% POPG. Horwitz and co-workers have provided experimental evidence that sonication of mixed PC/PG dispersions led to an asymmetric distribution of PG molecules (Michaelson et al., 1974). Since the electrostatic repulsion of PG molecules is larger on the more densely packed inner leaflet, relatively more PG molecules reside on the outside than on the inside of the vesicle.

Figure 3 further demonstrates that at low concentrations of melittin the binding of the peptide to negatively charged vesicles is enhanced compared to a partition equilibrium with no charge effects. On the other hand, once the membrane surface charge is neutralized, further melittin binding is difficult because now the repulsion of like charges becomes the dominant mechanism. In terms of conventional binding mechanisms which do not take into account electrostatic effects, this would correspond to a transition from positive cooperativity to negative cooperativity. For neutral POPC vesicles, electrostatic repulsion is effective even at the lowest melittin concentrations.

Molecular Interpretation of Melittin Partitioning. The melittin partition constant of 4.6×10^4 M^{-1} corresponds to a free energy of $\Delta G = -RT \ln 55.5K_P = -8.7$ kcal/mol. Upon binding to the membrane surface, melittin loses 1 translational and 2 rotational degrees of freedom, requiring a ΔG of about 12 kcal/mol (Janin & Chotia, 1978; Figure 3). In contrast, the transition from a disordered conformation in water to a helical peptide in the membrane entails only a rather small energy change since intermolecular hydrogen bridges are replaced by intramolecular ones (Jähnig, 1983). Energy balance hence requires that the hydrophobic effect provides at least a free energy change of $\Delta G = -20.8$ kcal/mol. Since the transfer of a nonpolar protein from water into a nonpolar environment yields a free energy of −15 to −20 cal per 1 Å^2 surface area (Chotia, 1974; Richards, 1977), we estimate a buried surface area of 1000–1400 Å^2. The total accessible surface area of melittin can be calculated from the molecular weight, M (2846), according to $A_s = 11.12M^{2/3}$ [cf. Richards (1977)], yielding $A_s = 2235$ Å^2. Hence, 40–60% of the total surface area of melittin can be expected to be submerged in the hydrocarbon layer.

The binding of melittin to the nonsonicated POPC bilayer can also be described by a partition equilibrium but is characterized by a 20-fold smaller partition constant of 2.1×10^3 M^{-1} corresponding to $\Delta G = -7.0$ kcal/mol (Kuchinka & Seelig, 1989). This can be explained by a tighter lipid packing so that it is more difficult for the peptide to penetrate between the lipid molecules. However, the energy *difference* between melittin binding to SUV and planar bilayers is only 1.8 kcal/mol. Following the same reasoning as outlined above, it can be concluded that only 40–50% of the melittin surface is embedded in the hydrophobic part of planar POPC bilayers.

Melittin–Phospholipid Head-Group Interaction. The binding of melittin to mixed POPC–POPG bilayers induces a conformational change at both the choline and the glycerol head group. Qualitatively, this result is in agreement with previous studies on the interaction of melittin with DMPC (Dempsey & Watts, 1987), POPC (Kuchinka & Seelig, 1989), and DMPC/DMPS (Dempsey et al., 1989) membranes. The driving force for the conformational change is the positive electric charge brought onto the membrane surface (Seelig et al., 1987): the larger the surface charge, the larger are the changes in the quadrupole splittings. This effect is not limited to melittin but can be induced by all positively charged agents which bind to the membrane surface such as metal ions, charged local anesthetics, or positively charged amphiphiles [see Seelig et al. (1987), Roux et al. (1989), and Scherer and Seelig (1989) and references cited therein]. Negatively charged molecules have the opposite effect on the head-group conformation; i.e., they increase the α splitting and decrease the β splitting (Macdonald & Seelig, 1988).

Studies with positively charged amphiphiles have shed light on the molecular nature of this conformational change (Scherer & Seelig, 1989). While the choline dipole is approximately parallel to the membrane surface in the neutral phosphatidylcholine membrane, incorporation of positively charged amphiphiles rotates the choline moiety out of the membrane plane with the N^+ end of the dipole pointing toward the water phase. At the largest amphiphile concentration, this angular deviation amounts to about 30°. The behavior of the phosphocholine head group in the presence of melittin parallels exactly that induced by the positively charged amphiphiles. However, since the positive charge at the membrane surface is much smaller, the variation of the dipole orientation is less pronounced.

Negative and positive charges counteract each other in the POPG–POPC–melittin system. Mixing POPC bilayers with negatively charged phosphatidylglycerol first rotates the N^+ end of the choline dipole toward the bilayer interior; on the other hand, addition of melittin induces the opposite rotation, reversing the negative charge effect. A zwitterionic POPC membrane without electric charge is characterized by quadrupole splittings of $\Delta\nu_\alpha = 6.0$ kHz and $\Delta\nu_\beta = 5.1$ kHz (at 25 °C). Figure 6 then demonstrates that for a POPC/POPG membrane with 20 mol % POPG almost identical splittings are obtained when 23 mmol of melittin is bound per mole of total lipid. This amount of melittin is however insufficient to fully compensate the negative charge of phosphatidylglycerol even if one assumes that melittin carries its maximum possible charge of 5+ to 6+. Hence, an apparently "neutral" choline dipole orientation can be obtained with a net negative charge on the membrane surface. A similar observation has been made for the ternary system DMPC–DMPS–melittin (Dempsey et al., 1989), and these findings require a refinement of the charge concept.

While the electric charge is the main driving force for the dipole reorientation, substance-specific variations are introduced by the fact that different agents occupy differential spatial positions with respect to the choline dipole. Hydrophobic residues may anchor a molecule deep in the membrane interior; bulky side groups may restrict it from approaching laterally the $^-P–N^+$ residue. Hence, not only the total charge but also the height of the charged particles with respect to the membrane surface as well as their lateral separation from either end of the $^-P–N^+$ dipole is of importance in determining the choline dipole orientation.

As has been pointed out previously, melittin is one of the most effective lipid head-group modulators, changing the quadrupole splitting of the α segment by about −90 kHz per mole of bound melittin (Kuchinka et al., 1989), a result confirmed by this study and also by Dempsey et al. (1989). Taking into account the effective charge of $Z_P = 1.9$, the variation of the quadrupole splitting is −45 kHz per effective charge, which is comparable to charged local anesthetics (Boulanger et al., 1981; Seelig et al., 1988) or hydrophobic ions. At the β segment of the choline head group, the influence of melittin appears to depend on the membrane composition. The maximum variation of the quadrupole splitting is observed

for the POPG/POPC membrane with a slope of 72.8 kHz/mol, reducing to 44 kHz/mol for pure POPC (Kuchinka & Seelig, 1989) and reaching a minimum value of 20 kHz/mol for the DMPS/DMPC membrane (Dempsey et al., 1989).

Conclusions

Previous melittin binding studies have been interpreted in terms of a chemical model, using the equation $K_A = [PL_n]/([P][L])^{-1}$ where [P] and $[PL_n]$ represent the concentration of free peptide and of occupied peptide binding sites, respectively. The size of the peptide "binding site" was found to vary considerably between $n \simeq 60$ lipids per melittin for neutral membranes (Vogel, 1981; Batenburg et al., 1988), $n \simeq 10$ using a more refined statistical treatment (Stankowski, 1983a), $n \simeq 3$–4 for negatively charged phosphatidylserine (Dufourcq & Faucon, 1977), and $n \simeq 2$ for cardiolipin membranes (Batenburg et al., 1987a). Likewise, the binding constant varied by 2 orders of magnitude between 10^6 and 10^8 M^{-1}. In contrast, the present analysis considers electrostatic effects and explicitly calculates the concentration of the peptide immediately above the plane of binding. With this correction, the binding of melittin can be described by a surface partition equilibrium, yielding the same surface partition coefficient of $K_P = 4.5 \times 10^4$ M^{-1} for vesicles with and without negative surface charge. Peptide partitioning is accompanied by a membrane expansion, and roughly 50% of the melittin surface is estimated to be in contact with the hydrocarbon layer. The same analysis applied to nonsonicated POPC bilayers yielded a smaller surface partition constant of 2×10^3 M^{-1} and a 10% smaller contact area (Kuchinka & Seelig, 1989).

Negatively charged phosphatidylglycerol and positively charged melittin have opposite effects on the orientation of the phosphocholine dipole: melittin rotates the N^+ end toward the water phase and POPG toward the hydrophobic interior. The extent of head-group reorientation is controlled not only by the total surface charge density but also by the specific spatial positioning of the charged molecules with respect to the choline dipole.

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Registry No. POPC, 26853-31-6; melittin, 20449-79-0; 1-palmitoyl-2-oleoyl-*sn*-3-phosphoglycerol, 81490-05-3; phosphocholine, 107-73-3.

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