Anjaneyulu, P. S. R., Beth, A. H., Sweetman, B. J., Faulkner, L. A., & Staros, J. V. (1988a) *Biochemistry 27*, 6844-6851.
Anjaneyulu, P. S. R., Beth, A. H., Juliao, S. F., Sweetman, B. J., & Staros, J. V. (1988b) *Biophys. J. 53*, 529a.
Beth, A. H., & Robinson, B. H. (1989) *Biol. Magn. Reson. 8*, 179-253.
Beth, A. H., Conturo, T. E., Venkataramu, S. D., & Staros, J. V. (1986) *Biochemistry 25*, 3824-3832.
Beth, A. H., Conturo, T. E., Abumrad, N. A., & Staros, J. V. (1987) in *Membrane Proteins: Proceedings of the Membrane Protein Symposium* (Goheen, S. C., Ed.) pp 371-382, Bio-Rad Laboratories, Richmond, CA.
Cabantchik, Z. I., & Rothstein, A. (1974) *J. Membr. Biol. 15*, 207-226.
Curtis, O. E., Jr., Sandri, J. M., Crocker, R. E., & Hart, H. (1958) *Org. Synth. 38*, 19-21.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606-2617.
Hartley, D. (1962) *J. Chem. Soc.*, 4722-4723.
Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314-326.
Jennings, M. L., & Nicknish, J. S. (1985) *J. Biol. Chem. 260*, 5472-5479.
Keana, J. F. W., Keana, S. B., & Beetham, D. (1967) *J. Am. Chem. Soc. 89*, 3055-3056.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
Pariza, R. J., Kuo, F., & Fuchs, P. L. (1983) *Synth. Commun. 13*, 243-254.
Staros, J. V. (1982) *Biochemistry 21*, 3950-3955.
Staros, J. V. (1988) *Acc. Chem. Res. 21*, 435-441.
Staros, J. V., & Kakkad, B. P. (1983) *J. Membr. Biol. 74*, 247-254.
Staros, J. V., & Anjaneyulu, P. S. R. (1989) *Methods Enzymol. 172*, 609-628.
Steck, T. L., Ramos, B., & Strapazon, E. (1976) *Biochemistry 15*, 1154-1161.
Stetter, H., & Rauhut, H. (1958) *Chem. Ber. 91*, 2543-2548.
Sweetman, B. J., & Blair, I. A. (1988) *Biomed. Environ. Mass Spectrom. 17*, 337-340.
Willingham, G. L., & Gaffney, B. J. (1983) *Biochemistry 22*, 892-898.

Interaction of Melittin with Mixed Phospholipid Membranes Composed of Dimyristoylphosphatidylcholine and Dimyristoylphosphatidylserine Studied by Deuterium NMR

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ABSTRACT: The interaction of bee venom melittin with mixed phospholipid bilayers composed of dimyristoylphosphatidylcholine deuterated in the α- and β-methylenes of the choline head group (DMPC-d_4) and dimyristoylphosphatidylserine deuterated in the α-methylene and β-CH positions of the serine head group (DMPS-d_3) was studied in ternary mixtures by using deuterium NMR spectroscopy. The changes in the deuterium quadrupole splittings of the head-group deuteriomethylenes of DMPC-d_4 induced by DMPS in binary mixtures [DMPC-d_4:DMPS (80:20 mol/mol)] were systematically reversed by increasing concentrations of melittin, so that at a melittin concentration of 4 mol % relative to total lipid the deuterium NMR spectrum from DMPC-d_4 in the ternary mixture was similar to the spectrum from pure DMPC-d_4 bilayers. This concentration of melittin is sufficient to neutralize the excess negative charge from DMPS. The absence of deuterium NMR signals arising from melittin-bound DMPS in ternary mixtures containing DMPS-d_3 indicates that the reversal by melittin of the effects of DMPS on the quadrupole splittings of DMPC-d_4 results from the response of the choline head group to the net surface charge rather than from phase separation of melittin–DMPS complexes. In mixtures containing deuterated DMPS [DMPC:DMPS-d_3 (50:50 mol/mol)] melittin caused systematic changes in the quadrupole splittings of the DMPS head-group deuterons that closely matched effects observed for a cationic transbilayer polyleucyl peptide ($K_2GL_{20}K_2A$) in similar ternary mixtures [Roux, M., Neumann, J. M., Hodges, R. J., Devaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313-2321]. The similarity in the effects of the two cationic but otherwise dissimilar peptides indicates that the DMPS head group responds to the surface charge resulting from the presence in the bilayer of charged amphiphiles, in a manner analogous to the response of the choline head group of phosphatidylcholine to the bilayer surface charge. The presence of DMPS greatly stabilized DMPC bilayers with respect to melittin-induced micellization, indicating that the latter effect of melittin may not be important for the hemolytic activity of the peptide.

Bee venom melittin (Habermann & Jentsch, 1967) is a basic, hydrophobic peptide of 26 amino acids that binds to model and cell membranes, causing their lysis through as yet unknown mechanisms. Melittin associates with membranes as an amphiphilic α-helix; the aggregation state of the peptide and the orientation of the helix with respect to the membrane remain undetermined [see Altenbach and Hubbel (1988) for a recent discussion]. In model membranes, melittin induces

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changes in lipid membrane morphology, causing reversible transitions between bilayers and micellar disks in saturated phosphatidylcholine membranes (Dufourc et al., 1986a,b; Dempsey & Watts, 1987; Dempsey et al., 1987), stabilizing bilayer structure in mixtures of DMPC and its hydrolytic products (Dempsey & Watts, 1987) and in phosphatidylethanolamine membranes (Batenburg et al., 1988), and inducing inverted lipid structures in negatively charged membranes (Batenburg et al., 1987a–c). The relationship between the effects of melittin on lipid organization and bilayer structure and its hemolytic activity is not established. It has been suggested that the ability of melittin to solubilize phospholipids as membrane disks may underlie the lytic activity of the peptide (Dufourc et al., 1986a) and that the similarity in the effects of melittin and lysophosphatidylcholine on membrane morphology in saturated phosphatidylcholine membrane may indicate common mechanisms of membrane disruption for melittin and lysoPC (Dempsey & Watts, 1987).

The affinity of melittin (net charge of +5 at pH 7.5) for negatively charged lipids is about 2 orders of magnitude greater than for zwitterionic lipids (Batenburg et al., 1988), and there is evidence for phase separation of melittin–lipid complexes in bilayers containing negatively charged lipids when the membranes are cooled into the gel phase (Faucon et al., 1981; Bernard et al., 1982; Lafleur et al., 1989). The potential ability of melittin to induce phase separation of negatively charged lipids, together with its selective effects on the organization of different lipid types, leads to the possibility that its hemolytic activity results from selective binding and phase separation of negatively charged membrane lipids with local changes in lipid morphology resulting in membrane disruption. These possibilities are explored here by using deuterium NMR to study the effects of melittin on mixed bilayers composed of head-group-deuterated DMPC and DMPS. The use of separate membrane preparations in which either the DMPC or the DMPS is deuterated allows independent determination of the effects of the peptide on each of the membrane components with the possibility of determining phase separation, in fluid-phase membranes, on the time scale of the deuterium NMR spectral anisotropy.

Materials and Methods

Materials. Melittin was purified from lyophilized bee venom (Bulgarcoop, Sofia, Bulgaria) by forced dialysis, ion-exchange chromatography on Sephadex G-25, and gel filtration as described (Gauldie et al., 1976; Dempsey & Watts, 1987). Residual phospholipase A_2 activity in melittin preparations was inhibited by inclusion of 5 mM EDTA in all buffers. Lipid integrity was determined by TLC after all NMR experiments (Dempsey & Watts, 1987). In none of the lipid preparations for which data are presented in this paper was there detectable formation of lysolipid or free fatty acid that would indicate lipid hydrolysis.

DMPC was purchased from Fluka. Head-group-deuterated DMPC (DMPC-d_9 and DMPC-d_4) were prepared as described previously (Sixl & Watts, 1982). DMPS and DMPS-d_3 (deuterated in the α- and β-positions of the head group) were synthesized by the method of Roux and Neumann (1986).

Preparation of Lipid Samples for ESR and NMR Experiments. Lipids were mixed in $CHCl_3$–MeOH in the appropriate weight ratios, and the solvent was removed in a stream of dry nitrogen and then by high vacuum (10^{-2} Torr) overnight. Samples containing DMPS were mixed in $CHCl_3$–MeOH with 5% water to aid solubilization of DMPS. These samples were lyophilized to minimize the selective precipitation of DMPS from DMPC during removal of the organic solvent. The dried lipid was resuspended by vortexing above the phase transition temperature of DMPS (45 °C) in an excess of 50 mM Tris-HCl and 5 mM EDTA, pH 7.5, made in deuterium-depleted water (Aldrich Chemical Co.) and containing an appropriate amount of melittin to give the desired melittin:lipid ratio. The pH was adjusted back to pH 7.5 if neccessary. The lipid was pelleted by centrifugation (70000g for 30 min at room temperature) and the supernatant removed and retained. The lipid samples were then placed in 5 mm × 30 mm NMR tubes and the samples freeze–thawed three times. The amount of free melittin in the supernatant was determined by UV spectroscopy using an extinction coefficient ϵ_{280} = 5600 M^{-1} cm^{-1} (Quay & Condie, 1983), and the melittin:lipid ratio in the pellet was calculated by adjusting the starting ratio to account for unbound melittin. The melittin:lipid ratios quoted here refer to lipid-bound melittin. The amount of unbound melittin in the DMPS-containing samples was negligible.

NMR Measurements. Deuterium NMR spectra were obtained at 55.3 MHz by using a Nicolet spectrometer with an Oxford Instruments magnet operating at 360 MHz for protons and using a solenoid probe (Bruker) tuned to the deuterium frequency. Spectra were collected by using pulse rotation angles between 60° and 90° in the single pulse mode in 1K data points over a spectral width of 40 kHz with a minimum preacquisition delay of 12 μs following the pulse. The signal:noise ratio was improved by exponential multiplication of the free induction decay (FID) and the block size increased to 2K by zero-filling before Fourier transformation. The 90° pulse for deuterium was around 11 μs. Spectra taken later in the study were obtained by using a quadrupole echo pulse sequence (Davis, 1983) with 7-μs 90° pulses (obtainable by using a 300-W power amplification), digitizing the signal with a Nicolet digital oscilloscope (Model 2090-111A) using a dwell time of 2 μs. Phosphorus NMR spectra were obtained at 145.9 MHz over a spectral width of 50 kHz without proton decoupling.

Results

DMPS:DMPC-d_4 Mixtures. Figure 1 illustrates the effects of DMPS on the deuterium NMR spectra of DMPC-d_4 at 25 and 40 °C and the effects of melittin at various lipid:peptide molar ratios on the spectra of DMPC-d_4:DMPS (80:20 mol/mol) mixtures. As previously shown (Sixl & Watts, 1983), inclusion of DMPS in bilayers with DMPC results in an increase in the value of the quadrupole splitting of the α-CD_2 groups and a decrease in $\Delta\nu_q$ for the β-CD_2 methylenes (Figure 1B) when compared with pure DMPC-d_4 bilayers (Figure 1A). Melittin was previously shown to have the opposite effect in bilayers of DMPC-d_4, reducing the quadrupole splitting of the α-CD_2 methylenes by 60% at a melittin:DMPC ratio of 4 mol % and marginally increasing (by 8%) the splitting of the β-methylenes (Dempsey & Watts, 1987). Successively increasing the concentration of melittin in mixed DMPC-d_4:20% DMPS bilayers results in a continuous reversal of the effects of DMPS on the head-group splittings of DMPC-d_4 (Figure 1). A spectrum similar to that obtained from pure DMPC-d_4 is obtained at a molar ratio (melittin:DMPS) of 1:5 (Figure 1D), which is approximately equivalent to a neutralization by melittin (5 or 6 positive charges/molecule, depending on the ionization state of the N-terminal amino group) of the excess surface charge resulting from incorporation of DMPS at 20 mol % into DMPC bilayers.

All the spectra obtained from DMPC-d_4:DMPS (20 or 50 mol %) mixtures with or without melittin are characteristic of single components with no evidence for DMPS-induced or melittin-induced phase separation of subpopulations of

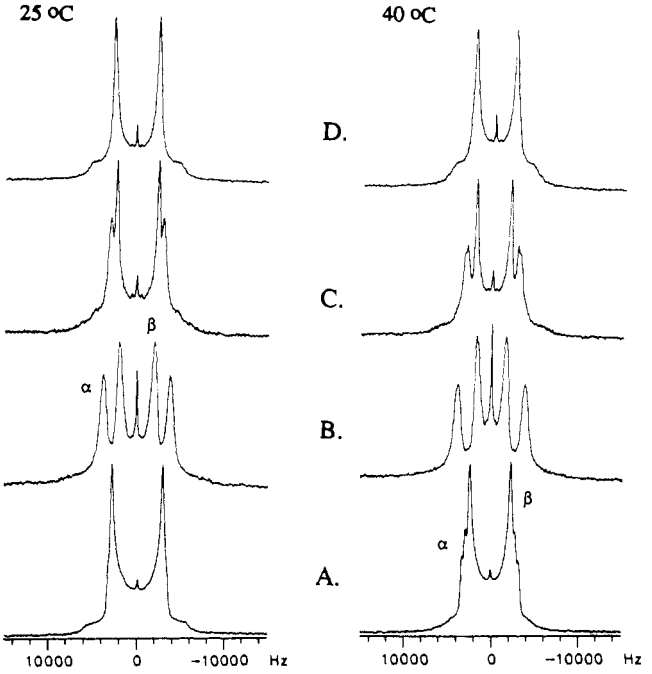

FIGURE 1: Deuterium NMR spectra of DMPC-d_4 in single-lipid bilayers (A), in mixed bilayers containing 20% DMPS (B), and in mixed DMPC-d_4:20% DMPS bilayers containing melittin at 2 mol % (C) and 4 mol % (D) relative to total lipid. The lipid was suspended in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA.

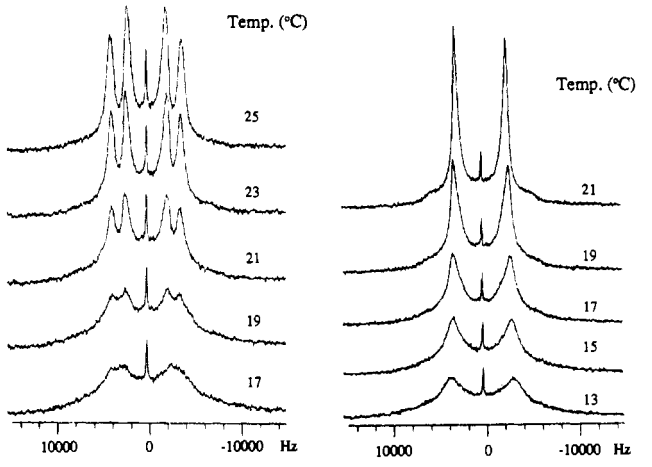

FIGURE 2: Variation of the deuterium NMR spectra of mixed bilayers containing DMPC-d_4:20 mol % DMPS (left) and DMPC-d_4:20 mol % DMPS:4 mol % melittin (right) as the temperature is lowered through the gel to liquid-crystalline phase transition of the lipid.

DMPC-d_4 having lifetimes greater than 10^{-4}–10^{-5} s (the time scale required to average the residual deuterium quadrupolar anisotropy). Similarly, the DMPC-d_4 quadrupole splittings increase and the spectral lines broaden continuously as the temperature is lowered through the liquid-crystalline–gel phase transition of the lipid in DMPC-d_4:DMPS mixtures with and without melittin (Figure 2), indicating the absence of phase separation at the phase transition. The lipid phase transition temperatures, estimated by deuterium NMR from the marked onset of spectral broadening (Figure 2), were always similar to phase transitions measured by ESR tempo partitioning (not shown).

In Figure 3 the temperature dependencies of the quadrupole splittings of the α- and β-methylenes of DMPC-d_4 are plotted for pure DMPC-d_4 bilayers and for DMPC-d_4 bilayers containing 20% DMPS with and without melittin. As previously observed (Browning & Seelig, 1980), the quadrupole splittings

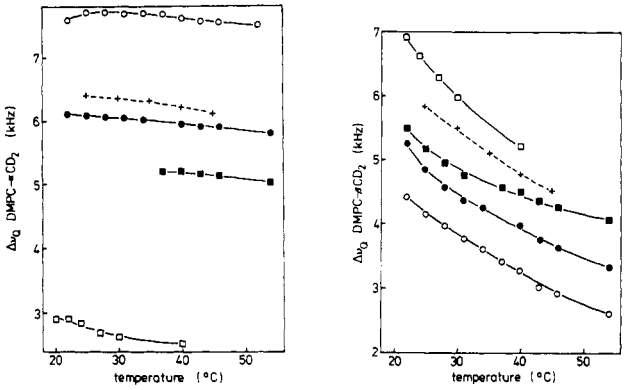

FIGURE 3: Temperature dependence of the deuterium NMR quadrupole splittings ($\Delta\nu_q$) for the choline head group α-deuteriomethylenes of DMPC-d_4 (left) and the β-deuteriomethylenes (right) in 50 mM Tris-HCl, pH 7.5. (+) Pure DMPC-d_4 bilayers; (□) DMPC-d_4:4 mol % melittin; (○) DMPC-d_4:20 mol % DMPS; (●) DMPC-d_4:20 mol % DMPS:2 mol % melittin; (■) DMPC-d_4:20 mol % DMPS:4 mol % melittin.

of the α-deuteriomethylenes are relatively insensitive to temperature, whereas the β-deuteriomethylenes show a large temperature dependence in $\Delta\nu_q$. These temperature characteristics are largely maintained in the presence of DMPS and in ternary mixtures containing DMPS and melittin despite very large chains in the values of $\Delta\nu_q$ induced by these compounds.

Figure 3 shows that the ability of melittin to reverse the effect of DMPS in increasing the splitting of the α-CD_2 group of DMPC-d_4 in mixed bilayers is much more effective than the reversal of the effect of DMPS on the β-splitting. Thus, the incorporation of melittin at 2 mol % in DMPC:20% DMPS bilayers overcompensates the effect of DMPS on the α-CD_2 groups, while at 4 mol % melittin the effect of DMPS on the β-splitting still dominates. Rather than simply canceling the effect of DMPS on the splittings of the α- and β-deuteriomethylenes of DMPC-d_4, the effects of melittin and DMPS are roughly additive.

DMPC:DMPS-d_3 Mixtures. Dilution of DMPS with equimolar DMPC results in a reduction of the deuterium quadrupolar splittings of the DMPS head group by about 60% (Browning & Seelig, 1980; Roux & Neumann, 1986). Further dilution of DMPS with DMPC has little effect on the DMPS head-group quadrupolar splittings, so that their magnitudes are little different for mixtures of DMPC and DMPS-d_3 containing 50% or 80% DMPC [compare our Figure 5 with Figure 6 of Roux et al. (1989)]. This finding indicates that the large quadrupole splittings (and short T_1 relaxation times) observed in pure DMPS result from strong intermolecular interactions between the PS head groups (Browning & Seelig, 1980) and that these interactions are almost fully suppressed by dilution of DMPS with equimolar DMPC.

Addition of melittin to bilayers composed of equimolar DMPS-d_3 and DMPC causes successive decreases in the β and the α_1 quadrupole splittings of the serine head group, whereas the inner α_2 splitting is relatively unaffected by the presence of melittin at concentrations of up to 8 mol % relative to total lipid (16 mol % relative to DMPS; Figures 4 and 5). The value of the quadrupolar splitting of the outer α_1-CD_2 splitting presumably changes sign, going through zero at around 6 mol % melittin (Figure 4), an observation confirmed in Figure 5, which shows the variation of the values of the quadrupolar splittings of the DMPS-d_3 head-group deuterons as a function of the melittin:lipid ratio in the equimolar DMPS-d_3:DMPC bilayers.

All of the spectra recorded from these ternary mixtures indicate the presence of a single DMPS-d_3 spectral component

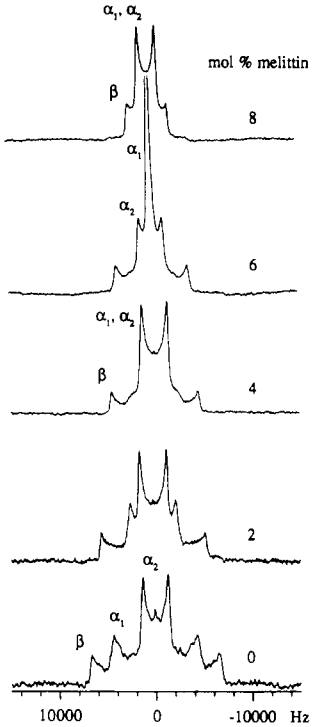

FIGURE 4: Deuterium NMR spectra at 35 °C of DMPS-d_3 in mixed bilayers with 50 mol % DMPC (bottom spectrum) and in mixed DMPS-d_3:DMPC (50:50 mol/mol) bilayers containing increasing amounts of melittin. The lipids were suspended in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA.

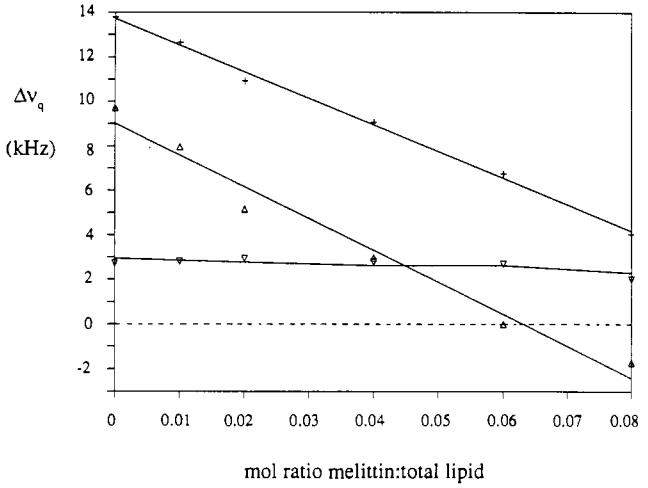

FIGURE 5: Variation of the deuterium NMR quadrupole splittings ($\Delta\nu_q$) of the serine head group of DMPS-d_3 in mixed bilayers with DMPC (DMPS-d_3:DMPC, 50:50 mol/mol) on increasing the concentration of melittin. (+) β-CD; (Δ) α_1-CD_2; (∇) α_2-CD_2. Measurements were made at 35 °C.

with no evidence for phase separation on the NMR time scale (10^{-4}–10^{-5} s). Spectra obtained over a bandwidth of 0.5 MHz by using quadrupole echo excitation optimized to detect broad spectral components showed no components other than those observed in single-pulse excitation (Figure 6), confirming that melittin does not form long-lived complexes with DMPS in mixtures with DMPC. Similarly, the quadrupole splittings for the deuterated serine head group increase and broaden continuously when the temperature is lowered through the phase transition temperature, which is reduced to about 23 °C in the ternary mixture containing DMPC, DMPS-d_3, and melittin (50:50:4 mol/mol/mol), indicating the absence of phase separation of DMPS and DMPC at the gel–liquid-crystalline phase transition of the lipid (Figure 7).

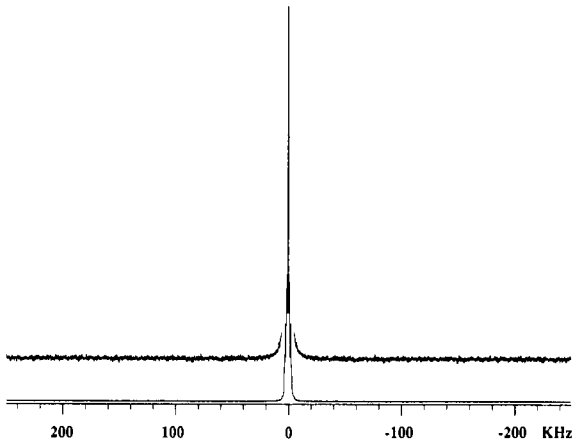

FIGURE 6: Deuterium NMR spectrum of a ternary mixed bilayer of DMPS-d_3:DMPC:melittin (50:50:6 mol/mol/mol) obtained with a quadrupole echo pulse sequence (7-μs 90° pulse; 30-μs interpulse delay; 2-μs dwell time). The upper spectrum is a 10-fold vertical expansion of the lower spectrum.

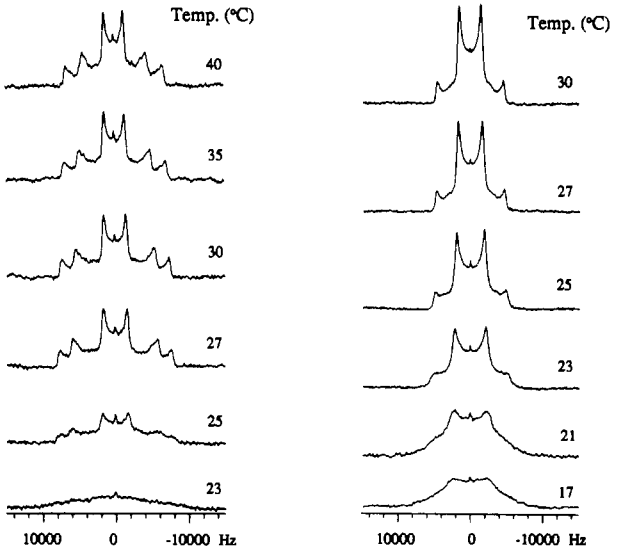

FIGURE 7: Variation of the deuterium NMR spectra of mixed bilayers of DMPS-d_3:DMPC (50:50 mol/mol) (left) and DMPS-d_3:DMPC:melittin (50:50:4 mol/mol/mol) (right) as the temperature is lowered through the gel to liquid-crystalline phase transition temperature of the lipid mixture.

Melittin-Induced Lipid Polymorphism. In bilayers composed of saturated phosphatidylcholines, melittin induces transitions between fluid-phase extended bilayers and gel-phase micellar disks at the lipid phase transition, an effect observable by the collapse of the phosphorus and deuterium fluid-phase powder patterns to narrow lines below T_c characteristic of isotropic reorientation of the gel-phase lipid particles on the NMR time scale (Dufourc et al., 1986a,b; Dempsey & Watts, 1987). DMPC is particularly sensitive to melittin-induced bilayer disruption, significant gel-phase micellization occurring for melittin concentrations above about 2–3 mol % (Dempsey & Watts, 1987). In neither the ternary mixtures containing deuterated DMPC (Figure 2) nor deuterated DMPS (Figure 7) was there any collapse of a proportion of the deuterium powder pattern into narrow lines when the mixtures were cooled through the lipid phase transition temperature into the gel phase that would indicate melittin-induced micelle formation. Similarly, the ^{31}P spectra (not shown) showed that all the lipid was in a bilayer phase at all temperatures and melittin/lipid compositions studied at melittin concentrations of up to 6 mol % relative to total lipid (12% relative to DMPC in DMPC/DMPS equimolar mixtures).

T_1 Relaxation Data. The spin–lattice (T_1) relaxation times measured at 42 °C for the α-deuteriomethylenes (27 ± 2 ms) and β-deuteriomethylenes (31 ± 2 ms) were not significantly affected by the presence of either DMPS at 20 or 50 mol %, melittin at 4 mol %, or mixtures of DMPS (20 mol %) and melittin (4 mol %). Similarly, there was no significant effect of melittin at 8 mol % in DMPC/DMPS-d_3 mixtures on the T_1 relaxation times of the DMPS head-group deuterons, which were 9.3 ± 0.3 ms for α-CD_2 and 9.4 ± 0.3 ms for β-CD at 35 °C.

Electrostatic Contributions to the Phosphatidylcholine Head-Group Conformation. It has been shown that the quadrupole splittings of the α- and β-deuteriomethylenes of PC vary linearly when groups conferring either a negative or positive potential to the membrane surface are titrated into a PC bilayer, an observation interpreted in terms of a charge-induced variation in the orientation of the choline head-group dipole [reviewed in Seelig et al. (1987)]. The deuterium quadrupole splitting of the α-CD_2 group increases when the negative potential increases, and decreases (less markedly) as the surface potential becomes increasingly positive. The quadrupole splitting of the β-CD_2 group varies in the opposite way (Seelig et al., 1987). The effects of charged amphiphiles on the measured quadrupole splittings of the DMPC-d_4 deuterons are characterized by fits to empirical equations of the form

$$\Delta\nu_q(A) = \Delta\nu_q(0) + xc_A \quad (1)$$

where $\Delta\nu_q(A)$ is the measured quadrupole splitting in the presence of the charged amphiphile, $\Delta\nu_q(0)$ is the measured quadrupole splitting of pure lipid in the absence of the amphiphile, and c_A is the mole fraction of the amphiphile (Altenbach & Seelig, 1984; Seelig et al., 1987). Derivation of similar equations for the quadrupole splittings of the α- and β-deuterons of DMPC-d_4 (at 40 °C) in the presence of melittin at mole ratios up to 4 mol % (Dempsey & Watts, 1987; this study) and in mixtures with DMPS at mole ratios up to 20 mol % (Sixl & Watts, 1983; this study) yields

$$\Delta\nu_q\alpha(\text{melittin}) = 6200 - 920\text{cML} \quad (2)$$

$$\Delta\nu_q\beta(\text{melittin}) = 4800 + 200\text{cML} \quad (3)$$

and

$$\Delta\nu_q\alpha(\text{DMPS}) = 6200 + 70\text{cPS} \quad (4)$$

$$\Delta\nu_q\beta(\text{DMPS}) = 4800 - 80\text{cPS} \quad (5)$$

where cML and cPS are the mole percentages of melittin and DMPS, respectively.

If the measured quadrupole splittings of the DMPC-d_4 deuteriomethylenes in the ternary mixtures examined here are a function of the residual surface charge, then the splittings should return to the values of pure DMPC-d_4 when the excess surface charge from DMPS is neutralized by melittin. A similar conclusion is made by assuming an ideal situation in which phase separation of melittin–DMPS complexes occurs with a defined stoichiometry (1:5; melittin–DMPS most closely satisfies the results obtained here). However, neither phase separation nor the response of the DMPC-d_4 choline head group to the net surface charge is consistent with the data of Figure 3, which indicates that the effects of melittin and DMPS are additive. The quadrupole splittings might then be characterized by relationship of the form

$$\Delta\nu_q\alpha(\text{melittin,DMPS}) = 6200 - 920\text{cML} + 70\text{cPS} \quad (6)$$

$$\Delta\nu_q\beta(\text{melittin,DMPS}) = 4800 + 200\text{cML} - 80\text{cPS} \quad (7)$$

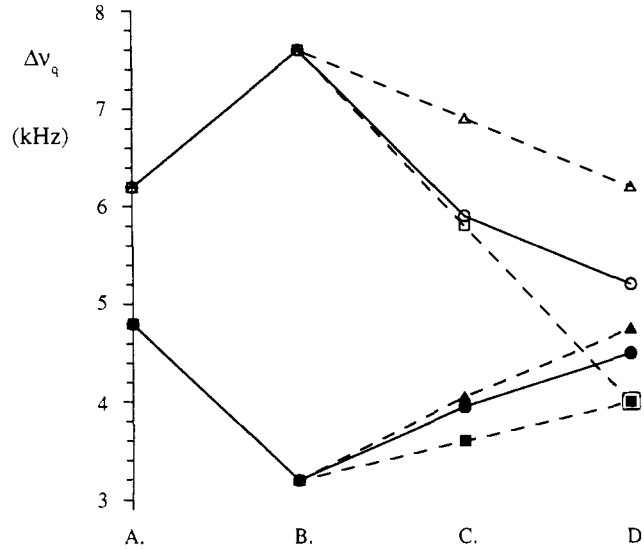

FIGURE 8: Deuterium NMR quadrupole splittings ($\Delta\nu_q$) of the choline head group α-CD_2 group (open symbols) and β-CD_2 group (solid symbols) in pure DMPC-d_4 bilayers (A), DMPC-d_4:20 mol % DMPS (B), DMPC-d_4:20 mol % DMPS:2 mol % melittin (C), and DMPC-d_4:20 mol % DMPS:4 mol % melittin (D). The experimental data at 40 °C (taken from Figure 3) are denoted by squares. Broken lines denote values calculated by assuming (a) an additive electrostatic contribution of melittin and DMPS to the DMPC-d_4 head group quadrupole splittings according to eq 6 and 7 (circles) and (b) phase separation of DMPS–melittin complexes from the mixed-lipid phase with stoichiometry of 5:1 (DMPS:melittin) (triangles). See text for details.

Figure 8 is a plot of the experimental variation of the values of the quadrupole splittings of the DMPC-d_4 head group (solid lines) together with the values of the quadrupole splittings calculated by assuming (1) melittin–DMPS phase separation occurs with a stoichiometry of 1:5 (melittin:DMPS) and (2) an additive effect of melittin and DMPS on the choline head-group quadrupole splittings according to eq 6 and 7. Neither the simple analyses based on melittin–DMPS phase separation nor the electrostatic contribution to the values of the head-group quadrupole splittings gives a preferred fit to the experimental data.

Discussion

The absolute magnitudes of the quadrupole splittings of the choline head-group α-CD_2 and β-CD_2 deuterons have contributions both from the orientation of the C–D bonds with respect to the major axis of motional averaging (the director axis which is parallel to the bilayer normal) and from the amplitude of motions that are fast on the time scale of the quadrupole coupling constant ($\gg 10^{-6}$ s^{-1}) and motionally average the anisotropy inherent in bilayer membrane structure (Davis, 1983). The effect of DMPS in promoting an increase in the magnitude of the α-CD_2 quadrupole splitting and a decrease in the magnitude of the β-CD_2 quadrupole splittings of the choline head group (Sixl & Watts, 1983; Figure 1) indicates that the negatively charged lipid induces a change in the average orientation of the head group. A general disordering of the head group, characterized by an increased amplitude of fast motions, would generated decreased values of the quadrupole splittings at all sites (Seelig & Seelig, 1980). In the same way, the ability of melittin to counteract the effects of DMPS on the quadrupole splittings of the choline head group in the ternary mixtures demonstrates that the average conformation of the head group is affected by the presence of melittin, returning toward a conformation close to that of

the pure lipid (pure DMPC) at a melittin:DMPS ratio of about 1:5 (mol/mol; Figure 1).

There are two possible explanations for the effect of melittin in DMPS–DMPC-d_4 mixtures. The first arises from reports of the ability of melittin to induce phase separation of subpopulations of negatively charged lipids (Faucon et al., 1981; Bernard et al., 1982; Lafleur et al., 1989) by the formation of melittin–lipid complexes. In the present study the effects of DMPS on the head-group splittings are "neutralized" by melittin at a ratio of melittin to DMPS of 1:5 (mol/mol). Melittin has a net charge of +5 at pH 7.5 in membranes, and the ability of each molecule of melittin to remove five molecules of DMPS from the mixed phase could give rise to the effects seen in Figure 1.

Phase separation of long-lived melittin–DMPS complexes on a time scale likely to be significant with respect to macroscopic effects such as hemolysis or disk micelle formation can, however, be ruled out on the following grounds. It is unlikely that any DMPS complexed with melittin in a phase-separated mixture would have identical conformational and dynamic properties compared with DMPS in the mixed phase with DMPC in the absence of melittin. At melittin:DMPS ratios lower than those required for stoichiometric complexation of all the DMPS, a two-component deuterium NMR spectrum containing separate signals from DMPS-d_3 complexed with melittin and uncomplexed DMPS-d_3 would be expected. However, in all mixtures studied, spectra characterized by only a single component of DMPS-d_3 were observed (Figure 4). Spectra obtained by using quadrupole echo detection (Davis, 1983) optimized to detect broad signals with short T_2 relaxation times (Figure 6) also failed to reveal components other than those obtained with single-pulse excitation, indicating that rapid exchange of DMPS-d_3 between all sites was manifest on the time scale of the deuterium quadrupole coupling constant (lifetimes of specific complexes of less than about 10^{-5} s). Similarly, there was no effect of melittin on the T_1 relaxation times of the head-group deuterons of DMPS in the presence of melittin that would indicate an alteration of the rapid head-group motions either on binding to the peptide or through formation of a DMPS-rich phase having properties similar to those of pure DMPS (Browning & Seelig, 1980). Thus, it is unlikely that phase separation of discrete melittin–DMPS-d_3 complexes presents a reasonable explanation for the melittin-induced reversal of the effects of DMPS on the quadrupole splittings of the choline head group of DMPC-d_4 in the ternary mixtures examined here.

Despite the absence of spectral components characteristic of discrete melittin–DMPS complexes, it is reasonable to assume that on some time scale a preferential interaction of melittin (a positively charged amphiphile) with negatively charged lipid occurs. Lateral diffusion of lipid molecules is characterized by a site to site hopping rate of 10^7 s^{-1} [Devaux & McConnell, 1972; Trauble & Sackmann, 1972; so that the lifetime of each lipid–lipid interaction is about 10^{-7} s], whereas an upper limit for the lifetime of the melittin–DMPS interaction is about 10^{-5} s (as required from the observation of single-component, unbroadened D NMR spectra). Thus, it is possible that a substantial preference of DMPS for melittin with relative association constant ($K_{DMPS:ML}/K_{DMPS:DMPC}$) of up to 100 may occur while satisfying the condition of fast exchange on the time scale of the deuterium quadrupolar anisotropy. Such a preferential interaction could make a significant contribution to the reversal of the magnitude of the quadrupole splittings of the choline head group by melittin in the ternary mixtures. Due to the rather slow time scale of NMR relative to the lifetimes of lipid–lipid and protein–lipid associations, it is not possible to determine the contribution of preferential interactions to the effects of melittin on the DMPC head-group quadrupole splittings in the ternary mixtures.

The observation that all components of the ternary mixture DMPC–DMPS–melittin are in rapid diffusional exchange on the time scale of the static quadrupole coupling constant of $\sim 10^5$ Hz requires that the contribution of the net surface charge to the conformation of the choline head group be considered. It is well established that the α- and β-quadrupole splittings of the choline head group respond to the net surface charge in a predictable manner (Seelig et al., 1987) due to systematic changes induced in the conformation of the head group. The effects of DMPS in inducing a concentration-dependent increase and decrease in the magnitudes of the DMPC-d_4 α- and β-splittings, respectively (Sixl & Watts, 1983; this study) and of melittin in promoting the opposite effects on the choline head-group splittings (decreasing the α-splitting and increase the β-splitting; Dempsey & Watts, 1987; this study) are fully consistent with the contribution of these charged amphiphiles to the net surface charge (Seelig et al., 1987). In ternary mixtures containing DMPC, DMPS, and melittin in fast diffusional exchange, the conformation of the choline head group is expected to respond to the *net* surface charge. The melittin-induced reversal of the effects of DMPS on the choline head-group splittings is consistent with this expectation, as is the observation of a neutralization of the effects of DMPS at a concentration of melittin (1:5 mol/mol, melittin:DMPS) sufficient to neutralize the excess negative surface charge contribution of DMPS. The response of the choline head-group conformation to the surface charge contributions of melittin, DMPS, and mixtures of the two charged amphiphiles is a sufficient explanation fot the effects of these molecules on the choline head-group quadrupole splittings.

The observation that the effects of melittin fit neither the result expected for a response of the PC head group to the total excess surface charge (Figure 8) nor the summed contribution to each of melittin and DMPS (Figure 8) indicates that these simple formalisms are insufficient to account quantitatively for the effects of the two components. This may not be surprising considering their empirical nature and the neglect of potential nonadditive contributions arising from interactions between the components of the ternary mixture such as variations in the location of the fixed charges on melittin or PS (Roux et al., 1989). Also neglected are nonelectrostatic contributions to head-group conformation that may arise from the presence of melittin in the membrane as seen in the dramatic effects of melittin on the macroscopic structure of saturated phosphatidylcholine membranes at peptide concentrations similar to those used here (Dufourc et al., 1986; Dempsey & Watts, 1987). Nonelectrostatic (steric) perturbations of the DMPC head-group conformation may be responsible for the rather small effect of melittin on the β-CD_2 quadrupole splitting in DMPC bilayers (Dempsey & Watts, 1987) compared with the effects of other positively charged amphiphiles (Akutsu & Seelig, 1980; Seelig et al., 1987; Roux et al., 1989). In bilayer membranes composed of choline-deuterated palmitoyloleoylphosphatidylcholine (POPC-d_4), a lipid less susceptible to melittin-induced bilayer disruption, the effect of melittin on the value of the β-CD_2 quadrupole splitting of the POPC-d_4 head group is much greater than observed here for the β-CD_2 quadrupole splitting of DMPC-d_4 and similar to the effects of other positively charged amphiphiles (Ku-

chinka & Seelig, 1989). The effects of melittin on the magnitudes of the α-CD_2 quadrupole splitting of POPC-d_4 and of DMPC-d_4 are very similar.

The effects of melittin on the values of the head-group quadrupole splittings of DMPS in the ternary mixtures with DMPC similarly result from monotonic changes in the conformation of the DMPS head group as the melittin concentration increases; the reversal of the sign of the α_1 quadrupole splitting precludes a general disordering of the head group (Figure 4). The effects of melittin on the DMPS head-group quadrupole splittings are very similar to the effects of a cationic polyleucyl peptide ($K_2GL_{20}K_2A$) in ternary mixtures with DMPC and head-group-deuterated DMPS [compare our Figure 5 with Figure 6 of Roux et al. (1989)]. Each peptide causes a concentration-dependent decrease in the magnitude of the β- and α_1-splittings (a slightly larger effect being observed on the α_1-splitting), whereas the α_2-splitting is relatively unaffected by the presence of either peptide in the mixed bilayer. The observation that the three separate splittings of the DMPS head group change in the same manner indicates that each peptide induces similar effects on the DMPS head-group conformation, despite the absence of sequence homology and the likely difference in membrane orientation (Davis et al., 1983; Terwilliger et al., 1982) of the two peptides. The common feature of the two peptides is their high net positive charge (+5 for melittin; +4–5 for $K_2GL_{20}K_2A$), and the common effects of the peptides on the conformation of the DMPS head group probably indicates that the DMPS head group responds to the surface charge induced by charged amphiphiles in a manner analogous to the response of the DMPC head group (Seelig et al., 1987).

In conclusion, we have shown that there is no detectable tendency for melittin to induce phase separation of negatively charged lipid in ternary mixtures with DMPC and DMPS. The effects of the peptide on the head-group conformation of head-group-deuterated DMPC and DMPS in mixtures of the two lipids are consistent with a major contribution from the net charge induced by melittin at the bilayer surface, although the peptide may have additional steric contributions to the head-group conformation. We propose that the marked similarities in the effects of melittin and of the transbilayer peptide $K_2GL_{20}K_2A$ on the DMPS head-group splittings in ternary mixtures with DMPC (Roux et al., 1989; this study) indicate that the DMPS head-group conformation responds to the surface charge in a manner analogous to the response of the DMPC head group (Seelig et al., 1987). Finally, we have shown that the additions of DMPS to DMPC bilayers greatly stabilizes the bilayers with respect to melittin-induced micellization. At melittin concentrations of up to 6 mol % relative to total lipid in equimolar DMPC–DMPS or DMPC–DMPS (80:20 mol/mol) there is no detectable micellization of lipid in either the fluid (Figure 1 and 4) or gel phases (Figure 2 and 7), whereas at a concentration of 3 mol % melittin in DMPC substantial gel-phase micellization of lipid occurs (Dempsey & Watts, 1987; C.D., unpublished results). Melittin at 6 mol % relative to lipid is a larger concentration than is required for substantial lysis of erythrocytes (Tosteson et al., 1985). If the absence of melittin-induced phase separation of negatively charged lipid, together with the stabilization of bilayers to the effects of melittin by negatively charged lipid, is maintained in biological membranes, then the significance of melittin-induced disk micelle formation (Dufourc et al., 1986a,b; Dempsey & Watts, 1987) with respect to the hemolytic activity of the peptide may be questioned.

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References

Altenbach, C., & Seelig, J. (1984) *Biochemistry 23*, 3913–3920.

Altenbach, C., & Hubbel, W. L. (1988) *Proteins 3*, 230–242.

Batenburg, A. M., Hibbeln, J. C. L., & de Kruijff, B. (1987a) *Biochim. Biophys. Acta 903*, 155–165.

Batenburg, A. M., Hibbeln, J. C. L., Verkleij, A. J., & de Kruijff, B. (1987b) *Biochim. Biophys. Acta 903*, 142–154.

Batenburg, A. M., van Esch, J. H., Leunissen-Bijvelt, J., Verkleij, A. J., & de Kruijff, B. (1987c) *FEBS Lett. 223*, 148–154.

Batenburg, A. M., van Esch, J. H., & de Kruijff, B. (1988) *Biochemistry 27*, 2324–2331.

Bernard, E. Faucon, J. F., & Dufourcq, J. (1982) *Biochim. Biophys. Acta 688*, 152–162.

Browning, J. L., & Seelig, J. (1980) *Biochemistry 19*, 1262–1270.

Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.

Davis, J. H., Clare, D. M., Hodges, R. S., & Bloom, M. (1983) *Biochemistry 22*, 5298–5305.

Dempsey, C. E., & Watts, A. (1987) *Biochemistry 26*, 5803–5811.

Dempsey, C. E., Cryer, G. D., & Watts, A. (1987) *FEBS Lett. 218* 173–177.

Devaux, P. F., & McConnell, H. M. (1972) *J. Am. Chem. Soc. 94*, 4475–4481.

Dufourc, E. J., Smith, I. C. P., & Dufourcq, J. (1986a) *Biochemistry 25*, 6448–6455.

Dufourc, E. J., Faucon, J. L., Fourche, G., Dufourcq, J., Gulik-Krzywicki, T., & LeMaire, M. (1986b) *FEBS Lett. 201*, 205–209.

Faucon, J. F., Bernard, E., Dufourcq, J., Pezolet, M., & Bougis, P. (1981) *Biochimie 63*, 857–861.

Gauldie, J., Hanson, J. M., Rumjanek, F. D., Shipolini, R. A., & Vernon, C. A. (1976) *Eur. J. Biochem. 61*, 369–376.

Kuchinka, E., & Seelig, J. (1989) *Biochemistry 28*, 4216–4221.

Lafleur, M., Faucon, J.-F., Dufourcq, J., & Pézolet, M. (1989) *Biochim. Biophys. Acta, 980*, 85–92.

Quay, S. C., & Condie, C. C. (1983) *Biochemistry 22*, 695–700.

Roux, M., & Neumann, J. M. (1986) *FEBS Lett. 199*, 33–38.

Roux, M., Neumann, J. M., Hodges, R. S., Devaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313–2321.

Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.

Seelig, J., MacDonald, P. M., & Scherer, P. G. (1987) *Biochemistry 26*, 7535–7541.

Sixl, F., & Watts, A. (1982) *Biochemistry 21*, 6446–6452.

Sixl, F., & Watts, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1613–1615.

Terwilliger, T. C., Weissman, L., & Eisenberg, D. (1982) *Biophys. J. 37*, 353–361.

Tosteson, M. T., Holmes, S. J., Razin, M., & Tosteson, D. C. (1985) *J. Membr. Biol. 87*, 35–44.

Trauble, H., & Sackmann, L. (1972) *J. Am. Chem. Soc. 94*, 4499–4510.