Molecular Details of Melittin-Induced Lysis of Phospholipid Membranes As Revealed by Deuterium and Phosphorus NMR

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ABSTRACT: Solid-state deuterium and phosphorus-31 nuclear magnetic resonance (^{2}H and ^{31}P NMR) studies of deuterium-enriched phosphatidylcholine ([3′,3′-$^2H_2$]DPPC, [*sn*-2-$^2H_{31}$]DPPC) and ditetradecylphosphatidylglycerol (DMPG-diether), as water dispersions, were undertaken to investigate the action of melittin on zwitterionic and negatively charged membrane phospholipids. When the lipid-to-protein ratio (R_i) is greater than or equal to 20, the ^{2}H and ^{31}P NMR spectral features indicate that the system is constituted by large bilayer structures of several thousand angstrom curvature radius, at $T > T_c$ (T_c, temperature of "gel-to-liquid crystal" phase transition of pure lipid dispersions). At $T \approx T_c$, a detailed analysis of the lipid chain ordering shows that melittin induces a slight disordering of the "plateau" positions concomitantly with a substantial ordering of positions near the bilayer center. At $T \gg T_c$, an apparent general chain disordering is observed. These findings suggest that melittin is in contact with the acyl chain segments and that its position within the bilayer may depend on the temperature. On a cooling down below T_c, for $R_i > 20$, two-phase spectra are observed, i.e., narrow single resonances superimposed on gel-type phosphorus and deuterium powder patterns. These narrow resonances are characteristic of small structures (vesicles, micelles, ... of a few hundred angstrom curvature radius) undergoing fast isotropic reorientation, which averages to zero both the quadrupolar and chemical shift anisotropy interactions. On an increase of the temperature above T_c, the NMR spectra indicate that the system returns reversibly to large bilayer structures. Longitudinal deuterium relaxation times show that, above T_c, melittin ($R_i = 20$) lowers the activation energy of the acyl chain motions (those on the nanosecond time scale) and increases it immediately below T_c. Experiments carried out at $R_i = 4$ exhibit isotropic ^{2}H and ^{31}P NMR lines, above and below T_c, indicating that melittin, at these concentrations, precludes the formation of large lamellar lipid phases. Relaxation measurements (T_{1z}, T_2) demonstrate that lipids are still organized, as in bilayers, within the resultant very small structures. The formation of these small structures upon addition of the direct-lytic factor melittin to lipid dispersions is proposed as a mechanism for the lysis of biological membranes, the supralysis.

Melittin is a 26 amino acid polypeptide that represents about 50% of bee venom (dry weight). It possesses two major roles: (i) the lysis of natural and artificial lipid membranes (Haberman, 1972; Weissmann et al., 1969; Olson et al., 1974; Hegner et al., 1968; Sessa et al., 1969) and (ii) the potentiation of the activity of phospholipases A_2 found in bee venom (Mollay et al., 1976, 1974; Yunes et al., 1977). Numerous physicochemical studies using a wide variety of techniques have shown that melittin can exist in both monomeric and tetrameric forms (Faucon et al., 1979; Lauterwein et al., 1980; Brown et al., 1980; de Bony et al., 1979; Talbot et al., 1979) and that both forms may interact with lipids (Talbot et al., 1982; Coddington et al., 1983). Fluorescence (Dufourcq & Faucon, 1977) and recent ^{13}C NMR[1] (Coddington et al., 1983) studies have shown that melittin has significant binding with phosphatidylcholine (PC) lipids but binds preferentially to negatively charged lipids. Although high-resolution ^{1}H NMR studies seem to demonstrate that melittin is monomeric in lysolipid micelles (de Bony et al., 1979; Lauterwein et al., 1980), it is still unclear what is the state of association of the peptide in the lipid bilayer. This information is of major importance in the understanding of the lysis mechanism.

Solid-state deuterium and phosphorus-31 nuclear magnetic resonance (^{2}H and ^{31}P NMR) have proven to be powerful techniques in probing the structural and dynamical properties of biological membranes (Seelig, 1977; Smith, 1982; Davis, 1983; Cullis & de Kruijff, 1976) and are shown herein to yield very important information on melittin–phospholipid interactions (vide infra).

Materials and Methods

Lysopalmitoylphosphatidylcholine was purchased from Sigma Chemical Co. (St. Louis, MO); perdeuterated palmitic acid and [3,3-$^2H_2$]palmitic acid were generous gifts from Dr. B. Perly (CEN Saclay, France) and Dr. A. P. Tulloch (NRC Saskatoon, Canada), respectively. Deuterium-depleted water was obtained from Aldrich Chemical Co. (Milwaukee, WI) and DMPG-diether purchased from Medmark (Munich, West Germany).

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[1] Abbreviations: NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPG-diether, 1,2-ditetradecyl-*rac*-glycero-3-phosphoglycerol; R_i, lipid-to-protein molar ratio; TLC, thin-layer chromatography; T_c, gel-to-fluid phase transition temperature; τ_c, correlation time; ω_0, Larmor angular frequency; T_{1z}, longitudinal relaxation time; T_2, transverse relaxation time; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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1-Palmitoyl-2-[$^2H_{31}$]palmitoyl-*sn*-glycero-3-phosphocholine ([*sn*-2-$^2H_{31}$]DPPC) and 1-palmitoyl-2-[3′,3′-$^2H_2$]palmitoyl-*sn*-glycero-3-phosphocholine were synthesized according to standard procedures (Dufourc et al., 1983; Perly et al., 1984). Melittin was purchased from Bachem A. G. (Switzerland) and utilized without further purification. Control experiments using highly purified melittin (Dasseux et al., 1984) gave identical results.

Model membranes (multilamellar dispersions) were obtained by hydrating lipids with a buffered solution (20 mM Tris, 2 mM EDTA, and 100 mM NaCl in deuterium-depleted water adjusted to pH 7.5 by dropwise addition of concentrated HCl) on a vortex mixer as described previously (Dufourc et al., 1983). Lipids and buffer solution were in 1:5 (w/w) ratio, respectively. ^2H and ^{31}P NMR control runs were performed to ensure that model membranes were composed of very large liposomes. Melittin was dissolved in the same buffer solution up to a concentration of 15 mM; the peptide is tetrameric under such conditions (Faucon et al., 1979; Talbot et al., 1979; Brown et al., 1980). This solution was then added to bilayers, at desired lipid-to-protein incubation ratios (R_i) and below T_c, the temperature of the "gel-to-liquid crystalline" phase transition of pure lipids. The sample was then shaken a few seconds and placed in the NMR spectrometer. Possible phospholipid degradation was checked by thin-layer chromatography (TLC) as previously described (Dufourc et al., 1983). No lysophosphatidylcholine was detected in any samples after completion of NMR experiments.

Deuterium NMR spectroscopy was performed at 30.7 MHz. Spectra were acquired on resonance, with quadrature detection, by means of the quadrupolar echo sequence (Davis et al., 1976). Spin–lattice (Zeeman) relaxation times, T_{1z}, were obtained by the standard inversion recovery sequence coupled with the quadrupolar echo sequence (Dufourc et al., 1984a). Phosphorus-31 NMR signals were acquired at 121.5 MHz with the use of a phase-cycled Hahn echo sequence (Rance & Byrd, 1983). Quadrature detection and broad-band proton decoupling were utilized. Data treatment was accomplished on Nicolet 1280 and Vax 11 computers; spectral dePaking and T_{1z} analyses were performed as described by Bloom et al. (1981) and Dufourc et al. (1984a), respectively.

Samples were allowed to equilibrate at least 30 min at a given temperature before the NMR signal was acquired. The temperature was electronically regulated to ±0.5 °C. Viscosity measurements were performed with an Ostwald-type viscosimeter immerged in a bath whose temperature was regulated to ±1 °C.

Results

Membrane Organization. Figure 1 shows deuterium powder spectra of [*sn*-2-$^2H_{31}$]DPPC dispersions in the presence of melittin (R_i = 50) as a function of temperature; the arrows indicate the way the temperature was varied [i.e., a spectrum was taken at 25 °C (after melittin addition), the temperature was brought to 65 °C, and a spectrum was taken at each temperature step]. The spectrum at 25 °C is typical of a pure lipid lamellar gel phase in which the acyl chains are almost in all-trans conformation (Davis, 1983); i.e., there is no effect of melittin detectable. Above T_c = 41 °C, the spectra exhibit axially symmetric line shapes characteristic of lipids undergoing fast reorientation around their long molecular axes in a uniaxial mesophase (Seelig, 1977). When the temperature is lowered below T_c (e.g., spectra at 38, 30, and 0 °C), isotropic central lines superimposed on gel-type powder patterns are observed. These lines are indicative of lipids undergoing isotropic reorientation (vide infra). The observation of two

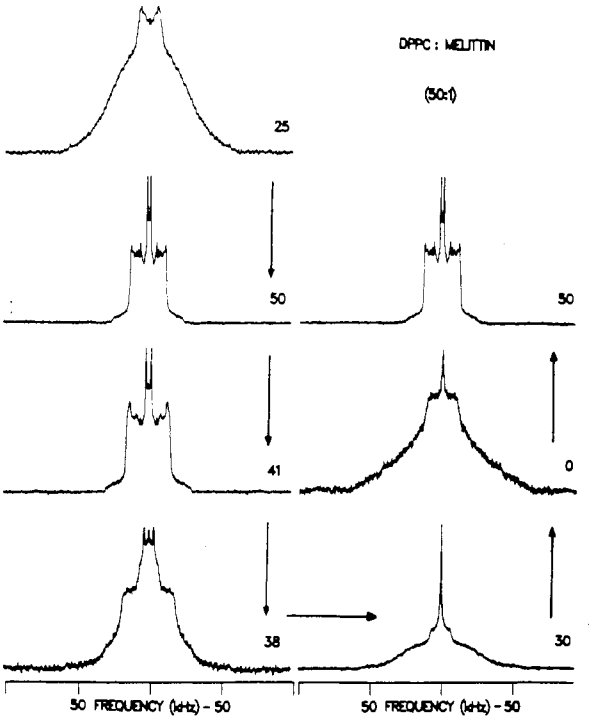

FIGURE 1: Temperature variation of ^2H NMR powder spectra of aqueous dispersions of [*sn*-2-$^2H_{31}$]DPPC in the presence of melittin (R_i = 50). Temperatures (°C) are indicated on the right side of each spectrum. The arrows show the direction of temperature variation during the experiment, after toxin addition at 25 °C. Typical experimental parameters: spectral window, 250 kHz; 90° pulse width, 5 μs; delay between two 90° pulses, 40 μs; recycle time, 1–1.5 s; 1800 accumulations.

subspectra indicates that the lipid molecules are in two distinct environments and that their rate of exchange from one region to the other is slow on the time scale of solid-state deuterium NMR (≈10^{-6} s). When the temperature is raised again above T_c, the two region spectra disappear and one observes axially symmetric powder patterns identical with those obtained in the first runs above T_c, at corresponding temperatures. Reproducible spectral shapes with isotropic lines, at corresponding temperatures, were also obtained on going below T_c again. The transition from an axially symmetric fluid phase to gel plus isotropic phases is thus reversible when the system melittin–lipid dispersions, mixed below T_c, has been brought once above T_c.

The experiments were repeated with increasing melittin concentration (R_i = 20, Figure 2). The temperature behavior of this system is qualitatively similar to that observed with R_i = 50. However, one notices that there is more isotropic line below T_c for R_i = 20 than for R_i = 50. For example, at 30 °C this line dominates the spectrum; the gel phase is nonetheless observed on increasing the vertical scale (Figure 2, 30 °C spectrum). It is interesting to notice that the spectrum at 38 °C is composed of isotropic and axially symmetric subspectra; i.e., there is no trace of gel-phase spectrum as in Figure 1, same temperature. Above T_c, the deuterium spectra lose their shoulders, compared to the spectra originating from pure lipid lamellar dispersions (Figure 3), at corresponding temperatures.

Figure 4 shows representative spectra when R_i = 4. The experimental procedure described above was also followed. One notices a gel-type powder pattern at 25 °C, after melittin addition, when the system has never been brought above T_c. On increasing the temperature above T_c, a single isotropic line is observed. This line is also observed on lowering the temperature again below T_c.

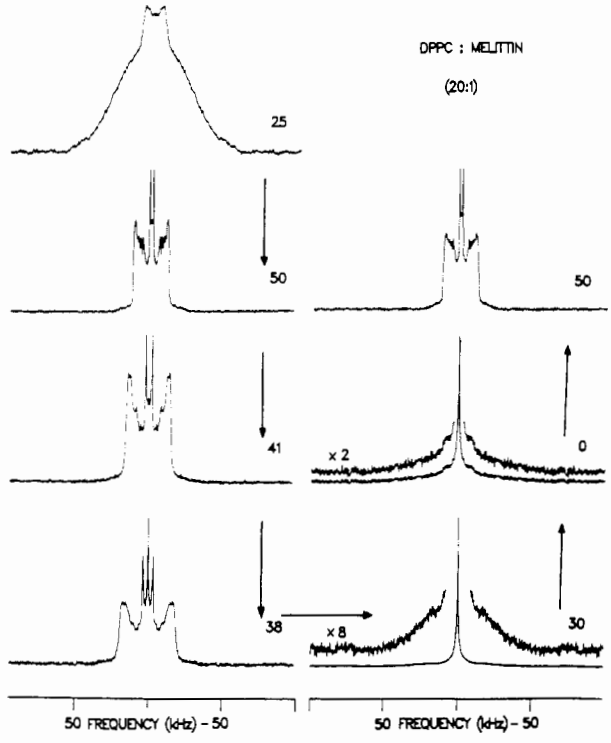

FIGURE 2: As in Figure 1, except $R_i = 20$.

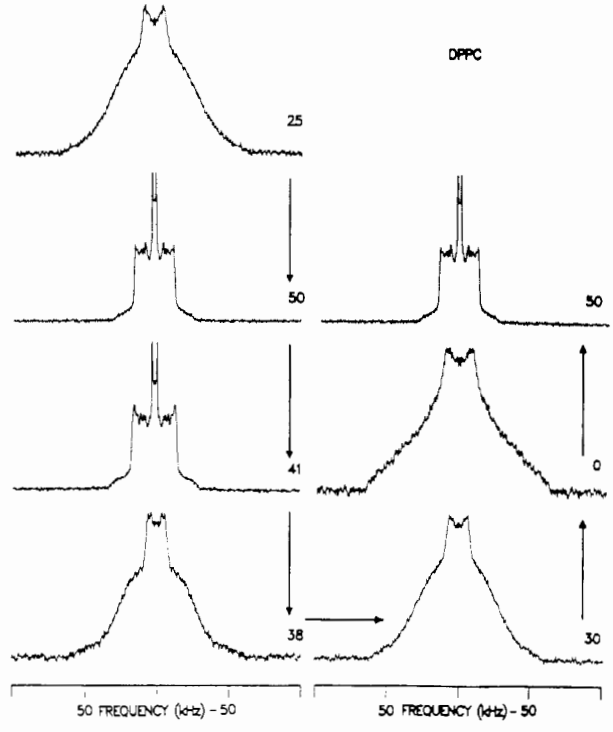

FIGURE 3: As in Figure 1, except $R_i = \infty$.

First-moment, M_1, calculations (Davis, 1979) of the variable-temperature series were performed for $R_i = \infty$ (pure lipid), $R_i = 50$, and $R_i = 20$. The resulting M_1 values are plotted in Figure 5 and clearly show that the T_c value of pure lipids is well determined from the marked discontinuity of the first moment as a function of temperature. Moments for spectra taken below T_c, in the presence of melittin and when the system has never been brought to $T > T_c$, are identical, within the experimental error, with those of pure lipid spectra, at corresponding temperatures (not shown in Figure 5). Below T_c, where two NMR regions are observed (vide supra) in the presence of melittin, the first moment exhibits the weighted

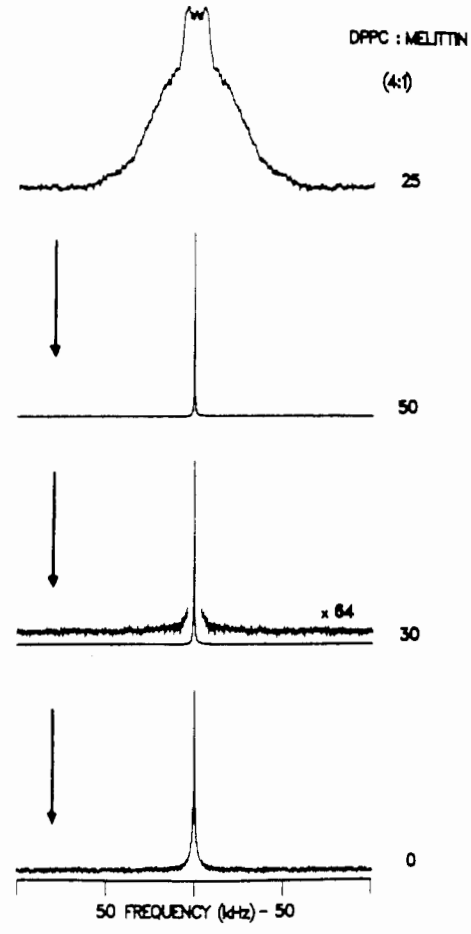

FIGURE 4: As in Figure 1, except $R_i = 4$.

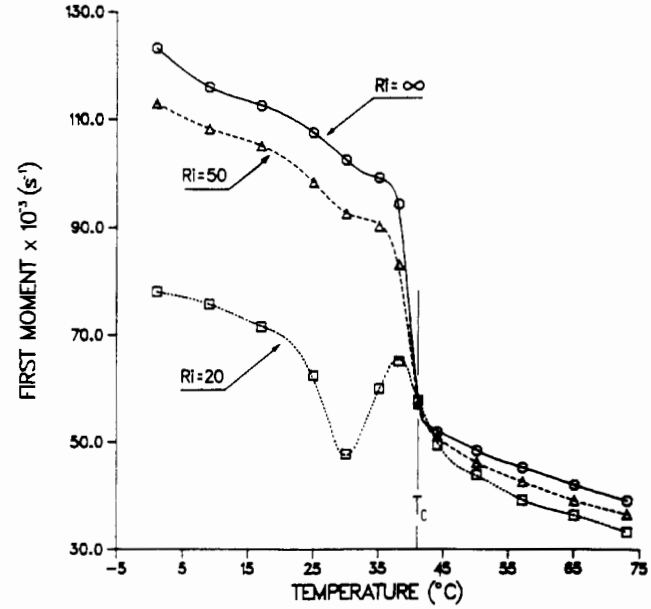

FIGURE 5: Temperature dependence of first moments, M_1, of spectra of [*sn*-2-$^2H_{31}$]DPPC in the presence and absence of melittin. The amount of melittin is indicated on each curve by means of R_i. Symbols give an estimate of the error.

average of the first moments of each individual region; this explains why M_1 in the presence of toxin is smaller than M_1 of pure lipid. One notices that below T_c the first moment goes through a minimum at $T = 30$ °C (for $R_i = 20$); i.e., the amount of isotropic line is maximum. A maximum in the amount of isotropic line is also observed at 30 °C, for $R_i = 50$. At this temperature, the area of each subspectrum was

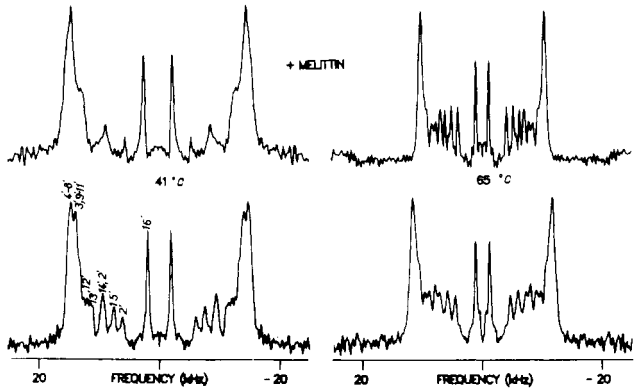

FIGURE 6: DePaked spectra of [*sn*-2-$^2H_{31}$]DPPC dispersions without (bottom spectra) and with (top spectra) melittin (R_i = 20), at 41 °C (left-hand side spectra) and 65 °C (right-hand side spectra). Primed numbers indicate assignments of deuterium-labeled positions on the *sn*-2 chain.

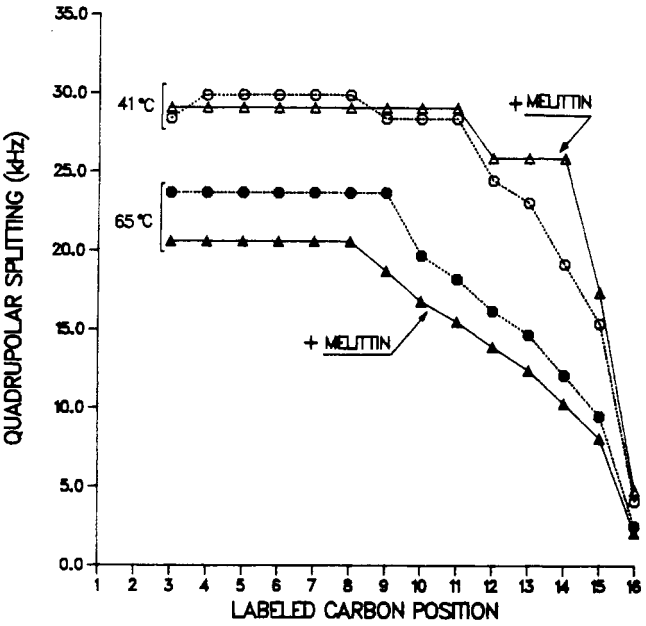

FIGURE 7: Quadrupolar splittings from dePaked spectra of [*sn*-2-$^2H_{31}$]DPPC in the presence and absence of melittin (R_i = 20) at 41 and 65 °C as a function of labeled position on the *sn*-2 chain. Symbols give an estimate of the error.

determined according to Dufourc et al. (1984b). The isotropic line represents about 20% and 65% of the total spectral area for R_i = 50 and 20, respectively. For $T \gg T_c$, the more melittin present, the more the first moment decreases. It is also interesting to notice that although they behave differently below and above T_c, first moments for $R_i = \infty$, R_i = 50, and R_i = 20 are equal, within the experimental error, at 41 °C. This indicates that these systems in their temperature variation all pass through a same dynamical stage in which the global bilayer ordering is approximately the same.

Above T_c and for $R_i = \infty$, 50, and 20, the powder spectra exhibit axially symmetric line shapes and were "dePaked" according to Bloom et al. (1981) to give spectra similar to those of oriented samples. Figure 5 shows such dePaked spectra for $R_i = \infty$ and R_i = 20 at 41 and 65 °C; measurement of the quadrupolar splittings $\Delta\nu_Q$ from the resolved doublet separation given by each labeled carbon position is thus very easy. Positions of labeling are indicated in Figure 6 with primed numbers and were assigned from singly labeled experiments (not shown) or from already published results (Seelig & Seelig, 1974; Davis, 1983). It was assumed that the presence of melittin did not change attributions of labeled positions. This

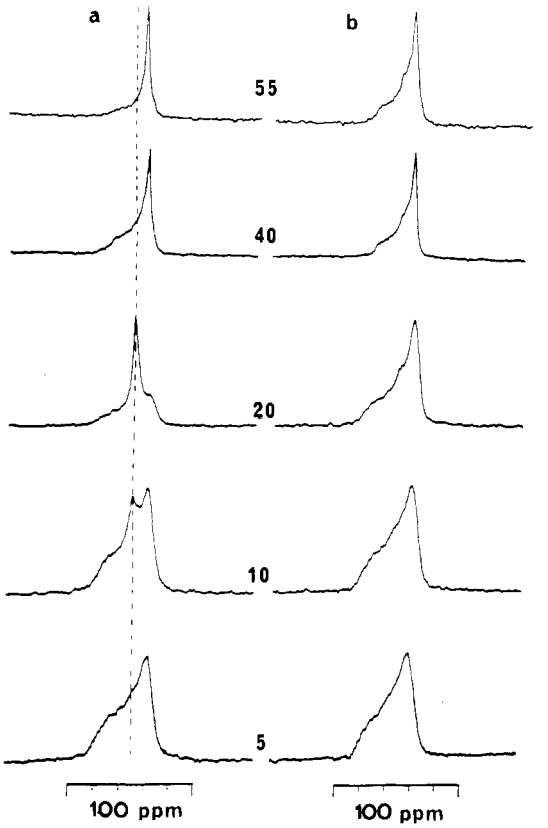

FIGURE 8: Temperature dependence of ^{31}P NMR powder spectra of DMPG-diether–water dispersions in the presence, R_i = 20 (a), and absence (b) of melittin. Temperatures (°C) are indicated on spectra. Typical experimental parameters: spectral window, 125 kHz; 90° pulse width, 10 μs; delay between pulses to form the echo, 60 μs; recycle time, 2 s; 500–1500 accumulations. All spectra were obtained in the presence of broad-band proton decoupling.

hypothesis was nonetheless checked with some selectively labeled lipids. Figure 7 shows quadrupolar splittings plotted as a function of the labeled carbon position. One notices in this figure that melittin induces a general decrease in $\Delta\nu_Q$ of the DPPC acyl chain, at 65 °C; the magnitude of this effect appears to be greater at the "plateau" level (labeled positions 3–9) than near the center of the bilayer. At 41 °C, melittin leads to a slight decrease of the plateau quadrupolar splittings concomitantly with a marked increase of those for positions near the end of the acyl chains, leading to an extension of the plateau character. The above behavior was also observed for R_i = 50 with, however, a lesser magnitude (not shown).

Figure 8 shows phosphorus-31 powder spectra of DMPG-diether (water dispersions) in the presence (R_i = 20) and absence of melittin, as a function of temperature. Observations similar to those obtained with ^2H NMR (same R_i) can be made, i.e., coexistence of two NMR phases—namely, lamellar gel plus isotropic—below T_c (T_c of DMPG-diether is about 25 °C at pH 7.5) and mixing of these phases above T_c to give ^{31}P NMR powder spectra characteristic of molecules undergoing axially symmetric motions in a uniaxial liquid crystalline medium. It is interesting to notice that the narrow single resonance completely disappears at low temperatures (i.e., 5 °C). Furthermore, for $T \gg T_c$, the spectra differ markedly in shape; the low-field shoulder of the powder spectrum at 55 °C, in the presence of melittin, has almost disappeared. As will be discussed later, this might be an indication of a bilayer size effect on the powder pattern line shape (Burnell et al., 1980). Experiments carried out for R_i = 4 (not shown) gave rise to an isotropic ^{31}P NMR line above and below T_c. The DPPC–melittin system previously studied by ^2H NMR was

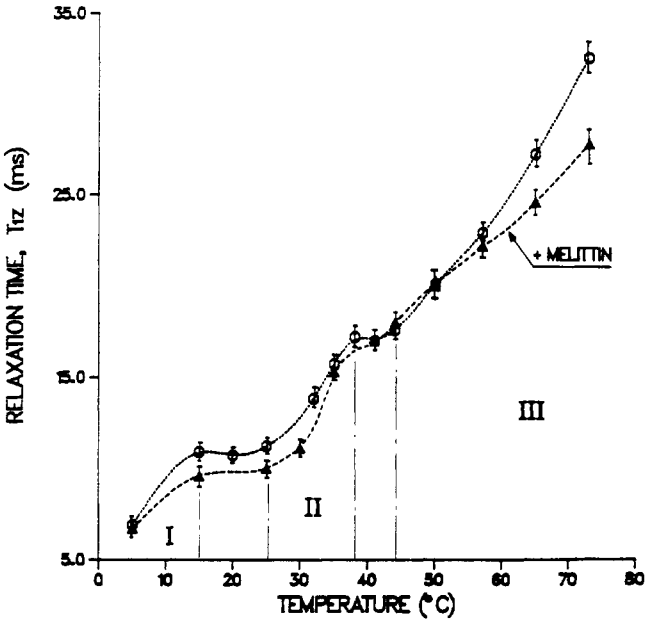

FIGURE 9: Temperature dependence of deuterium longitudinal relaxation time, T_{1z}, for aqueous dispersions of [3′,3′-$^2H_2$]DPPC in the presence (R_i = 20) and absence of melittin. Bars and symbols give an estimate of the experimental error.

also investigated by ^{31}P NMR (not shown) and yielded the same results as found for DMPC-diether–melittin.

Membrane Dynamics. Longitudinal deuterium relaxation time (T_{1z}) measurements were performed on [3′,3′-$^2H_2$]DPPC water dispersions with and without melittin (for R_i = 20 and 4). The analysis was carried out as described by Dufourc et al. (1984a). Variation of T_{1z} across the individual powder patterns was not detected, within the experimental error. When the spectra exhibited a superposition of an isotropic line and a powder pattern, e.g., R_i = 20 (vide supra), the T_{1z} values of each of these components were determined by fitting separately the equation $M(\tau_1) = M(0)[1 - A \exp(-\tau_1/T_{1z})]$ to the integrated areas of the narrow line and of the powder spectrum (Dufourc et al., 1984a). $M(\tau_1)$ and $M(0)$ represent the longitudinal magnetization at times τ_1 and $\tau_1 = 0$, respectively, where τ_1 is the variable delay between the 180° inverting pulse and the first 90° pulse of the echo sequence. The adjustable parameter A was used to account for the imperfections of the inverting pulse. In all cases, the T_{1z} of the line and of the powder pattern were found to be identical within the experimental error, and the spin–lattice relaxation process was characterized by a single exponential. Figure 9 shows the temperature dependence of T_{1z} for [3′,3′-$^2H_2$]DPPC in the presence (R_i = 20) and absence of melittin. The plots of Figure 9 were separated into three regions (namely, I, II, and III) in which the T_{1z} value increases markedly with temperature. This temperature dependence suggests that the relevant molecular motions of DPPC at position 3′, in these three ranges of temperature, are in the regime of short correlation time ($\omega_0^2\tau_c^2 \ll 1$). Assuming an Arrhenius-type dependence for motions in each of the temperature ranges, one can estimate activation energies (E_a) from the data of Figure 9. This yields E_a = 18 ± 1, 25 ± 3, and 29 ± 4 kJ mol^{-1} for regions III, II, and I, respectively, in the absence of melittin and E_a = 14 ± 1, 32 ± 3, and 23 ± 4 kJ mol^{-1} for the same regions, respectively, in its presence. It is interesting to notice in Figure 9 that for temperatures between regions I and II or between regions II and III T_{1z} does not vary appreciably with temperature in the absence of toxin. This is also true in the presence of melittin except around 41 °C where the marked step in T_{1z}, as seen for pure DPPC, is almost eliminated.

T_{1z} and line widths were also measured for [3′,3′-$^2H_2$]DPPC, R_i = 4. At 65 °C, the isotropic line was well approximated by a single Lorentzian line shape; the width at half-height W_e was 260 ± 20 Hz, and T_{1z} was 22.4 ± 1 ms. At 25 °C, the experimental line looked more like a superposition of several lines of different width and could not be fitted by a single Lorentzian function. A mixture of Gaussian and Lorentzian line shapes was needed to obtain a reasonable approximation of this line. The resulting line width was 780 ± 30 Hz; T_{1z} at 25 °C was 8.5 ± 1 ms. For all the above experiments the intrinsic line broadening due to inhomogeneities in the magnetic field and sample was less than 20 Hz.

Viscosity measurements were performed on DPPC–melittin samples (R_i = 4); η was found equal to 0.96 10^{-3} p, at 25 °C, and 0.50 10^{-3} p, at 65 °C. It has to be mentioned that due to the large quantity of solution required for these measurements, NMR samples were diluted twice in the buffer; η values must therefore be taken as rough estimates rather than as accurate determinations.

Discussion

In a lamellar mesophase (i.e., water–lipid dispersions), the molecular reorientations are not completely random in space; the molecules adopt a prefered orientation about which rapid motion of limited amplitude can occur. As a consequence, the static deuterium quadrupolar interaction is partially averaged, and residual quadrupolar splittings are observed in the NMR spectrum (for unoriented mesophases one obtains quadrupolar "powder" patterns). In isotropic fluids, the molecular reorientations are random in space, leading to a total averaging of the quadrupolar interaction; narrow single Lorentzian lines are then observed. However, in some cases, one may observe simple Lorentzian resonances even if the local molecular motions are anisotropic; this is encountered for example in very small (few hundred angstrom curvature radius) single bilayer vesicles or micelles (Stockton et al., 1976). In these cases, the characteristic liquid crystalline properties persist (e.g., phase transition), but the residual quadrupolar interaction is averaged to zero by isotropic reorientation of the spherical vesicles or by fast lateral diffusion of the phospholipid around the vesicle surface.

The above applies equally to ^{31}P NMR. The dominant interaction, the chemical shift anisotropy (CSA), is averaged by molecular motions in the mesophase. In systems where isotropic motional averaging, such as particle tumbling or phospholipid lateral diffusion, is negligible, the ^{31}P NMR spectra exhibit broad, asymmetric line shapes (CSA powder patterns) reflecting anisotropic motions of the molecules (Cullis & de Kruijff, 1976; Burnell et al., 1980) (e.g., Figure 8b).

Single resonances characteristic of isotropic phases, from both the ^2H and ^{31}P NMR viewpoints, are observed in our experimental spectral set (Figures 1, 2, 4, and 8). As discussed above, the narrow lines may be accounted for by fast isotropic tumbling of a molecule (case of pure liquid) or by tumbling of supramolecular aggregates of melittin and lipids (bilayer vesicles, mixed micelles, ...). Release of a fatty acid chain into the water medium due to possible phospholipid degradation could also lead to single ^2H NMR resonances; this possibility is discounted on the basis of the TLC control experiments (vide supra). If the observed narrow lines were reflecting total isotropic molecular tumbling (as in liquids), the deuterium T_{1z} and T_2 relaxation times would be equal (Abragam, 1961; Stockton et al., 1976). In contrast, it is found that T_{1z} is 20 times greater than T_2 at 65 °C, for R_i = 4. This indicates that the lipid chains possess local ordering, due to molecular

packing, as in bilayer vesicles or micelles. Stockton et al. (1976) proposed a formalism expressing T_2 of vesicle lipids as a function of T_{1z}, the local orientational order parameter S_{C-^2H}, and the rotational correlation time τ_v, of lipids in the small structure:

$$W_e = \frac{1}{\pi T_2} = \frac{1}{\pi T_{1z}} + \frac{9\pi}{20} A_Q^2 S_{C-^2H}^2 \tau_v \qquad (1)$$

In eq 1, A_Q is the quadrupolar coupling constant (A_Q = 170 kHz for methylene C–^{2}H groups; Seelig, 1977), and $\tau_v^{-1} = \tau_r^{-1} + \tau_d^{-1}$, where τ_r is the correlation time for tumbling of the small structure and τ_d is the correlation time for lateral diffusion of a phospholipid within the molecular aggregate. For usually observed lateral diffusion constants (ca. 1.10^{-8} cm^2 s^{-1}), it can be shown that τ_d does not contribute significantly to τ_v if the vesicle radius is less than 100 Å. Radii of the small structures at R_i = 4 have been determined from quasi-elastic light scattering experiments (Dufourcq et al., 1986): r_v = 80 Å at 25 °C, and r_v = 60 Å at 65 °C. Consequently, in the following discussion τ_d will be neglected. Correlation time $\tau_r \approx \tau_v$ is related to the radius of the vesicle or micelle, r_v, through the well-known Stokes–Einstein equation in which the viscosity of the medium (water in our case) and the temperature of the system are experimentally available. Thus, from the available experimental data one can calculate the local orientational order parameter, S_{C-^2H}, of [3′,3′-$^2H_2$]DPPC in the isotropic NMR phase. Using r_v = 80 Å and η = 0.96 10^{-3} P, at 25 °C, one obtains S_{C-^2H} = 0.19 and, for r_v = 60 Å and η = 0.50 10^{-3} p, at 65 °C, S_{C-^2H} = 0.25. Despite the poor accuracy of the calculated S_{C-^2H} values, principally due to the viscosity measurements (vide supra), the order of magnitude of the orientational order parameter indicates that the lipid acyl chains, within the small tumbling structures, are still organized. Unlike pure lipid preparations, the ordering does not change appreciably from 25 to 65 °C. This may be related to theories on lipid-to-protein interactions (Abney & Owicki, 1985), which predict that certain proteins could level the lipid ordering.

The formation of these small objects depends upon several factors: (i) the temperature history of the sample, (ii) the concentration of melittin in the system, and (iii) the temperature of the model membrane with respect to T_c. The first point is relatively clear, there is no modification of lipid powder spectra upon melittin addition below T_c. The invariance of the first moment in the presence of melittin, under such conditions and on comparison with data from pure lipids, is another way to monitor that there is no change of lipid spectra on the time scale of the NMR experiment, i.e. 1–2 h. The system must therefore be brought once above T_c to exhibit changes in the ^{2}H and ^{31}P NMP spectra. Same observations have been reported by Dasseux et al. (1984) using fluorescence measurements. For high protein concentration (R_i = 4), small structures are observed above and below T_c (once having been above T_c). Melittin thus prevents the formation of large lamellar lipid dispersions. It can therefore be postulated that the mechanism of membrane lysis, as induced by melittin, is linked to the formation of small vesicles or micelles; i.e., melittin would play the role of a detergent, at high concentrations.

When the peptide concentration is relatively low (R_i = 20 and 50), the formation of small objects is only observed below T_c. Moreover, the proportion of narrow line varies with temperature; a maximum is observed at ca. 30 °C for R_i = 20 and 50, with DPPC. Assuming that all melittin molecules are complexed with DPPC lipids giving rise to narrow lines, at 30 °C, one may calculate (from R_i and the percentage of narrow line at 30 °C) that the lipid-to-protein molecular ratio in the complexes (R_c) is ca. 10–13. Given the above assumptions, a melittin concentration such that $R_c \leq$ 10–13 would be the requirement to have formation of very small structures. Such behavior is also found for the negatively charged lipids (DMPG-diether) with ^{31}P NMR data. If the above assumption is not verified, that is, if there is still melittin not complexed with lipids (e.g., free melittin exchanging with bound melittin), at the temperature of the maximum of narrow line, the numbers given for the lipid-to-protein ratio in the complexes must be taken as lower limits. The temperature variation of the percentage of NMR isotropic phase might be an indication of such an exchange between free and bound melittin.

For R_i = 20 and 50, axially symmetric deuterium and phosphorus powder spectra characteristic of a bilayer phase are observed above T_c. It must be emphasized that under these conditions only a single lipid phase is present (as detected by NMR). The small structures seen below T_c have remixed, on increasing the temperature above T_c, to give the lipid bilayer fluid phase. Orientational order parameters, S_{C-^2H}, can be obtained by measurement of residual quadrupolar splittings, $\Delta\nu_Q$ [$\Delta\nu_Q = (3/4)A_Q S_{C-^2H}$; Seelig, 1977; Stockton et al., 1976], and lead to detailed information about the amplitude of segmental motions (Dufourc et al., 1984a,b), at the position of labeling. Assuming that the axis of motional averaging of the lipids (usually taken to be the bilayer normal) does not change upon addition of relatively low amounts of toxin (R_i = 20 and 50), one observes (Figure 7) that melittin leads to a differential ordering depending upon temperature and bilayer depth. At high temperatures, a disordering is induced by the presence of melittin, for all labeled positions. The decrease of the first moment at high temperatures (Figure 5) is also an indication of an increase of acyl chain disorder (the first moment of [*sn*-2-$^2H_{31}$]DPPC spectra represents, when the system comprises only one phase (as detected by NMR), the overall acyl chain orientational order parameter). Figure 5 shows that at high temperatures the more melittin present, the greater the disorder. Moreover, the effect appears to be larger at the plateau positions (carbons 3–9) than near the center (carbons 10–16) of the bilayer. At 41 °C, one notices in Figure 7 that little disorder is induced at the plateau level whereas the center of the bilayer is markedly more ordered in the presence of toxin than in its absence.

As mentioned under Results the influence of bilayer size might come into play at high temperatures and could explain the observed apparent disordering induced by melittin (at R_i = 20). Stockton et al. (1976) and Burnell et al. (1980) performed simulations of deuterium and phosphorus spectra, respectively, to take account of the effect of bilayer vesicle size on spectral shapes. Their conclusions were that powder patterns due to both quadrupolar and chemical shift anisotropy interactions begin to lose their shoulders when the vesicle radius is lower than 2500–3500 Å. A collapse towards a symmetric narrow single resonance is observed when the radius is lower than 500 Å. However, both predict that collapsing happens concomitantly with a very important broadening of deuterium and phosphorus line shapes. Figures 6 and 8 show that at high temperatures (65 and 55 °C, respectively) the lines have about the same width in the presence of melittin, compared to those in its absence. Hence, it is unclear if bilayer size effects can solely account for the observed spectral line shapes (at R_i = 20, principally), at high temperatures.

The following will try to rationalize the observed differential ordering upon consideration of melittin location with regard to the lipid bilayer. The peptide can possibly occupy three

average positions with respect to the bilayer depth: (i) at the bilayer surface, capping the polar head groups, i.e., extrinsic; (ii) inserted in to the top region of the bilayer, i.e., acting as a spacer of head groups and glycerol backbone regions; (iii) in contact with the fatty acyl chains throughout the entire bilayer thickness. A situation in which melittin caps (i) the lipid heads would lead to greater chain packing, which should in turn be reflected by the same relative increase in ordering at all labeled positions. Conversely, if melittin were acting as a spacer of head groups (ii) (Terwilliger et al., 1982), the chains would have lesser contacts, especially near the center of the bilayer where more space would be available for methylene and methyl motions. This should be reflected by a greater disorder, i.e., weaker chain packing, and should be more pronounced near the bilayer center. Contact with acyl chains (iii) may increase or restrict the methylene and methyl motions depending upon the position of melittin with respect to fatty acyl chain segments. Situations ii and iii could account for the observed general disordering at elevated temperatures. However, near the transition temperature, the peptide induces an increase in degree of ordering at the center of the bilayer with practically no change at the "plateau" positions. Location iii, i.e., melittin in contact with the acyl chains down to the core of the bilayer, thus appears the more likely source of the observed differential ordering effect near T_c. In addition, one observes a brodening of spectra at 41 °C (e.g., Figure 6, R_i = 20), which could account for the presence of melittin inside the bilayer. Such broadening effects have been already shown to reflect the presence of proteins within membranes (Paddy et al., 1981). To summarize the discussion about melittin location, in the lipid fluid phase, it appears that the peptide may occupy several positions in the bilayer depending on the temperature with respect to T_c. Disordering at high temperatures and differential ordering near T_c may reflect the progressive penetration of melittin into the bilayer when the temperature is lowered toward T_c. It is interesting to remark that Coddington et al. (1983) found a reduction in the motional rate of the acyl chain segments of membrane phospholipids (as determined by ^{13}C NMR relaxation times) upon melittin addition. These authors seem to favor location iii to account for their results.

It is worthwhile mentioning that Vogel et al. (1983) conclude from polarized infrared spectra of melittin in macroscopically oriented lipid membranes that the peptide either spans the bilayer or is folded back within one monolayer, hence acting as a wedge. Although our findings concerning the peptide location seem to agree with theirs, care must be taken in comparing the two studies; their system is in the gel state (ordered according to their terminology) whereas we discuss the melittin location in the lipid fluid phase, near T_c. As was demonstrated herein, large membrane morphological changes occur in the gel phase upon melittin addition (when the system has been brought once in the fluid phase). Hence, the peptide may occupy very different positions with respect to the lipid bilayer depending upon the physical state (gel or fluid) of the latter.

The rates of T_{1z} relaxation, as well as the activation energies for lipid chain motions, in the presence of melittin, remain comparable to, although clearly experimentally different from, those measured in the absence of the peptide. Moreover, below T_c, at R_i = 20, the T_{1z} were identical for each of the two phase components (i.e., the single line and the powder pattern). These findings indicate that lipids in the small structures, although perturbed, retain most of the motionl properties of lipids in large multilayers.

The striking phenomenon of this study is the reversible transition from very small to very large structures (for R_i = 20 and 50). This transition occurs at about T_c and appears therefore to be triggered by the acyl chain melting. Although this phenomenon is not well understood, a tentative of explanation may be proposed. The entering into the lipid fluid phase, which represents a large increase in acyl chain dynamics, could allow the fusion of the small structures, hence the formation of very large objects. Conversely, on a cooling down below T_c, the dynamics of both proteins and lipids dramatically decrease. This could lead to a clustering of lipids and proteins to form small complexes that would separate from the bulk of uncomplexed lipids. However, it is clear that this phenomenon requires a theoretical basis to be correctly understood.

Conclusions

The study described herein illustrates the strength of deuterium and phosphorus solid-state NMR in revealing the organization and dynamics of membrane phospholipids upon addition of the principal bee venom toxin melittin. Phase changes, coexistence of two phases in the NMR time scale, proportion of lipids in a two-phase region, detailed order parameter changes as a function of bilayer depth, and changes in activation energies of motions are information that has been extracted from the NMR data. These results cast light on the mechanism of direct lysis induced by melittin.

The formation of very small structures (melittin–lipid complexes) upon addition of melittin to very large lipid bilayers is proposed as a mechanism for membrane lysis. This supralysis (or direct-lysis) mechanism may be viewed as a large change in the morphology of the biological membrane, which leads to membrane disruption and consequently to leakage of the cell contents. The above mechanism must be differentiated from the indirect-lysis or molecular-lysis process induced by phospholipases, which resides in the destruction, by chemical hydrolysis, of a molecular unit (the phospholipid). It is noteworthy that De Grado et al. (1982) have shown that low bulk concentrations of melittin coud lead to erythrocyte lysis for R_i ranging from 50 to 200, which do not differ markedly from R_i utilized herein. However, it must be emphasized that our findings apply to model membranes; further studies on natural membranes are clearly required to clarify the details of the lysis mechanism on live cells.

According to our proposition about melittin action on membranes, one may suggest an explanation for the potentiation produced by melittin (vide supra) on phospholipases A_2: the former would destabilize the bilayer in producing small supramolecular structures in which the lipids would be more exposed to the action of the latter. The potentiation could also work the other way around, the action of phospholipases, in fragilizing the bilayer, would facilitate the destabilization promoted by melittin. However, little is known about these mechanisms, at the molecular level. The determination of the melittin location in the small structures, as well as its state of association in the complex will certainly help in the understanding of such processes.

Although model and natural membranes are currently studied by NMR [for a review, see Bloom & Smith (1985)], the present work is the first to report important changes induced by a protein on the morphology, the local structure, and the dynamics of lipidic membranes. It is also noteworthy that a single ^2H or ^{31}P NMR resonance line shape does not necessarily mean disorder but may reflect macroscopic geometrical changes of the lipid–protein system (Bienvenue et al., 1982). Such a differentiation may be inferred from relaxation mea-

surements, as shown herein, or by use of complementary techniques such as freeze–fracture electron microscopy or light scattering (Faucon et al., 1985). Earlier experiments in which isotropic ^2H NMR lines appear upon protein addition to lipid membranes (Rice & Oldfield, 1979) might be reconsidered in the light of our findings.

We should emphasize that the apparently general observation that proteins do not modify greatly the structure and dynamics of lipid membranes, as seen by ^2H NMR, might be peculiar to certain systems. As an example, one may compare the present results with ^2H NMR data on other lipid–protein interactions (Oldfield, 1982). Melittin behaves very differently with lipid membranes than does ATPase or cytochrome *c* oxidase. In this respect, we differ with the conclusions of Jähnig et al. (1982). Care must be taken in generalizing the action of the few proteins studied so far to all lipid–protein interactions.

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