Modulation of Melittin-Induced Lysis by Surface Charge Density of Membranes

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ABSTRACT Phosphorus NMR spectroscopy was used to characterize the importance of electrostatic interactions in the lytic activity of melittin, a cationic peptide. The micellization induced by melittin has been characterized for several lipid mixtures composed of saturated phosphatidylcholine (PC) and a limited amount of charged lipid. For these systems, the thermal polymorphism is similar to the one observed for pure PC: small comicelles are stable in the gel phase and extended bilayers are formed in the liquid crystalline phase. Vesicle surface charge density influences strongly the micellization. Our results show that the presence of negatively charged lipids (phospholipid or unprotonated fatty acid) reduces the proportion of lysed vesicles. Conversely, the presence of positively charged lipids leads to a promotion of the lytic activity of the peptide. The modulation of the lytic effect is proposed to originate from the electrostatic interactions between the peptide and the bilayer surface. Attractive interactions anchor the peptide at the surface and, as a consequence, inhibit its lytic activity. Conversely, repulsive interactions favor the redistribution of melittin into the bilayer, causing enhanced lysis. A quantitative analysis of the interaction between melittin and negatively charged bilayers suggests that electroneutrality is reached at the surface, before micellization. The surface charge density of the lipid layer appears to be a determining factor for the lipid/peptide stoichiometry of the comicelles; a decrease in the lipid/peptide stoichiometry in the presence of negatively charged lipids appears to be a general consequence of the higher affinity of melittin for these membranes.

INTRODUCTION

Melittin, the major component of honeybee venom, is a basic amphiphilic peptide of 26 amino acids that binds spontaneously to biological and pure lipid membranes (Habermann, 1972). The similarity between its structure and that of the presequence of mitochondrion-destined proteins and signal peptides leading to the insertion of proteins into membranes, (Engelmann and Steitz, 1981; Von Heijne and Blomberg, 1979) makes the study of this peptide and its interactions with various types of membranes of particular interest. The effect of melittin on phospholipids has been studied extensively (for a general review, see Dempsey, 1990). One of its fascinating features is that, unlike gramicidin, which universally promotes inverted hexagonal phase (Killian and de Kruijff, 1988) or glycophorin, which stabilizes bilayers (Taraschi et al., 1982), the modulation of membrane organization by melittin depends strongly on the nature of the lipid component. Melittin causes the micellization of saturated phosphatidylcholine bilayers (Dufourcq et al., 1986), leading to the formation of small discoidal comicelles similar to lipoproteins. In the case of phosphatidylethanolamine, melittin has a bilayer stabilizing effect, as it inhibits the formation of hexagonal phase (Batenburg et al., 1988). Conversely, melittin induces the formation of inverted phases upon interaction with cardiolipin (Batenburg et al., 1987a), egg phosphatidylglycerol (eggPG), 1,2-dioleoyl-sn-glycero-3-phosphatidic acid (DOPA) and phosphatidylserine (PS) (Batenburg et al., 1987b). Therefore, it is clear that some physical properties of the lipid component dictate the architecture of the resulting lipid/melittin complexes.

Melittin-induced micellization of pure saturated phosphatidylcholine bilayers is well characterized. In the case of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) model membranes, melittin changes the lipid morphology by breaking up the membrane into small lipid/peptide particles (Dufourcq et al., 1986; Dufourc et al., 1986a). This disruption is referred to here as lysis or micellization. The resulting comicelles have been described as discoidal gel-phase bilayers of about 500 Å diameter surrounded by a layer of melittin (Dufourcq et al., 1986; Lafleur et al., 1987). The disruption of gel-phase DPPC bilayers into small comicelles is complete when the incubation lipid-to-peptide molar ratio (R_i) is equal to or less than 20. Upon heating above the gel-to-liquid crystalline phase transition temperature (T_m), the small particles refuse into extended bilayers, for $R_i > 5$. This temperature-dependent polymorphism is completely reversible (Dufourc et al., 1986a).

From the results reported in the literature, it is obvious that melittin does not interact with negatively charged lipid and zwitterionic phosphatidylcholine in the same fashion. No lysis is observed when melittin interacts with bilayers formed

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Abbreviations used: ATR-IR, attenuated total reflection infrared spectroscopy; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine]; DOTAP, 1,2-dioleoyl-3-(trimethylammonio)propane; DPTAP, 1,2-dipalmitoyl-3-(trimethylammonio)propane; LA, linoleic acid; NMR, nuclear magnetic resonance; OA, oleic acid; PA, palmitic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; R, lipid-to-peptide molar ratio; R_i, incubation lipid-to-peptide molar ratio; T_m, gel-to-liquid crystalline phase transition temperature.

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exclusively with negatively charged lipids (Batenburg et al., 1987b; Lafleur et al., 1991). In addition, the binding of melittin to charged lipids is two orders of magnitude stronger than that with zwitterionic phospholipids (Batenburg et al., 1987b; Dufourcq and Faucon, 1977). It is likely that electrostatic interactions between the positively charged residues of the peptide and the negatively charged lipid head groups play an important role. At this point, it is suggested that these interactions influence the insertion depth of the peptide into fluid lipid bilayers, causing a modulation of bilayer curvature. This modulation is proposed to dictate the polymorphism of the lipid/melittin macrostructures (Batenburg and de Kruijff, 1988).

Despite the fact that the studies of complexes formed by melittin with bilayers formed by a single lipid species provide useful information on lipid/peptide assemblies, investigations using more complex lipid matrices are necessary to improve our understanding of the interactions between peptides and lipids in biological systems. At this point, only a limited number of studies have examined the effects of melittin on binary lipid mixtures (Lafleur et al., 1989; Dempsey et al., 1989; Beschiaschvili and Seelig, 1990). An important contribution of these investigations is that melittin does not appear to cause phospholipid demixing in fluid bilayers formed by two phospholipids bearing different head groups.

In the present study, the importance of electrostatic interactions in the lytic activity of melittin has been investigated using binary lipid mixtures. We have characterized melittin-induced micellization observed with saturated phosphatidylcholine bilayers containing various charged species in limited concentrations. By contrast with systems that involve exclusively negatively charged lipids, the lytic activity of melittin should be maintained for these bilayers whose charged lipids are "diluted" in a DPPC matrix. We have therefore characterized the modulation of the lytic activity of the peptide by the presence of charges at the bilayer interface in physiological conditions; natural membranes contain about 10–20% charged species (Gennis, 1989). More precisely, we have determined how melittin lytic power is influenced by the presence of limited amounts of negatively charged species, either as phospholipids or deprotonated fatty acids: 1,2-dipalmitoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (DPPG), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (DMPS), palmitic acid (PA), oleic acid (OA), and linoleic acid (LA). To get more information on the charge density effect, we have also studied the lysis induced by melittin on DPPC in the presence of synthetic positively charged lipids: 1,2-bis(palmitoyl)-3-(trimethylammonio)-propane (DPTAP) and 1,2-bis-(oleoyl)-3-(trimethylammonio)propane (DOTAP). Even though positively charged lipids do not occur in biological membranes, the lytic activity of melittin on DPPC containing 10 mol % of positively charged lipid brings additional information on the role played by electrostatic interactions in the micellization induced by the peptide.

The lytic property of melittin can be easily studied by ^{31}P nuclear magnetic resonance (NMR) because extended bilayers and small comicelles have motions with very different correlation times (Watts and Spooner, 1991). Small particles resulting from the lysis lead to the averaging of the phosphorus chemical shift anisotropy, due to their fast tumbling on the NMR time scale; an isotropic signal is therefore observed. Intact lipid vesicles or extended bilayers formed upon refusion have a longer correlation time and their reorientation with respect to the magnetic field is slow. In this case, the interaction between the nucleus and the magnetic field is orientation-dependent, giving rise to a typical powder pattern (Seelig, 1978).

MATERIALS AND METHODS

DPPC, DPPG, DMPS, and DOTAP were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). DPTAP was synthesized according to the procedure described by Stamatatos et al. (1988). Deuterium oxide, palmitic acid, oleic acid and linoleic acid were obtained from Sigma (St. Louis, MO) and used without further purification. Melittin was purified from bee venom (Sigma, St. Louis, MO) by ion exchange chromatography on SP-Sephadex C-25, and desalted according to a high-performance liquid chromatography (HPLC) procedure (Lafleur et al., 1987). Samples were prepared by hydrating 30 mg lipid with 1 ml of 10 mM *N*-hydroxyethylpiperazine-N′-ethanesulfonic acid (HEPES) buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 100 mM sodium chloride, at pH = 7.5 or 9.5. The samples were then heated above T_m and vortexed. In the case of lipid mixtures, lipids were weighted in appropriate ratio, dissolved in benzene-methanol, and then lyophilized. The freeze-dried lipid mixtures were hydrated as described above. For lipid-melittin complexes, an aliquot of melittin solution in water was added to hydrated bilayers to obtain the desired R_i. The mixtures were vortexed, heated, and cooled several times through T_m before data acquisition. This treatment was necessary since it has been shown that DPPC/melittin samples must be incubated above the pretransition temperature in order to observe lysis (Monette et al., 1993). When necessary, pH adjustments were performed by adding small aliquots of dilute hydrochloride acid or sodium hydroxide aqueous solutions to the sample. Deuterium oxide was added to all samples to get a deuterium lock.

A Bruker WH-400 spectrometer was used to record the ^{31}P-NMR spectra with a single pulse of 20 μs (35°) and broadband decoupling. The relaxation delay was set to 1 s and the number of scans was typically 5400. Alternatively, a Bruker AMX_R-400 was used with the same conditions except for proton decoupling which was achieved with composite pulse decoupling (GARP pulse sequence). The sample temperature was regulated with a Bruker variable temperature controller.

In the binding experiment, the phospholipid amount was determined according to the method published by Fiske and SubbaRow (1925); melittin concentration was directly determined by measuring the absorbance at 280 nm (ϵ_{280} = 5600 $M^{-1}cm^{-1}$) (Dufourcq et al., 1986)

RESULTS

First, we have studied the effect of increasing amount of negatively charged DPPG in DPPC bilayers on the micellization induced by melittin. Fig. 1 shows the ^{31}P-NMR spectra of different DPPG/DPPC mixtures (ranging from 0 to 50 mol % DPPG in DPPC), at 26°C, with a constant R_i of 40. Under these conditions, pure DPPC gives rise to a dominant isotropic signal, resulting from the almost complete disruption of the large vesicles, and the formation of small discoidal particles, in agreement with previous results (Dufourcq et al., 1986; Dufourc et al. 1986a). As the amount of DPPG increases from 0 to 15 mol %, Fig. 1 shows a progressive reduction of the isotropic signal intensity relatively to the powder pattern. For mixtures containing more than 15 mol

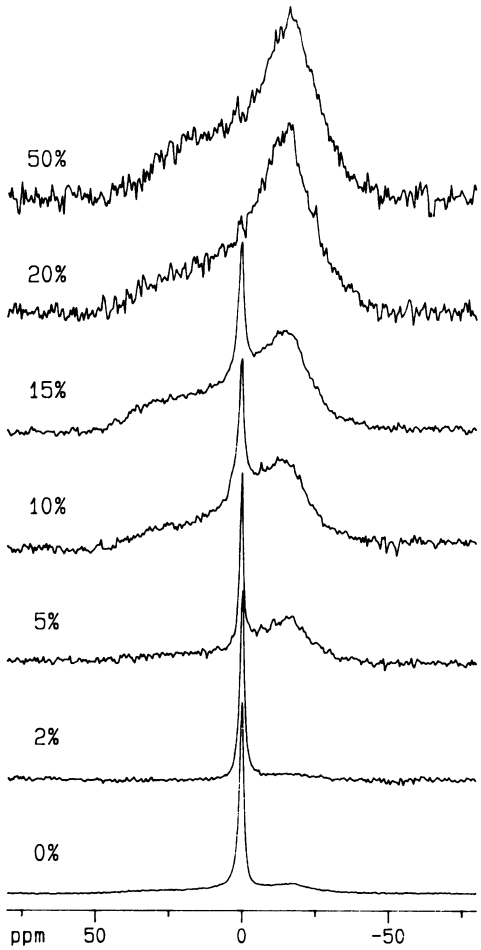

FIGURE 1 ^{31}P-NMR spectra of DPPG/DPPC mixtures in the presence of melittin at R_i = 40, pH = 7.5, at 26°C. DPPG molar concentration is indicated for each spectrum.

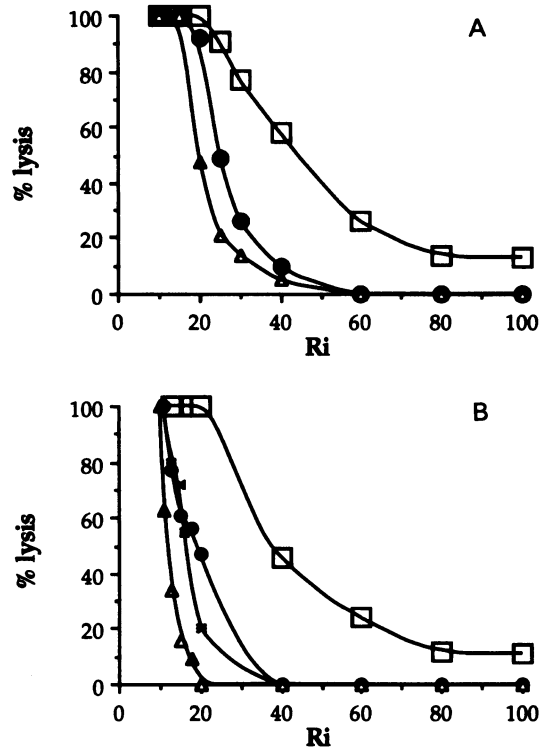

FIGURE 2 Relative amount of lysis with respect to the lipid/peptide R_i at 26°C for 10 mol % DPPG (▲) or DMPS (●) in DPPC and pure DPPC (□) at pH = 7.5 (*A*), and for 10 mol % palmitic acid (▲), oleic acid (*), or linoleic acid (●) in DPPC and pure DPPC (□) at pH = 9.5 (*B*).

% DPPG, the isotropic signal has completely vanished. In these conditions, the presence of DPPG in DPPC bilayers in proportion comparable to the physiological proportion of negatively charged species in various membranes is therefore sufficient to inhibit completely the lysis induced by melittin.

If electrostatic interactions between the basic peptide and the charged interface are responsible for the increased stability of the bilayer to micellization, a similar resistance can be expected for DPPC bilayers containing other negatively charged species. Fig. 2 displays the percentage of lysis as a function of the R_i, at 26°C, for mixtures of DPPC containing 10 mol % of negatively charged phospholipids or fatty acids, compared to pure DPPC. The % lysis is estimated from the ratio of the area of the isotropic signal over the total area of the ^{31}P-NMR spectrum (isotropic signal + powder pattern). Even though ^{31}P-NMR spectra were not acquired in ideal conditions for quantitative analysis, these calculated ratios are good estimates of the sample compositions. Fig. 2 *A* shows the % lysis measured as a function of R_i for the mixtures containing 10 mol % negatively charged phospholipids (DPPG or DMPS), at 26°C, with a constant pH of 7.5. These two phospholipids have been selected because their T_m's (41 and 37°C for DPPG and DMPS, respectively) (Marsh, 1990) are close to that of DPPC (41°C), favoring lipid mixing. For pure DPPC bilayers, the proportion of lysed vesicles increases as the R_i decreases, as expected. When the ratio reaches 20, the lysis is complete, in agreement with previous results (Dufourcq et al., 1986). The presence of 10 mol % charged phospholipid in DPPC bilayers shifts significantly these curves toward lower R_i values. Consequently, more melittin is required to achieve a certain proportion of lysis when the bilayer contains 10 mol % charged phospholipids with respect to pure DPPC bilayers. For example, 50% of pure DPPC bilayers are lysed for R_i = 45. A R_i of about 20–25 is required to achieve the same fraction of lysed vesicles for DPPC bilayers containing 10 mol % of DMPS or DPPG. This represents an increase of about 30% in peptide concentration. This inhibition of the lytic activity of melittin is substantial considering that the proportion of negatively charged phospholipids is only 10 mol %. The presence of 20 mol % DMPS in DMPC bilayers has also been reported to have a stabilizing effect with respect to the melittin-induced lysis (Dempsey et al., 1989).

Similar results are obtained if the charges are introduced by using 10 mol % deprotonated fatty acids. The lysis induced by melittin on pure DPPC and on DPPC bilayers containing palmitic, oleic, or linoleic acid is compared in Fig. 2 *B*. For these samples, the pH was carefully set at 9.5 and rigorously checked before and after data acquisition to

ensure the deprotonation of the fatty acids into the bilayer; the pK_a of fatty acids incorporated in a phosphatidylcholine bilayer is estimated to 8.5 (Kantor and Prestegard, 1978; Schullery et al., 1981). Fatty acids should therefore be mostly deprotonated at pH = 9.5. It is also worth noting that, for pure DPPC, the proportion of lysis observed at pH = 7.5 and pH = 9.5 is comparable, indicating that the peptide inherent ability to cause lysis is not pH-dependent over the investigated range. Similarly to the effect of charged phospholipids, the presence of deprotonated fatty acids in the bilayer inhibits the lytic activity of melittin, relative to pure DPPC. Although there are small differences between their respective ability to stabilize the bilayer, the observed inhibition is a general trend for all three fatty acids.

As stated above, pH control is essential to maintain a constant charge density at the bilayer surface, especially when the source of negative charge is a deprotonated fatty acid. It has been shown that the pK_a of palmitic acid in phosphatidylcholine bilayers is around 8.5 (Schullery et al., 1981). Consequently, a change of pH in this region has an important influence on the proportion of protonated/unprotonated fatty acid, affecting therefore the surface charge density. To support our conclusion that reduced lysis is due to the presence of negative charges at the bilayer interface, we have monitored the lytic activity of melittin on a DPPC/10 mol % palmitic acid mixture as a function of the pH. Fig. 3 displays the different ^{31}P-NMR spectra obtained at several pH for the same DPPC sample containing 10 mol % palmitic acid, with a constant R_i = 60, at 26°C. At pH of 8.6 and above, Fig. 3 shows a complete inhibition of the lysis, in agreement with the results presented in Fig. 2. A decrease of 0.5 pH unit leads to the formation of some small particles giving rise to an isotropic signal coexisting with the powder pattern. At pH 7.5, the disruption of the lipid bilayer is even more important: the proportion of lysed vesicles is estimated to be greater than 60%. Decreasing the pH down to 6.5 does not modify considerably this percentage (data not shown).

Melittin-induced lysis has been characterized in more details for the sample containing 10 mol % palmitic acid in DPPC. This system has been selected because all the lipid acyl chains are identical and saturated. The presence of unsaturation in the hydrophobic core has some effect of the lytic activity of melittin (M. Monette and M. Lafleur, to be published); the DPPC/PA systems avoids the possibility of combined effects from the surface charge density and acyl chain unsaturation. Fig. 4 represents the relative amount of lysed vesicles as a function of melittin concentration (mol %) - for (*A*) pure DPPC and (*B*) DPPC with 10 mol % palmitic acid (pH = 9.5). Melittin concentration is expressed as mol %, i.e., number of melittin molecules/100 phospholipids. The region for which the % lysis varies from 10 to 100% is considered as linear for purposes of comparison between pure DPPC and the DPPC/10 mol % PA mixture; the corresponding least-square fit equations are presented in Fig. 4. The slope represents the amount of lysis induced by the addition of 1 mol % melittin, in the linear range; this value is similar for both lipid systems, indicating that the addition of 1 mol % melittin causes about 25% lysis for pure DPPC and the

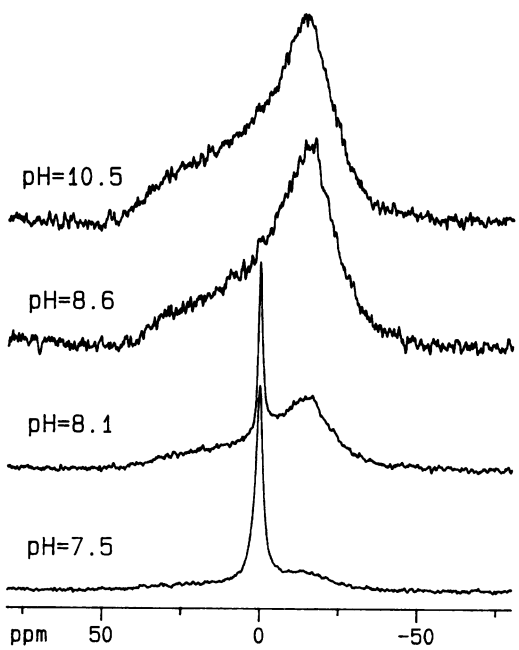

FIGURE 3 ^{31}P-NMR spectra of a DPPC sample containing 10 mol % palmitic acid in the presence of melittin at R_i = 60 at 26°C as a function of the pH.

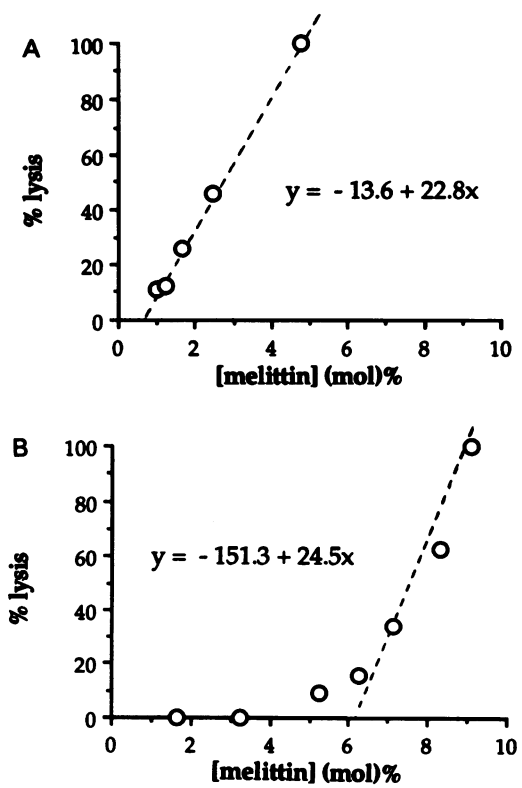

FIGURE 4 Compared lytic activity of melittin as a function of the peptide concentration at 26°C and pH = 9.5, for pure DPPC (*A*) and DPPC/10 mol % palmitic acid system (*B*).

DPPC/10 mol % palmitic acid system. On the other hand, the x-intercept is significantly different for sample containing 10 mol % palmitic acid compared with pure DPPC. This extrapolated parameter represents the maximum amount of melittin that should not cause lysis, based on the behavior in the linear region. This value is 0.6% for pure DPPC and increases to 6.2% when the lipid bilayer contains 10 mol % palmitic acid. These results indicate that more melittin can be accommodated in a negatively charged bilayer without causing lysis, compared with a zwitterionic bilayer.

In order to verify that the reduction of lysis observed in the presence of charged lipids is not due to a reduced binding of the peptide, we have determined the proportion of bound melittin to DPPC bilayers containing 10 mol % palmitic acid at pH = 9.5. Several phospholipid/melittin ratio were examined (R_i = ∞, 100, 80, 60, 50, 40, 30, and 25). No detectable lysis occurred, as monitored by NMR, in agreement with Fig. 2 *B*. After centrifugation at 2500 × g for 30 min, the amount of residual melittin and phospholipids were determined in the supernatant. No melittin was detected in the supernatant except for the sample containing the largest amount of melittin (R_i = 25). For this particular sample, we found <2% of the initial amount of melittin in the supernatant. The phosphorus concentration in this phase was below the detection limit for all samples. These assays confirm that almost all of the peptide interacts with the negatively charged lipid bilayers without causing their disruption.

Next, we have studied the effect of positively charged lipid on the lytic activity of melittin. Fig. 5 shows the ^{31}P-NMR spectra for a sample containing 10 mol % DPTAP in DPPC at constant pH of 7.5. As for the other samples, the lipid mixture gives rise to a powder pattern characteristic of bilayers in the gel phase at room temperature, while the spectrum obtained at higher temperature (55°C) is typical of a fluid phase. The addition of a small amount of melittin (R_i = 100) has a dramatic effect on these positively charged bilayers. The spectrum obtained is dominated by a very large isotropic signal (width at half height of 1 kHz). Subsequent additions of melittin lead to the narrowing of the isotropic signal. Similar to the behavior of DPPC/melittin complexes, the small particles obtained at R_i = 30 refuse back to extended bilayers upon raising the temperature to 55°C. Therefore, the presence of DPTAP in DPPC bilayers enhances the lytic activity of melittin compared to that observed with pure DPPC bilayers (cf. Fig. 2 *A*). A similar promotion of the lytic activity is also obtained in the presence of DOTAP in DPPC bilayers (data not shown).

The study of the lysis induced by melittin was exclusively performed in the gel phase, due to the enhanced stability of the comicelles below T_m. Throughout this study, we have monitored the effect of melittin on various types of membranes in the liquid crystalline phase and the results confirm the formation of extended bilayers above T_m, for large R_i. This result implies that the presence of negatively charged species in DPPC bilayers does not modify the nature of the polymorphism induced by melittin but only the extent of the micellization. In this paper, the isotropic line observed by NMR spectroscopy is associated with small lipid/peptide

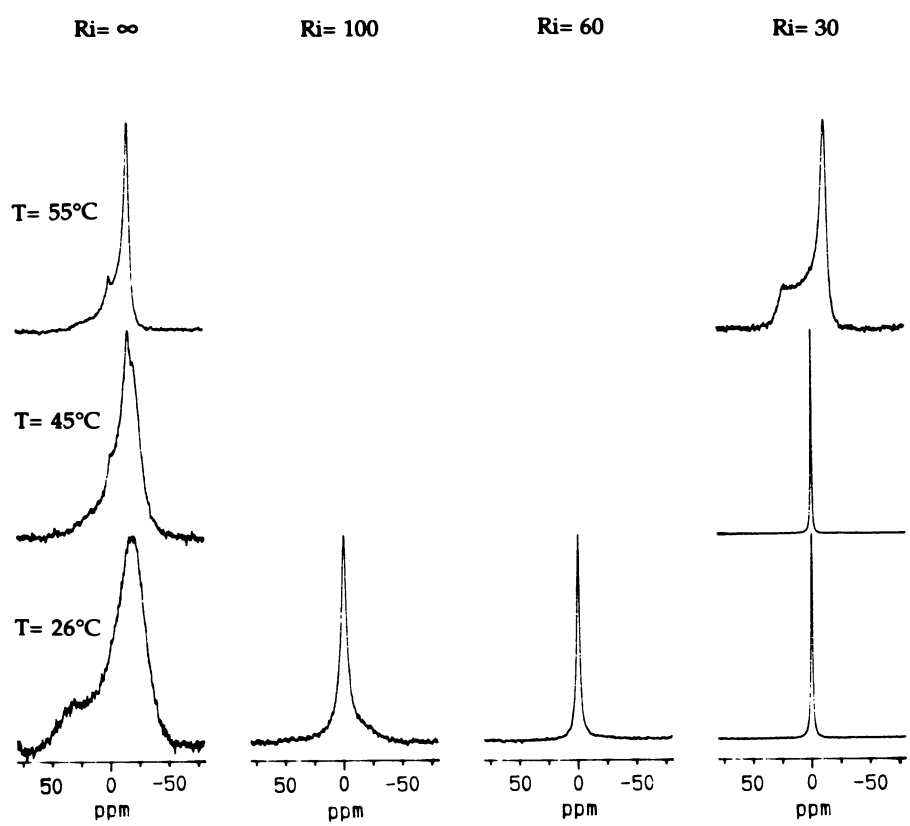

FIGURE 5 ^{31}P-NMR spectra of a DPPC/10 mol % DPTAP mixture at various temperatures (26, 45, and 55°C) and various lipid/melittin R_i's at pH = 7.5.

comicelles, although other types of structures (cubic phase, for example) also give rise to isotropic lines. As an example, the ^{31}P-NMR spectra of eggPG/melittin system show an isotropic signal assigned to non-lamellar phases as ascertained by electron microscopy (Batenburg et al., 1987b). Similarly, we have observed that the interaction between melittin and DPPG bilayers leads to the immediate precipitation of the lipid/peptide assemblies; these structures give rise to a broad isotropic signal in the ^{31}P-NMR spectra in the gel and the liquid crystalline phase. In our study on binary lipid mixtures, the fact that powder patterns are observed in the liquid crystalline phase strongly support the assignment of the isotropic line to small comicelles. In addition, the samples giving rise to isotropic lines were transparent.

DISCUSSION

From the results presented in Figs. 1 and 2, it can be stated that the presence of negatively charged phospholipids or unprotonated fatty acids inhibits severely the lytic activity of melittin. Moreover, the reduction of lysis is proportional to the amount of these charges at the bilayer interface. Because this inhibition appears to be independent of the nature of the molecule carrying the negative charge, we suggest that electrostatic interactions between the positively charged peptide and the negatively charged bilayer are responsible for this phenomenon. An additional evidence is presented in Fig. 3, showing that the variation of pH leads to a variation of the susceptibility of the membrane to undergo lysis. The pH-dependent polymorphism of DPPC/10 mol % palmitic acid/melittin system is directly related to the protonation of the palmitic acid into the bilayer. At high pH values, palmitic acid is mostly deprotonated, which leads to a net negative charge density at the bilayer interface. The inhibition of the lysis induced by melittin is therefore comparable to the one resulting from the presence of a negatively charged phospholipid in the bilayer. A reduction of pH leads to the protonation of palmitic acid, giving rise to a reduced charge density of the bilayer surface; the lysis is observed again. Conversely, the presence of a positively charged phospholipid in the membrane leads to the promotion of the lysis, as shown in Fig. 4. This result is rather surprising since it could be expected that the repulsive interaction between the peptide and the charged bilayer should lead to reduced lysis, as a consequence of reduced affinity between the charged species. All these results indicate clearly that melittin-induced lysis can be controlled by interfacial charge density.

A qualitative model for the lysis mechanism

To rationalize these results, we have focused our attention on two key steps of the lysis mechanism. Initially, melittin interacts with the lipid surface, leading to its partial insertion into the interfacial region. The affinity of melittin for membranes can be rationalized on the basis of hydrophobic interaction between the nonpolar amino acids and the phospholipid hydrocarbon chains (Dufourcq et al., 1986; Lafleur et al., 1987). When melittin interacts with zwitterionic lipid bilayer, the apolar face of the peptide is in contact with the acyl chains of the bilayer, reducing unfavorable interactions with the water. This organization allows the polar face of melittin to interact with the polar head region or the water (Terwilliger et al., 1982). When melittin interacts with negatively charged membranes, the driving force for peptide adhesion is enhanced by the electrostatic interaction between the species of opposite charge. The importance of this contribution is shown by the higher affinity of melittin for negatively charged bilayers, relative to zwitterionic phosphatidylcholine bilayers (Dufourcq and Faucon, 1977). This first step is supported by results obtained by polarized attenuated total reflection infrared spectroscopy (ATR-IR), showing that the interaction between melittin and fully hydrated bilayers in the liquid crystalline phase leads to partial insertion of the peptide with the helix axis parallel to the bilayer surface, irrespective of the surface charge density (Frey and Tamm, 1991).

The second key step is the redistribution of the peptide in the lipid assembly resulting in the disruption of the membrane. The way this redistribution proceeds is not yet determined but it is clear that the peptide is transferred from the interface to the apolar core of the bilayer since, in the resulting comicelles, the apolar face of the helical peptide is in direct contact with the phospholipid acyl chains (Dufourc et al., 1986a). This peptide translocation has been proposed to be triggered by the liquid crystalline-to-gel phase transition (Dempsey and Watts, 1987). The partial penetration of melittin into the bilayer leads to a volume expansion in the polar head region that can be accommodated in the liquid crystalline phase by the fluid chains. Such an expansion at the interface level is difficult in the gel phase, since the acyl chains are more rigid. The inability of the gel phase bilayer to accommodate melittin in the polar region is proposed to cause the redistribution of the peptide in the lipid bilayer leading to the micellization (Dempsey and Watts, 1987). This redistribution can be associated with the deeper penetration of melittin into the bilayer observed as the temperature is lowered below the transition temperature (Dufourc et al., 1986b).

The effect of surface charge density on melittin-induced lysis can be rationalized on the basis of these two key steps. In the presence of negatively charged lipids, a severe reduction of the lysis is observed irrespective of the nature of the negative charge. The binding experiment shows that almost all of the peptide interacts with the negatively charged bilayers, in agreement with previous results (Dempsey et al., 1989). This result confirms that the inhibition is not related to a reduced interaction of the peptide with the bilayer (step 1). We propose that the redistribution of melittin in the lipid assembly (step 2) is inhibited in the presence of negatively charged lipids, likely due to the electrostatic anchoring of the positively charged melittin to the negatively charged bilayer interface.

The increase in lysis observed for a positively charged membrane can be rationalized on the same basis. In this case,

hydrophobic interactions drive the adsorption of melittin at the bilayer surface. However, the repulsive electrostatic interactions between the positively charged bilayer interface and the toxin favor the redistribution of the peptide in the bilayer and, as a consequence, promote the micellization of the membrane. Because of unfavorable electrostatic interactions, the membrane cannot accommodate even small amounts of melittin and its disruption is observed even for very high lipid/peptide ratio (Fig. 4). If the geometry of the DPPC/DPTAP/melittin comicelles is similar to the one proposed for the discs formed with DPPC/melittin, the lipid/melittin ratio in the comicelles is proportional to the radius of the discoidal particle (r). This relation is due to the fact that the number of lipids is proportional to the disc area (αr^2), whereas the number of peripheral melittins is related to the circumference (αr). Since complete lysis is observed even for very low peptide concentration, the resulting complexes should have a high lipid/melittin ratio and, consequently, a large radius. This deduction is in agreement with the NMR results since the comicelles formed in the presence of positively charged lipids give rise to a very broad isotropic line, implying that their correlation time is sufficiently short to allow the averaging of the phosphorus chemical shift anisotropy but long enough to observe a significant line broadening. The line width decreases with the increase in the peptide concentration, suggesting that the assemblies are further broken into smaller ones, as expected for a decreased lipid/melittin ratio.

In our interpretation, the position of melittin relative to the gel phase bilayer is essentially controlled by electrostatic interactions and modulates the lytic properties of the peptide. This conclusion is analogous with a previous model proposing that electrostatic interactions between melittin and negatively charged bilayers affect the relative depth of the peptide in the bilayer, in the liquid crystalline phase (Batenburg and de Kruijff, 1988). This rationale combined with the shape-structure concept was proposed to explain the polymorphism of these complexes with melittin (Batenburg and de Kruijff, 1988).

A quantitative model for the lysis mechanism

To investigate further the relation between electrostatic interactions and lytic activity, a quantitative analysis of DPPC/10 mol % palmitic acid system and pure DPPC was performed. A very interesting feature of the graphs of % lysis versus melittin concentration obtained for these systems is the almost identical slope in their linear region (Fig. 4). This result indicates that the same amount of added melittin causes the same proportion of lysis, suggesting that lysis takes place in a similar fashion in both cases. Second, the x-intercept is very different for two systems: 0.6 mol % for pure DPPC and 6.2 mol % for DPPC containing 10 mol % deprotonated palmitic acid (one order of magnitude higher). This parameter represents the extrapolated amount of melittin that interacts with the membrane without causing lysis, based on the behavior in the linear region. In the case of the DPPC/PA mixture, the lack of lytic activity of these melittin molecules is proposed to be mainly due to the electrostatic anchoring of the peptide at the interface, as discussed in the previous section. Even though these melittin molecules do not affect the morphology of the lipid bilayer, their charged residues should lead to a reduction of the effective charge density of the interface. This result combined with the similar slope obtained for the charged and neutral membranes leads us to suggest that the first fraction of melittin is adsorbed at the bilayer interface and reduces the surface charge density; when a sufficient amount of melittin is adsorbed at the surface so as to reach electroneutrality, the bilayer undergoes lysis upon addition of melittin the same way neutral bilayers do.

The effective charge of melittin can be evaluated based on the assumption that electroneutrality is reached before lysis occurs and that all the melittin molecules are bound to the vesicles. This latter premise is sensible considering that the binding constant of melittin to DPPC has been estimated to $6 \times 10^4\ M^{-1}$ (Beschiaschvili and Seelig, 1990; Beschiaschvili and Baeuerle, 1991) and is even more important in the presence of negatively charged lipids (Dufourcq and Faucon, 1977; Beschiaschvili and Seelig, 1990). This almost complete binding is also supported by our assay, showing that melittin was essentially all bound to DPPC/10 mol % PA bilayers. Our results suggest that 5.6 mol % melittin (6.2–0.6 mol %) are required to neutralize the negative charges present at the bilayer interface. Since the bilayer contains 10 mol % of negatively charged lipids, the calculated effective charge for melittin is +2.0. This value is comparable to the one obtained previously by different approaches (+1.5 to +1.9) (Beschiaschvili and Baeuerle, 1991). The effective charge is lower than the total charge of melittin, which is +6. The origin of the reduction has been discussed elsewhere (Beschiaschvili and Baeuerle, 1991); among many reasons are the large size of melittin compared to the Debye length and the fact that charged amino acids of the peptide can be located rather far from the bilayer surface.

Using a similar approach, the lipid-to-peptide stoichiometry in the comicelles can also be estimated. The slope was defined earlier as the percentage of lysed vesicles caused by the addition of 1 mol % melittin, expressed as percentages relative to total phospholipids. For DPPC, the addition of 1 melittin drags 23 lipids from the extended bilayers into the comicelles. Since some melittin is already bound to the vesicles before lysis occurs, this contribution, estimated from the x-intercept (x_{int}), has been taken into account; it is calculated as follows:

$$[\text{melittin}]_{\text{bound}} = x_{\text{int}} \times \text{slope}/100. \quad (1)$$

The lipid/peptide ratio in the comicelles (R) is calculated as follows:

$$R = \text{slope}/(1 + [\text{melittin}]_{\text{bound}}) \quad (2)$$

According to these calculations, the discs formed with pure DPPC contain approximately 20 lipid molecules per melittin. A similar stoichiometry was previously obtained using a

chromatographic approach (Dufourcq et al., 1986). The calculated ratio (R) goes down to 10 lipid molecules per melittin for the DPPC/10 mol % palmitic acid mixture. This result is the first indication that the lipid/peptide ratio in comicelles is decreased when DPPC bilayers contain negatively charged lipids. A parallel can be made with the lipid/peptide ratio obtained for melittin interacting with pure negatively charged lipids, keeping in mind that, in these cases, melittin does not cause micellization but leads to the formation of nonlamellar structures. The lipid/peptide stoichiometries reported are: 4 cardiolipin/melittin (Batenburg et al., 1987a), 2 DOPA/melittin, and 4 eggPG/melittin (Batenburg et al, 1987b). In addition, it has been shown that pure PS vesicles can bind up to 8 times more melittin that PC vesicles (Dufourcq and Faucon, 1977). We conclude that the increase in melittin proportion in the complexes formed with lipid matrices containing negatively charged species (micellar or inverted structures) is a general trend. The reduced lipid/melittin ratio deduced from our results seems to be an additional manifestation of the enhanced affinity of melittin for membrane containing negatively charged species, even when they are minor species.

The remarkable agreement between our results and previous values of effective charge of melittin and the stoichiometry of DPPC/melittin in the comicelles provides us confidence about the validity of our conclusions obtained by the present model.

CONCLUSION

To conclude, some biological implications of the results presented are discussed. This study reveals that melittin-induced lysis can be controlled by the introduction of a limited amount of negatively charged lipid in the DPPC matrix. This inhibition is certainly less drastic than the complete modification of the morphology observed when melittin interacts with pure negatively charged lipids. However, the role played by a limited concentration of charged species is substantial and may be relevant from a biological point of view. The surface charge density of a membrane may play a role in peptide binding but may also control the insertion of peptides in membranes; as a consequence, it modulates the lytic properties of amphipathic peptides. The lytic activity of some peptides or proteins has been reported to be inhibited by negatively charged lipids. A recent study (Roux et al., 1994) has shown that myelin basic protein, the major protein in myelin, disrupts PC bilayer membranes into small comicelles, similarly to melittin. This micellization is severely inhibited when PC bilayers contain 20 mol % PS. Similarly, nisin, a lantibiotic, causes the release of the content of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles; this leakage, characterized by fluorescence techniques, is also reduced when anionic lipids are introduced in PC membranes (Garcera et al., 1993). From our study and these recent results, a lysis-inhibiting character of the negatively charged lipids seems to emerge. It is too early to conclude that this feature is universal, but the extrapolation of the understanding gained from the present study to other systems must be examined.

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