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Effect of cholesterol on the polymorphism of dipalmitoylphosphatidylcholine/melittin complexes: an NMR study

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In order to get insights into the effects of cholesterol on protein activity, the lytic power of melittin on 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/cholesterol mixtures was studied using solid-state deuterium and phosphorus-31 nuclear magnetic resonance spectroscopy (^{2}H and ^{31}P-NMR). After incubation, melittin disrupts pure DPPC vesicles, leading to the formation of small lipid/peptide complexes below the phase transition temperature (T_m), whereas large bilayer assemblies are reformed above T_m; the transition between these two species is thermally reversible. This study reveals that cholesterol modifies this thermal behavior and that this modulation of the lytic power of melittin is indirect, since it is essentially related to the original effect of the sterol on the thermotropism of pure lipid bilayers. It is known that melittin does not lyse gel phase DPPC bilayers spontaneously. Our study shows that the addition of large amounts of sterol (30 mol%) does not promote the spontaneous lysis at 26°C, despite the increased *fluidity* of the lipid system. The lysis takes place around 32°C, regardless of the cholesterol concentration. This study also shows that high concentrations of cholesterol ($\geq$ 30%) in DPPC bilayer inhibit the lysis. It is proposed that the tight lipid packing due to high cholesterol concentrations prevents the penetration of melittin into the bilayer. When melittin interacts with cholesterol-rich bilayers (30 mol%), the lysis is only partial, and leads to the formation of small cholesterol-depleted particles. Finally, DPPC which bears deuteriated acyl chains was used to determine the influence of melittin on the orientational order of the lipid chains in the large assemblies. The quadrupolar splittings obtained in the presence of melittin are not considerably different than those obtained in the absence of melittin.

Introduction

Cholesterol is an important component of eukaryotic membranes. This sterol is known to modify the physical properties of lipid bilayers which leads to membranes having a considerable strength but with a fluid apolar core. This unique combination of properties has been proposed as a critical trigger in cell evolution [1].

At this point, the influence of cholesterol on phospholipid bilayers has been well-studied, and our knowledge has been recently pieced together with the detailed study on phase equilibria of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/cholesterol mixtures from both experimental [2] and theoretical [3] points of view. One of the major conclusions is that cholesterol induces the formation of a particular phase, the β or liquid-ordered phase. This phase shows the following characteristics: lateral and rotational diffusion rates comparable to those of the liquid crystalline phase; highly ordered phospholipid acyl chains; bilayer thickness intermediate between those obtained for the gel and the liquid crystalline phases; elasticity modulus higher than that of the liquid crystalline phase. According to the phase diagram [2], a biphasic region composed of gel-ordered and liquid-ordered phases is found at low temperature ($T \leq 40$°C) when the cholesterol

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Abbreviations: CSA, chemical shift anisotropy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d_{62}, 1,2-bis(perdeuteriopalmitoyl)-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; lysoPPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; R_i, incubation lipid-to-protein molar ratio; T_m, gel-to-liquid crystalline phase transition temperature; $\Delta\nu_Q$, quadrupolar splitting; $\Delta\chi$, magnetic susceptibility anisotropy.

concentration varies from 5 to 22 mol%. A higher cholesterol concentration leads to the complete disappearance of the gel ordered phase, and the lipids form exclusively the β phase. Similar phase diagrams are observed for other phosphatidylcholine/cholesterol mixtures [4]. Taking into account the cholesterol content of plasmic membranes of eukaryotic cells (approx. 30 mol%) [5], it is likely that this liquid ordered phase is of biological relevance.

Even though the behavior of phosphatidylcholine/cholesterol bilayers is well-characterized, our knowledge of the effect of cholesterol on lipid/protein interaction is rather limited. It has been shown that cholesterol does not affect all proteins in a similar manner; actually, it can stimulate, inhibit or have no effect on protein activity [6]. The model proposed at this point to rationalize the modulation of protein activity by cholesterol is based on cholesterol–protein interactions [6,7]. In order to get wider insights into the role played by cholesterol in lipid–protein interactions, the effect of cholesterol on the polymorphism of amphiphilic peptide/phospholipid complexes was investigated. A wide variety of amphipathic helical peptides or proteins is found in nature including apolipoprotein, antibiotics and toxins. In the present investigation, melittin was used as a model for this type of peptides, and its interactions with model membranes composed of DPPC in the presence of cholesterol were studied.

Melittin is a basic and amphiphilic peptide of 26 amino acids that binds to model and cell membranes, causing their lysis through an unknown mechanism [8]. Upon interaction with a membrane-like interface, or when self-aggregated, melittin forms an α-helix which leads to an asymmetrical spatial distribution of polar and non polar amino acids: the non polar residues are located on one side of the helix rod while the polar or charged amino acids are on the other side. This amphiphilic character of melittin is greatly responsible for its various actions on membranes, as reviewed elsewhere [8].

In DPPC model membranes, melittin alters lipid organization by breaking up the membrane into small lipid/peptide particles, when the incubation lipid-to-peptide molar ratio (R_i) is between 5 and 50. This disruption of the bilayer is referred to here as lysis. The structure of these DPPC/melittin particles has been described as small discoidal bilayers with a diameter of 235 Å, whose edges are coated with a layer of helical melittin [9]. These particles are stable only at low temperatures corresponding to gel phase DPPC bilayers. The increase in temperature above the gel-to-liquid crystalline phase transition temperature (T_m) leads to the fusion of the small particles into extended bilayers. Consequently, this lipid phase transition is associated with a reversible transition from discs to large lipid/melittin assemblies [9]. However, the bilayer lysis induced by melittin does not occur spontaneously. When melittin is added to gel state bilayers, the peptide does not penetrate into the bilayers, and no lysis is observed [9,10]. It is only when the sample is brought in fluid phase that melittin penetrates into the bilayers to disrupt the lipid structure. After an initial incubation above T_m, melittin/DPPC complexes show the reversible behavior described above.

Polymorphic behavior of DPPC/melittin complexes can easily be monitored by NMR [11,12], since the different phases are characterized by different correlation times. Small particles, resulting from the lysis, experience fast tumbling leading to completely averaged interactions between the nucleus and the magnetic field; an isotropic signal is observed in that case. On the other hand, motions in extended bilayers such as multilamellar vesicles have longer correlation times than the NMR time-scale. In this case, the interactions between the nucleus and the magnetic field, which depend on the orientation of the bilayer with respect to the magnetic field, are only partially averaged, giving rise to typical powder patterns.

In this paper, the effect of cholesterol on lipid/melittin polymorphism was characterized using solid-state NMR spectroscopy. Cholesterol changes the properties of DPPC bilayers; how this change is reflected in the interaction between lipids and melittin was examined. In addition, the possibility of rationalizing the lysis induced by melittin was investigated in terms of the conformational freedom of the phospholipid acyl chains and the cohesive strength of the membrane, both modulated by cholesterol.

Material and Methods

DPPC and 1,2-bis(perdeuteriopalmitoyl)-*sn*-glycero-3-phosphocholine (DPPC-d_{62}) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Deuterium-depleted water and cholesterol were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Melittin was purified from bee venom (Sigma) by ion-exchange chromatography on SP-Sephadex C-25, and desalted according to the high-performance liquid chromatography (HPLC) procedure described in Lafleur et al. [13].

Samples were prepared by hydrating 30 mg lipid with 1 ml of 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 100 mM sodium chloride at pH 7.4. The sample was then heated and vortexed. Cholesterol/DPPC samples were prepared by mixing appropriate volumes of cholesterol and DPPC stock solutions in benzene. These solutions were lyophilized, then hydrated as described above. For the complexes, an aliquot of melittin solution in Hepes buffer was added to hydrated bilayers to obtain

the desired lipid-to-protein molar ratio. The mixture was then vortexed and was heated and cooled several times prior to data acquisition, except for the insertion experiment (Fig. 1), where spectra were acquired immediately after the addition of melittin.

A Bruker WH-400 spectrometer was used for the ^{31}P-NMR experiments, with a single pulse of 20 μs (approx. 35°) and a relaxation delay of 1 s. The sample temperature was regulated using a Bruker variable temperature (VT) controller. The number of scans was 5400. Alternatively, a Bruker AM-300 wide-bore spectrometer was used with a spin echo sequence where the 90° pulse was 5 μs, the pulse delay 60 μs and the relaxation delay 5 s. The number of scans was 1440. This spectrometer was also equipped with a Bruker VT controller.

^{2}H-NMR spectra were acquired on the Bruker AM-300 spectrometer with a home-built probe equipped with a 10-mm solenoid coil. The quadrupolar echo sequence was used with a 90° pulse of 4 μs, a pulse delay of 50 μs and a recycling delay of 0.5 s. After the second pulse, 8192 points were acquired in quadrature with a dwell time of 0.8 μs. The number of scans was typically 12000. Spectral dePakeing was performed as described by Sternin et al. [14].

The chemical analysis of the DPPC/cholesterol/melittin complexes was performed as follows: phosphorus was determined according to the method developed by Fiske and SubbaRow [15]; cholesterol was determined using the assay described by Rudel and Morris [16]; melittin was directly determined at 280 nm (ϵ_{280} = 5600 $M^{-1}cm^{-1}$) [9].

Results

The lysis of DPPC/cholesterol bilayers induced spontaneously by melittin was investigated first. As mentioned earlier, melittin does not induce lysis of gel-ordered phase bilayers if the sample has not previously been heated above its transition temperature [9,10]. Since cholesterol causes changes in molecular order and dynamics of the gel phase membrane, the effect of cholesterol on the spontaneous lysis of DPPC bilayers caused by melittin was examined. The formation of small lipid/peptide complexes with increasing amounts of cholesterol was monitored at different temperatures using ^{31}P-NMR (Fig. 1). In these samples, melittin was added at room temperature to hydrated DPPC/cholesterol mixtures at a phospholipid-to-peptide ratio of 20:1 (R_i = 20), and the spectra were

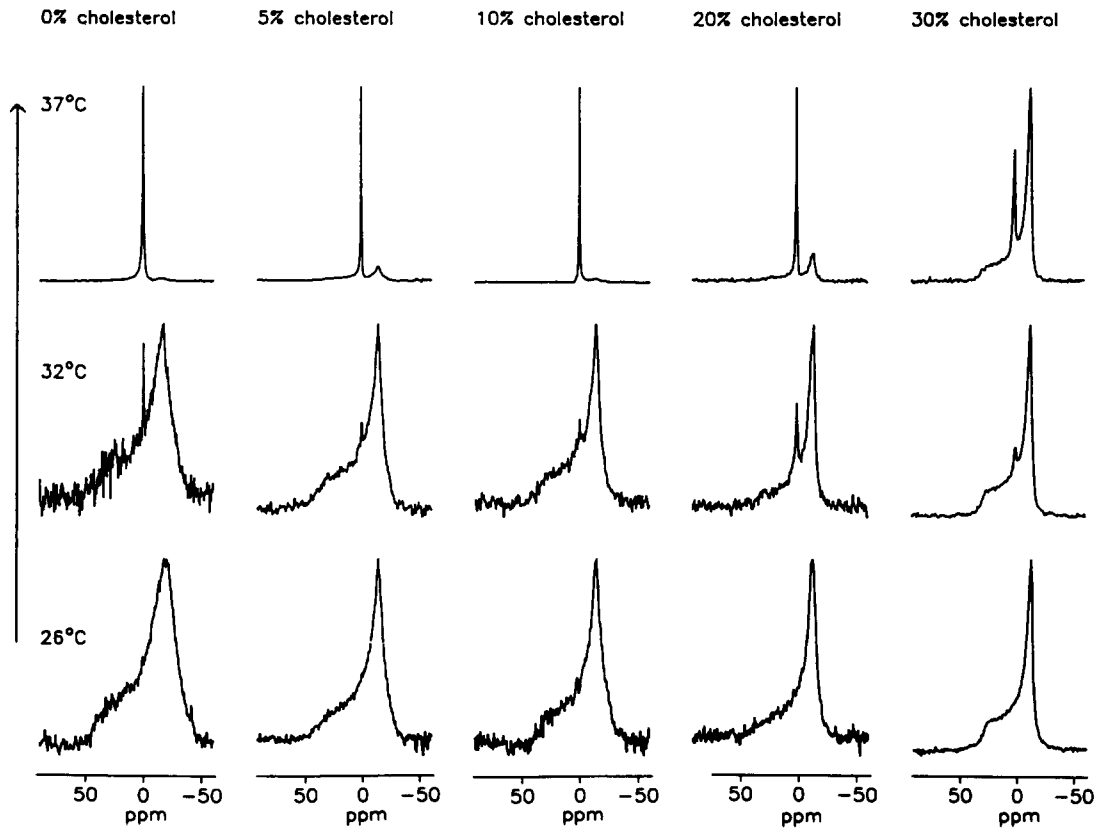

Fig. 1. ^{31}P-NMR spectra of aqueous dispersion of DPPC/cholesterol mixtures in the presence of melittin at R_i = 20. Cholesterol concentration is indicated at the top of each column. The arrow indicates the direction of the temperature variation. All spectra were acquired immediately after the addition of toxin, prior to any incubation at high temperature.

acquired prior to incubation at high temperature. The spectrum of pure DPPC/melittin complex shows the high-field peak and low-field shoulder of a signal resulting from partial averaging of the chemical shift anisotropy by fast axial motion of the lipids in the large structures [17]. This powder pattern indicates that there is no formation of small lipid/protein complexes, in agreement with previous results [11,18]. When the temperature is increased, the powder pattern is progressively transformed into an isotropic signal. This spectroscopic change is associated with the formation of small DPPC/melittin discs whose correlation time is short relative to the NMR time-scale [18]. For samples containing cholesterol, all spectra show a typical powder pattern at 26°C. This indicates that spontaneous formation of DPPC/melittin small complexes is not promoted by the presence of cholesterol. This resistance to lysis is observed even when the cholesterol proportion reaches 30 mol%, in which case the lipids form a liquid-ordered phase [2]. The lysis begins at about 32°C, around the pre-transition of pure DPPC, irrespective of the cholesterol content. Increasing the temperature from 32°C to 37°C leads to a larger proportion of the isotropic signal. This change in the lipid macrostructure is even observed for the liquid-ordered phase. The formation of small particles is however significantly inhibited in this latter case.

Next, the same samples were studied after being subjected to several heating and cooling cycles through the transition temperature, in order to reach a reversible thermal behavior. The polymorphism of these 'equilibrated' DPPC/cholesterol/melittin complexes at $R_i = 20$ was studied by ^{31}P-NMR (Fig. 2). At low temperature (26°C), an isotropic signal associated with small discs is observed, whereas above T_m, large bilayer assemblies are responsible for the observed chemical shift anisotropy (CSA). When the temperature is decreased back to 26°C, the isotropic signal reappears, in agreement with previous results [11,18]. The presence of cholesterol up to 20 mol% does not modify this behavior considerably. These samples show a dominant isotropic signal at 26°C and a signal manifesting CSA at high temperature (58°C). All of them also show a reversible temperature behavior. The spectra show that the increase in cholesterol content leads to the coexistence of small complexes and the large lipidic assemblies over a wider range of temperatures (Fig. 2). For the DPPC mixture containing 30 mol% cholesterol, there is coexistence of isotropic signal and powder spectrum for the whole temperature range studied (Fig. 2) and the ratio of small particles-to-large assemblies does not strongly depend on temperature, when compared with the mixtures containing a smaller proportion of cholesterol. It should be noted that some spectra show partial orientation of the lipid bilayer at 90° with respect to the magnetic field.

Since the phospholipids give rise to two distinct ^{31}P-NMR signals in the DPPC/30 mol% cholesterol mixture containing melittin, the lipid exchange between the two different macrostructures is slow on the NMR time-scale. This mixture was subjected to centrifugation at $2500 \times g$ for 30 min, in order to test the possibility of separating the small particles from the large macrostructures, and to get more information on

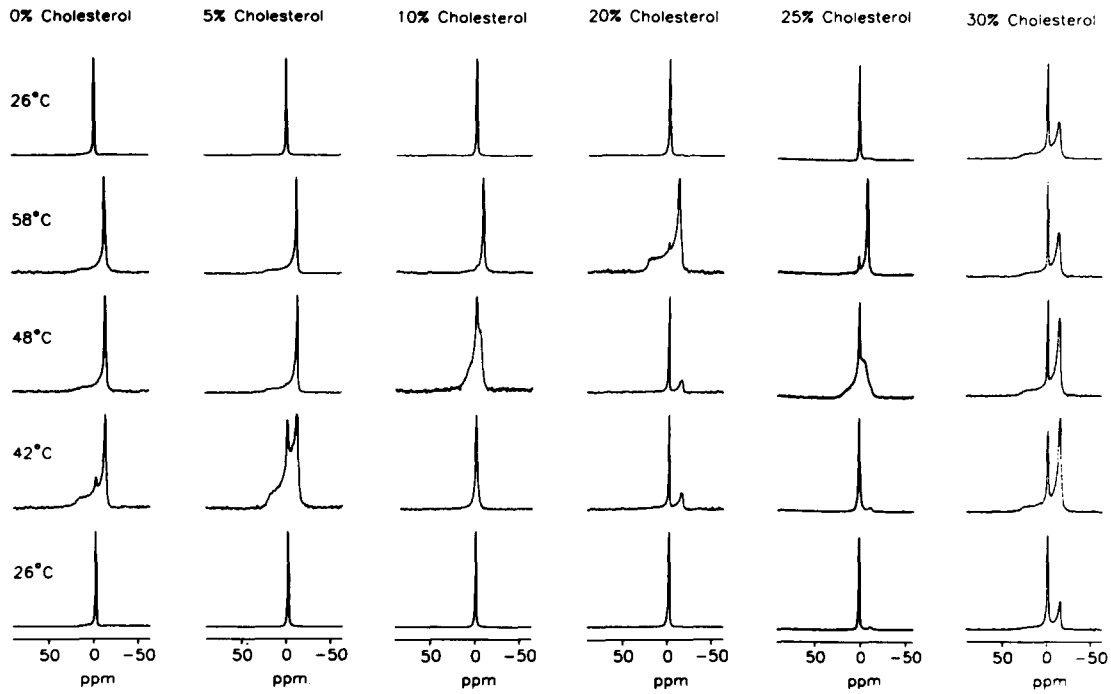

Fig. 2. Same as Fig. 1, except the samples were heated and cooled several times prior to acquisition.

their respective stability and composition. Several DPPC/30 mol% cholesterol/melittin mixtures with concentrations of melittin varying from $R_i = 20$ to $R_i = 5$ were submitted to centrifugation: some were done at 25°C, whereas the temperature was not controlled for others. No important differences were observed between these two sets of results. After centrifugation, two distinct phases were obtained: the lower phase was clear while the upper one was thick and white. After separation of these phases, each fraction was studied by ^{31}P-NMR (Fig. 3). Spectra 3a and 3b were acquired before centrifugation and attest the coexistence of small particles and large vesicles as seen earlier. Spectra 3c to 3e, obtained from the clear fraction, show exclusively an isotropic signal indicative of small complexes at all temperatures. The white dispersion contains exclusively large lipid bilayers as illustrated by the powder pattern (spectra 3f to 3h). No isotropic signal was observed in the latter fraction, even after several heating and cooling cycles. The chemical analysis of each phase was performed on samples of DPPC/30 mol% cholesterol. Almost all of the melittin ($\geq 90\%$) is concentrated in the clear phase. The phospholipid-to-peptide molar ratio was less than 10. It should be stressed that this ratio includes melittin interacting with the lipids, as well as free melittin. This phase also shows a lower cholesterol content, which ranges from 9 to 23 mol%, than the starting mixture (containing 30 mol% cholesterol). Conversely, the white dispersion phase is enriched in cholesterol, ranging from 36 to 39 mol%, in comparison with the starting mixture. It was impossible to assay the peptide in this phase because of the strong light diffusion. However, a rough estimate obtained by subtracting the amount of melittin in the clear phase from the total amount in the sample indicates a phospholipid-to-peptide ratio equal to or greater than 90:1.

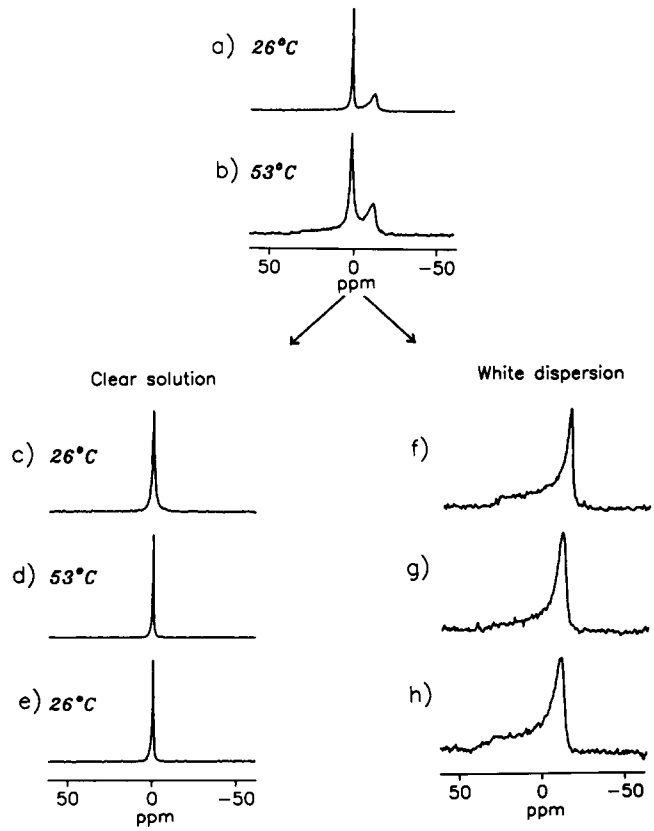

Fig. 3. ^{31}P-NMR spectra of DPPC containing 30 mol% cholesterol and melittin at $R_i = 20$ before centrifugation (a and b), and after centrifugation (c–h). Spectra c–e correspond to the clear solution and spectra f–h correspond to the white dispersion.

The DPPC/30 mol% cholesterol mixture is not completely lysed by melittin at $R_i = 20$. Two reasons can be evoked. First, more melittin might be necessary to form small particles in the presence of cholesterol, and the phospholipid-to-peptide ratio used for the experiment was perhaps not sufficient to cause total lysis. Second, the chemical analysis of the complexes showed an enrichment in cholesterol for the large assemblies; this high cholesterol content might also prevent the lysis of DPPC bilayers by melittin. To determine the origin of this phenomenon, the progressive lysis of DPPC/cholesterol bilayers induced by increasing amount of melittin, at 26°C, was examined (Fig. 4). The estimation of lysed vesicles is obtained from the ratio of the isotropic signal area over the total area of the spectrum (isotropic signal + powder pattern). Even though ^{31}P-NMR spectra were not acquired in ideal conditions for quantitative analysis, the calculated ratios presented here are representative of the sample compositions. When the melittin content varies from $R_i = 80$ to $R_i = 20$, the proportion of isotropic signal increases from 14% to 91% for pure DPPC bilayers. In the case of DPPC/30 mol% cholesterol mixtures, this proportion only varies from 7% to 17% for the same range in melittin concentration. Even at $R_i = 5$, the proportion of isotropic signal represents only 21% (data not shown). Therefore, the increase in the relative area for the isotropic line as a function of the melittin proportion is definitely less important for the cholesterol-rich bilayers than that for the pure DPPC. For a constant peptide concentration ($R_i = 20$), the increase in the cholesterol content also leads to an important reduction of the isotropic signal, this effect being non linear. The presence of 25 mol% cholesterol in DPPC prevents only 28% of the lysis. However, at 35 mol% cholesterol, the residual powder pattern correspond to 93% of the signal. At 40 mol% cholesterol, lysis is completely inhibited. These results indicate a high resistance of cholesterol-rich bilayers to the lytic power of melittin.

In order to get more insights into the conditions required for lysis of the lipid vesicles, the orientational order of the acyl chains was characterized by ^{2}H-NMR for DPPC-d_{62}/cholesterol/melittin systems. Fig. 5a

shows spectra obtained for DPPC-d_{62}/cholesterol mixtures in the same proportion as for Fig. 2, at 28 and 57°C. Below T_m, the spectrum of pure DPPC-d_{62} is characteristic of large vesicles in the ordered gel phase [19]. Above T_m, the spectrum is transformed into one which displays sharply resolved peaks with reduction of the quadrupolar splittings typical of the liquid-disordered phase [19]. A cholesterol content increase from 0 to 30 mol% modifies the powder spectra lineshape. At low temperature, increasing amounts of cholesterol lead gradually to axially symmetric powder spectra with large quadrupolar splittings, indicating a transition from a gel phase to a liquid-ordered phase, for which phospholipids experience rapid axial diffusion with little *trans-gauche* isomerisation of the hydrocarbon chain [2]. At a higher temperature (57°C), as the cholesterol content increases in the lipid bilayers, an increase in the quadrupolar splittings is observed, indicating a transition from a liquid-disordered phase to a phase with greater orientational order. All these results are in good agreement with the previous results of Vist and Davis [2]. Fig. 5b shows the corresponding ^{2}H-NMR spectra for the same mixtures as in Fig. 5a, but in the presence of melittin ($R_i = 25$). All the samples were incubated prior to data acquisition so that they display a reversible thermal behavior. The polymorphism observed by ^{2}H-NMR is in agreement with that determined by ^{31}P-NMR (Fig. 2). For cholesterol contents higher than 15 mol%, the increase in temperature from 28 to 57°C leads to a greater proportion of the powder spectra as that observed in Fig. 2. It should be noted that a smaller proportion of melittin was added to the lipid bilayers for these experiments ($R_i = 25$) than for those in ^{31}P-NMR ($R_i = 20$); this leads to a smaller relative intensity of the isotropic line. The quadrupolar splittings observed on the ^{2}H-NMR spectra is a measurement of the residual strength of the quadrupolar interactions [19]. In the case of the small complexes, the fast tumbling leads to complete averaging, and circumvents the determination of the orientational order of the acyl chain of the lipids forming these small particles. However, for the large lipidic assemblies, the quadrupolar splittings reflect the intramolecular averaging of the orientation-dependent interactions mainly by *trans-gauche* isomerization. The quadrupolar splittings of the lipid macrostructure in the presence of melittin (Fig. 5b) exhibit the same behavior as that of the DPPC/cholesterol mixture (Fig. 5a). The quadrupolar splittings ($\Delta\nu_Q$) were measured for spectra showing axial symmetry at the maximum intensity associated to the first segment of the acyl chains (the plateau region). The ^{2}H-NMR spectra of DPPC/cholesterol mixtures were dePaked in order to obtain $\Delta\nu_Q$ measured for the 90° orientation. For DPPC/choles-

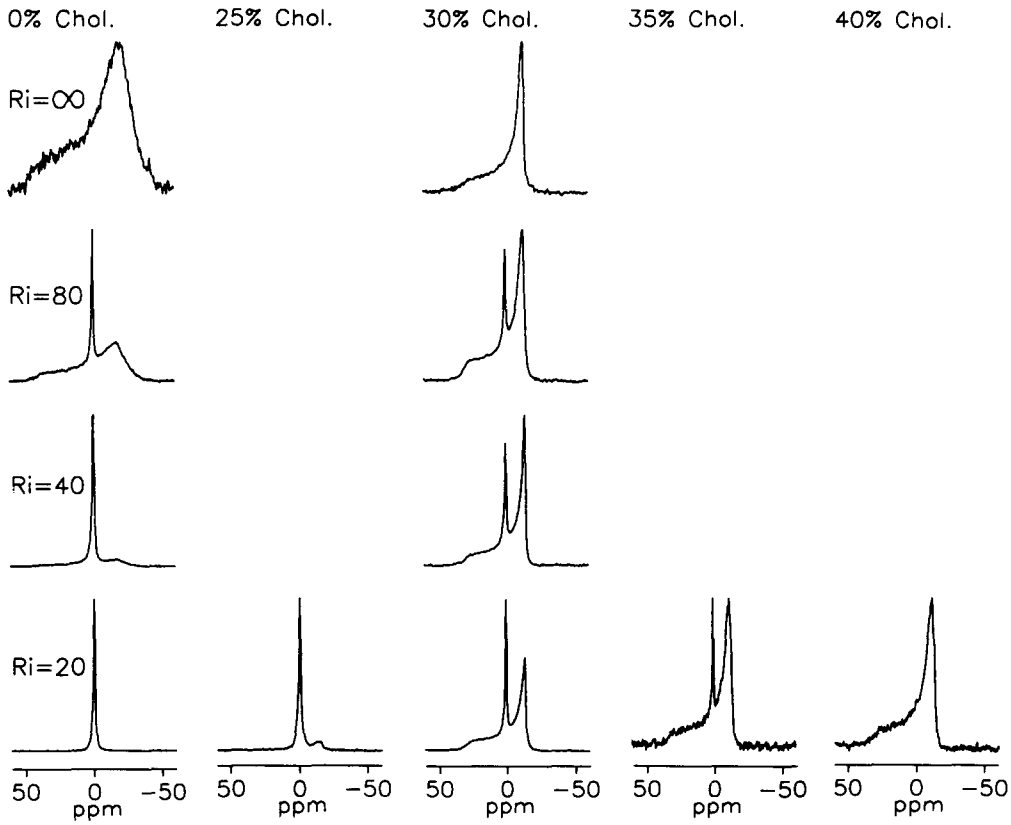

Fig. 4. ^{31}P-NMR spectra obtained at 26°C for several DPPC/cholesterol/melittin mixtures with increasing amount of melittin (from top to bottom), and for increasing amount of cholesterol (from left to right).

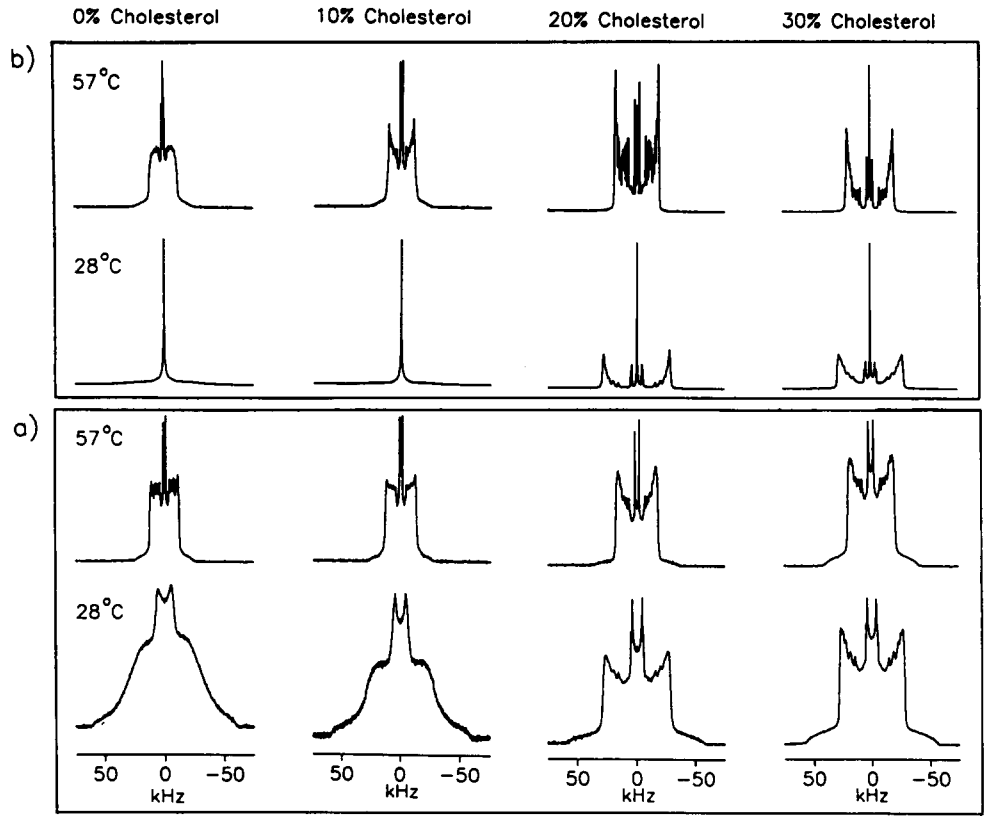

Fig. 5. (a) ^{2}H-NMR spectra of DPPC with various amounts of cholesterol; (b) ^{2}H-NMR spectra of samples in (a) in the presence of melittin, at $R_i = 25$. The cholesterol concentrations are indicated on top of each column and apply to samples with and without melittin. A small isotropic signal is still observed in the DPPC spectrum at 57°C, likely due to residual HOD in the sample.

terol/melittin samples containing more than 10 mol% cholesterol, ^{2}H-NMR spectra were already oriented at 90°, and the quadrupolar splitting was measured directly on the spectra. These measurements show that melittin does not alter the order parameters of the lipid acyl chains in the large bilayer assemblies by more than 5%, except for the sample containing 10 mol% cholesterol, which show a more pronounced effect (a decrease in order parameter of 17% at 57°C). However, this sample give rise to fuzzy lines (with and without melittin), and it is rather difficult to measure precisely the quadrupolar splittings. As already observed by ^{31}P-NMR, melittin can induce an important change in the shape of the large structure spectrum; there is a dramatic decrease in the intensity of the shoulders, suggesting the bilayer orientation in the magnetic field.

Discussion

The effect of cholesterol on the spontaneous incorporation of melittin followed by the disruption of the lipid bilayer will be discussed first. At low temperature ($T = 26$°C), no lysis is observed, even when the cholesterol concentration is as high as 30 mol%. It has been shown that cholesterol increases the phospholipid motion in the gel phase, as illustrated by the ^{2}H-NMR spectral lineshape showing axially symmetric reorientation (present results and Ref. 2) and by the increased lateral diffusion [20]. Despite this increase in mobility, the spontaneous membrane lysis induced by melittin is not observed. It can, therefore, be concluded that increased lateral and axial diffusions induced by the presence of cholesterol do not trigger the lysis of DPPC bilayers by melittin at low temperature. This resistance of the bilayer to the lytic power of melittin can be associated with high orientational order, high elasticity modulus and/or low compressibility, since these properties are common to both gel and liquid-ordered phases. It is also important to note that the presence of the cholesterol hydroxyl group located at the interface does not disturb the packing of lipids sufficiently to create defects which have been associated with the deep insertion of melittin in the bilayer leading to the lysis [21]. The spontaneous lysis has been previously associated with the gel-to-liquid crystalline phase transition [21] where the coexistence of both phases leads to several surface defects. However, the results presented here clearly demonstrate that the lysis occurs around the pre-transition ($T = 32$°C) where the thermal destabilization of the bilayer leads to the formation of ripples at the surface. These irregularities in the phospholipid packing are possibly responsible for the penetration of melittin into the bilayer and the subsequent formation of small particles.

Despite the abolition of the pre-transition induced by the presence of cholesterol [2], the spontaneous lysis is still observed around 32°C, regardless of the cholesterol content, indicating that a structural change must happen around 32°C. Recent results have shown that temperature can induce changes in the structural properties of the liquid ordered phase [22]. A change in the cholesterol position in the lipid bilayer and a disordering of the terminal segment of the lipid acyl chains are proposed in the temperature region between 30–45°C. The displacement of cholesterol toward the bilayer surface could possibly act as the trigger for the melittin-induced lysis. Therefore, the present results suggest that, even with a structure that permits rotational and lateral diffusion of the lipids as important as in the liquid crystalline phase, the liquid-ordered phase shows a resistance to melittin-induced lysis. If this lysis can be associated with a deep penetration of the toxin into the bilayer, then these results suggest that peptide or protein insertion can be controlled by the deformation of the interface.

This study reveals that cholesterol modulates the polymorphism of DPPC/melittin complexes as a function of temperature, and these changes can be directly correlated to the bilayer properties. It is well-established that cholesterol induces a progressive broadening of the gel-to-liquid crystalline phase transition, leading to the abolition of the transition when the cholesterol content reaches 25 mol% [2,23]. This is explained by the formation of a unique phase which exists over the whole temperature range, the liquid-ordered phase [2,4]. In parallel with the broadening of the DPPC transition induced by cholesterol, the ^{31}P-NMR spectra show that the small particles and large bilayer assemblies also coexist over a wider range of temperatures (42 to 48°C) as the cholesterol concentration varies from 5 to 20 mol%. In addition, when the mixture contains enough cholesterol so that the lipids remain in the liquid-ordered phase (30 mol% cholesterol), the cooperative small particles/large assemblies transition vanishes. Consequently, the effect of cholesterol on the polymorphism of DPPC/melittin macroassemblies originates essentially from its effect on the thermotropism of the lipid bilayers.

The interaction between melittin and DPPC bilayers containing 30 mol% cholesterol gives rise to two distinct phases that can be physically isolated and analyzed. The chemical analysis of each phase reveals an uneven distribution of cholesterol between them. The small particles resulting from the lysis are cholesterol depleted leading to a cholesterol enrichment of the remaining large bilayers. This result shows that melittin extracts selectively the lipids from the bilayer. This phenomenon can be rationalized on the basis of the small particle stability. It has been found that DPPC/melittin complexes at $R_i \geqq 5$ are not stable at temperatures above T_m [9,11,18]. At these temperatures, lipids form a fluid disordered phase where there is a large increase in lipid dynamics and decrease in the bending elastic modulus. The restricted area of the particles (diameter of 235 Å) may not be able to accommodate such fluid bilayers. Cholesterol leads to the fluidification of the gel phase lipids and, from this point of view, could limit the formation of small particles. Consequently, at equilibrium, small DPPC/cholesterol/melittin complexes with reduced cholesterol content would be favored. Alternatively, melittin insertion could be easier in regions where the cholesterol content is lower, leading also to selective lysis. A more detailed investigation is necessary to determine the origin of this selectivity.

The thermal stability of the small complexes containing about 15 mol% cholesterol (Fig. 3) can appear to be in disagreement with the behavior of mixtures containing a similar amount of cholesterol and showing a small particles/large assemblies phase transition, as for pure DPPC (Fig. 2). The chemical analysis indicates a very important increase of the DPPC-to-melittin ratio in the clear phase. This melittin concentration is likely high enough ($R_i \leqq 10$) to stabilize the small particles over the whole range of temperature. The formation of stable small particles has been observed for DPPC/melittin complexes [9] and for DPPC/20 mol% cholesterol mixture (data not shown) at $R_i = 5$.

It was impossible to achieve total lysis of DPPC/30 mol% cholesterol bilayers in spite of the large amount of melittin added to the sample. This can be explained by the increased cholesterol content resulting from the specificity in the lysis of the bilayer by melittin; this cholesterol-enriched bilayer is likely more resistant to the lysis as shown by the behavior of the DPPC/40 mol% cholesterol mixture. The lysis and the formation of small particles require conditions permitting the deep insertion of melittin into the bilayer. This is illustrated by the resistance of gel phase bilayers (which give rise to the formation of small particles upon incubation above the pre-transition temperature) to the lytic effect of melittin. It is possible that bilayers containing high concentrations of cholesterol have similar properties and become lysis-resistant. To address this possibility, a different procedure for sample preparation was used in order to bypass the peptide penetration into the preformed bilayers. An organic solution containing DPPC and cholesterol was divided into two aliquots. The first aliquot of the lipid mixture was co-lyophilized with melittin and then hydrated. The other one was treated as usual and used as a control. Fig. 6 shows the ^{31}P-NMR spectra of DPPC/40 mol% cholesterol/melittin sample ($R_i = 20$) obtained using this colyophilization procedure (spectrum b) and from the control sample (i.e., melittin in aqueous solution was added to the multilamellar dispersion) (Fig. 6,

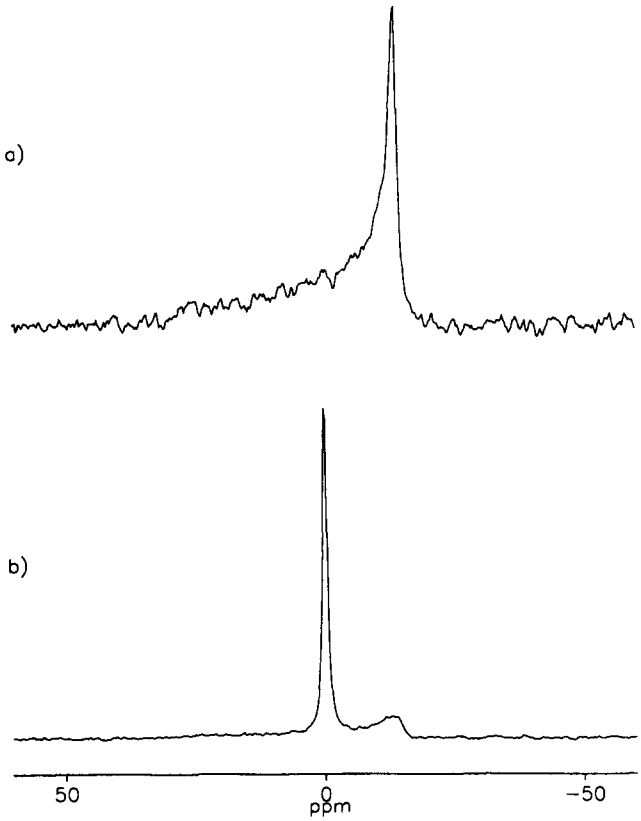

Fig. 6. ^{31}P-NMR spectra at 26°C of (a) DPPC/40 mol% cholesterol in the presence of melittin at $R_i = 20$. Melittin was added to prehydrated multilamellar dispersion. (b) Same composition as (a), except the lipids and melittin were co-lyophilized from an organic solution, then hydrated.

spectrum a). The spectra were acquired at room temperature after several heating and cooling cycles. It is clear from the large proportion of isotropic line in the spectrum obtained from the co-lyophilized sample that the peptide penetration is facilitated by the co-lyophilization, and the formation of small particles is then possible. In order to show that the co-lyophilization should not alter significantly the sample, the same experiment was performed on a DPPC/5 mol% cholesterol sample. After several heating and cooling cycles, the samples prepared by both methods give rise to an isotropic signal at 26°C, which is progressively transformed into a powder spectrum upon increasing temperature (data not shown), indicating that, in this case, the thermal behavior is independent of sample preparation. From these results, it can be stated that the tight lipid packing obtained with a high cholesterol content prevents the penetration of melittin in the bilayer, and therefore inhibits subsequent lysis.

The presence of melittin in the lipid bilayer does not affect dramatically the orientational order of the lipid acyl chains in DPPC/cholesterol mixtures, as shown by the ^{2}H-NMR spectra. At 57°C, a reduction in the quadrupolar splittings of about 3% is observed when going from DPPC bilayers to DPPC/melittin complexes. This is in qualitative agreement with previous results showing that melittin has a disordering effect on DPPC acyl chains [11]. The complexes containing at least 30 mol% cholesterol do not display a change in orientational order (less than 1%). Two factors may influence the order parameters: first, melittin can reduce slightly the order as in DPPC bilayers; second, the increase in cholesterol content in the residual bilayer as determined by the chemical assay should lead to an increase in the order parameters. The sum of these two opposing effects could account for the equivalence of lipid chain order observed between the DPPC/cholesterol bilayers with and without melittin.

Above T_m, several ^{31}P and ^{2}H-NMR spectra of DPPC/cholesterol mixtures in the presence of melittin at $R_i = 25$ do not show a typical powder pattern. They clearly indicate that the lipidic assemblies are oriented in the magnetic field, since the 90° orientation of the lipid director axis with respect to the magnetic field is overrepresented in the spectra. This phenomenon has already been observed with DMPC/melittin complexes [24]. The origin of this orientation is due to the diamagnetic susceptibility ($\Delta\chi$) of the complexes. Lipids themselves show a negative value of $\Delta\chi$ (based on the measurement on a single crystal of DPPC by Sakurai et al. [25]) and, when organized in a parallel manner, the resulting anisotropy can be important enough to orient the lipid bilayers in a magnetic field. This has been shown for DPPC with 30 mol% cholesterol [26] or with 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysoPPC) [27]. There is also the possibility of a contribution from the peptide, since it has been shown that α-helices can orient parallel to the magnetic field direction [28]. In the complexes with melittin, it is impossible to determine whether the peptide contribution is added to or overrun by that of the lipids, since the relative orientation of the melittin in the small complexes has not been determined yet. The orientation observed for DPPC/cholesterol/melittin complexes is very important, since the shoulders of the powder pattern have almost completely disappeared. It is definitively more drastic than the partial orientation observed for multilamellar vesicles of DPPC/30 mol% cholesterol in a magnetic field of 9.4 T, resulting from the vesicle deformation into an ellipsoidal shape [26]. Vesicle deformation is unlikely to be responsible for the severe orientation observed in the complexes with melittin, since it would imply regions with very high curvature joining the oriented areas. In the case of DMPC/melittin complexes, the orientation has been explained by the lateral fusion of the small discoidal particles above T_m, leading to large and flat bilayers which do not form closed vesicles [24]. This phenomenon can also be extended to the DPPC/cholesterol system. Alternatively, small particle stacking could also lead to oriented spectra. It should be

noted that oriented samples were not always obtained. Despite the fact that, at this point, the critical parameters leading to the orientation could not be identified clearly, it seems that the thermal history of the sample is crucial.

To conclude, some biological implications of the findings presented in this paper should be discussed. First, it is important to stress that despite its liquid-like dynamics, the liquid-ordered phase, which is likely found in biological membranes, is a structure that offers a certain resistance to the disruptive effect of amphiphilic peptide or protein. This is shown by the resistance of the liquid ordered phase to the lysis induced by melittin prior to incubation. This point is particularly important for the investigation of the signal peptide insertion leading to the incorporation of proteins into membranes. Second, the spontaneous lysis induced by melittin in DPPC bilayers with high cholesterol contents indicates that there are structural changes occurring in the liquid-ordered phase which are triggered by temperature. Similar changes based on the detailed investigation of DPPC/cholesterol system have been proposed [22]. As mentioned in that paper, the structural changes occur in a physiologically-relevant temperature domain, and the possible roles of such changes in biological membranes should be investigated. Indeed, the results presented here also show that the presence of cholesterol in the lipid bilayer can prevent the lysis induced by melittin; it is possible that the physiological concentration of cholesterol in the plasmic membrane plays a similar role with respect to analog amphiphilic peptides or protein. Finally, it was shown herein that the lytic properties of melittin are modulated by cholesterol via its effect on the lipid bilayer polymorphism. The modulation of protein activity by cholesterol is important since cholesterol is found in plasmic membranes. The effect of the sterol on the activity of some proteins is possibly due to direct interactions as proposed previously [6,7]. However, it is known that cholesterol modulates several physical properties of the bilayer including its thickness, elasticity modulus and lipid chain order, and protein activity may be sensitive to these properties. So, cholesterol can modulate the characteristics of the bilayers, and, *as a consequence*, the activity of certain peptides or proteins. The study presented here on the lytic power of melittin is a straightforward illustration of such an indirect effect.

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