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# Fluid or gel phase lipid bilayers to study peptide-membrane interactions?

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**Abstract** Since many studies on peptide-membrane interactions are carried out only with fluid phase lipid bilayers ($L_\alpha$-phase, absence of cholesterol) we have investigated whether this phase is really a suitable model for biological membranes. For this purpose the action of melittin on zwitterionic and negatively charged phospholipid bilayers, in the absence and presence of 30 mol% cholesterol, was investigated by solid state $^{31}$P-NMR. From the NMR point of view, it appears that systems composed of a single phospholipid best mimic the sterol-containing system a few degrees below the gel-to-fluid phase transition, i. e., in the rippled phase ($P_{\beta'}$). It is then proposed that a relatively rigid membrane containing local defects, rather than a $L_\alpha$-bilayer, is required as an appropriate model for natural membranes when probing the action of melittin. Such requirements might be crucial when studying peptide-lipid interactions.

**Key words** Melittin · Phosphatidylcholine · Phosphatidic acid · Cholesterol · Membrane defects · $^{31}$P-NMR

## Introduction

In recent years the study of peptide-membrane interactions has become an exciting research field. Although peptides are used as models for the more complex protein-membrane interactions, they also have very interesting properties by themselves. This is especially true for biological or synthetic antimicrobial and hemolytic peptides, as well as signal peptides, which act via the cell membrane and where lipids are involved in their action (Tamm 1991; Cornut et al. 1993; Saberwal and Nagaraj 1994; Maloy and Kari 1995). Of course, the most convenient way to investigate such biologically active peptides would be to study their interaction with natural membranes. Unfortunately the complexity of such systems usually precludes the use of biophysical techniques such as NMR and there is therefore no detailed understanding of their mechanism of action. Simplifications which result from working with model systems are thus necessary. Such an approach often requires the use of lipid bilayers composed of only one, or maybe two, lipids to model the lipid matrix of a real membrane. Such a simplification presents several drawbacks among which is the thermal sensitivity of such lipid bilayers. This is particularly important when working in the absence of physiological cholesterol concentrations (around 30 mol%), that is to say when the system can appear as lamellar gel ($L_{\beta'}$) or fluid ($L_\alpha$) phases depending on temperature. Such a situation is not found in eucaryotic plasma membranes (Nes and McKean 1977), which are less sensitive to thermal variations due, at least in part, to their elevated cholesterol concentrations. Obviously it is advantageous to incorporate physiological amounts of sterol in model membranes rather than dealing with pure phospholipids. When working in the absence of cholesterol, the question arises of whether one should work preferentially with gel or fluid phase bilayers. On the other hand, most natural phospholipids (an exception being sphingomyelin) only provide $L_\alpha$-bilayers at room temperature due to their very low transition temperature and it is often considered that fluid state membranes best mimic the properties of a biological membrane. In part this is due to the characteristic rapid translational movement of lipids in the fluid state that approaches much more the *fluid mosaic* of real membranes than the rigid organization of lipids in the gel state. Consequently numerous studies dealing with peptide-membrane interactions have been carried out only on fluid phase bilayers (see for example Saberwal and Nagaraj (1994) and referencs therein). Nevertheless it appears questionable whether an argument based on diffusion constants, which may well have its justification in the case of integral proteins, is also valid for peptides. The action of these peptides is usually linked to their association with, or insertion into, the membrane, i. e., a transient event that

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may be determined by effects other than lipid lateral diffusion.

We have therefore investigated the effects of melittin, a 26 amino acid amphipathic peptide, on dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidic acid (DMPA) (pH 4.2) model membranes (as being representative of zwitterionic and charged bilayers) in the absence and presence of 30 mol% cholesterol. As the specific hydrophobic interaction between cholesterol and phospholipids is of major importance for the proper function of natural plasma mebranes, we believe that the sterol-containing systems may give some indications about the interactions between peptides and biological membranes. We will thus test which phase (fluid, rippled or gel) best mimics the peptide action as compared to a cholesterol-containing bilayer. These findings will be discussed with respect to real membranes with the aim of better understanding the problem described above.

Melittin seems to be well suited for this purpose since it shows dramatic effects on real membranes (e. g. hemolysis of erythrocytes at concentrations $\leq 10^{-7}$ M). Furthermore it is one of the most extensively studied peptides with respect to peptide-membrane interactions (for a review see Dempsey 1990). Because of the remarkable ability of this peptide to induce morphological changes in lipid membranes, $^{31}$P solid state NMR was considered to be the method of choice because it has been proven to be a powerful tool to monitor lipid polymorphic behavior (Seelig and Seelig 1980). It has to be mentioned that the $^{31}$P-NMR data presented herein is solely used to probe macroscopic phase changes and that modifications of molecular order and dynamics within the bilayer core cannot be deduced from such measurements.

## Materials and methods

DPPC, cholesterol and DMPA were obtained from Sigma (France). Melittin was purchased from Serva (France). Preparation of multilamellar vesicles was performed as described elsewhere (Pott and Dufourc, 1995). In the case of DPPC a Tris buffer system at pH 7.5 was used. The negatively charged DMPA system was prepared in an acetate buffer at pH 4.2. All systems contained 100 mM NaCl and 2 mM EDTA. Thin layer chromatography showed the absence of sample degradation after completion of NMR experiments.

NMR measurements were performed either on a Bruker WH 270 spectrometer operating at 109.35 MHz or on a Bruker ARX 300 spectrometer operating at 121.49 MHz. $^{31}$P-spectra were obtained with a phase-cycled Hahn echo pulse sequence and gated proton decoupling as previously described (Pott and Dufourc, 1995). Samples were allowed to equilibrate for at least 30 min at a given temperature prior to acquisition. The temperature was regulated to ±1 °C. Experiments were carried out by decreasing the temperature, unless otherwise mentioned.

Spectral dePaking was performed as reported elsewhere (Bloom et al. 1981; Sternin et al. 1983). The deconvoluted spectra were calculated for a bilayer oriented at 90° with respect to the magnetic field direction. Percentages of spectral compounds were determined by simulation of the different subspectra and subsequent subtraction according to Pott and Dufourc (1995).

## Results

Figure 1 A and B shows selected $^{31}$P-NMR spectra of DPPC in the absence and presence of 30 mol% cholesterol as a function of temperature. At 20 °C the pure DPPC spectrum consists of a characteristic gel phase powder pattern with a large chemical shift anisotropy (CSA) and a broad axially symmetric lineshape. With increasing temperature a distinct spectral narrowing is observed. This illustrates nicely the temperature dependence of the sterol-free system, i. e., the onset of additional modes of motion of the phospholipid when passing from the gel, 20 °C, into the rippled phase, 35 °C, and thereafter into the fluid phase, 60 °C ($L_{\beta'} \rightarrow P_{\beta'} \rightarrow L_{\alpha}$). In the presence of cholesterol the spectra stay almost invariant with temperature (Fig. 1 B). Under these conditions it is known that this bilayer shows only very weak temperature dependence and that the DPPC main phase transition is abolished (Vist and Davis, 1990; McMullen and McElhaney, 1995).

The effects melittin (peptide-to-lipid molar ratio, $R_i$, of 20) on these systems are reported in Fig. 1 C and D. At this point one has to mention that the DPPC/melittin gel phase spectra, in contrast to the spectra published in Pott and Dufourc (1995), are acquired by increasing the temperature and that no incubation at high temperature was performed prior to measurements. In the absence of cholesterol and in the gel phase at 20 °C the toxin only slightly affects the spectral shape. When entering the rippled phase (35 °C) one detects the appearance of an isotropic line superposed on the powder pattern. The observed isotropic line stays stable for at least several hours and the formation of an isotropic phase at 35 °C was reproducible with freshly prepared samples. In contrast to the gel phase experiments, the spectra of the DPPC/melittin system in the fluid phase were obtained after incubation at high temperature outside the magnetic field. This was done because of the sensitivity of this system to effects triggered by the external magnetic field when passing from the gel into the fluid phase (Dempsey and Sternberg, 1991; Pott and Dufourc, 1995). With this precaution, the presence of the toxin results in a complete disappearance of the fluid state powder pattern and the appearance of a broad and slightly asymmetric isotropic line.

The DPPC/cholesterol/melittin system (Fig. 1 D) behaves quite differently. It is characterized by the superposition of a narrow isotropic line and a powder pattern with varying intensity depending on the temperature. The linewidth of this isotropic line is comparable to that obtained for the sterol-free system in the rippled phase.

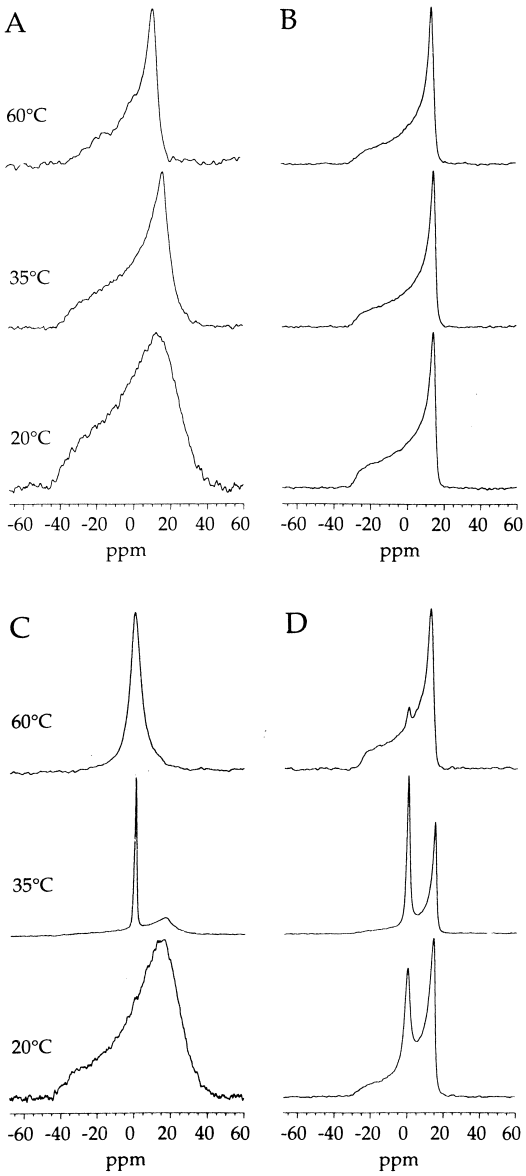


**Fig. 1** Selected solid state $^{31}$P-NMR spectra of DPPC in the absence (**A** and **C**) and presence (**B** and **D**) of 30 mol% cholesterol. Spectra are presented at temperatures that correspond to the three phases of pure DPPC dispersions: 20 °C, $L_{\beta'}$; 35 °C, $P_{\beta'}$; 60 °C, $L_{\alpha}$. **A** and **B**: Spectra in the absence of melittin. **C** and **D**: Spectra of the melittin-containing systems ($R_i = 20$)

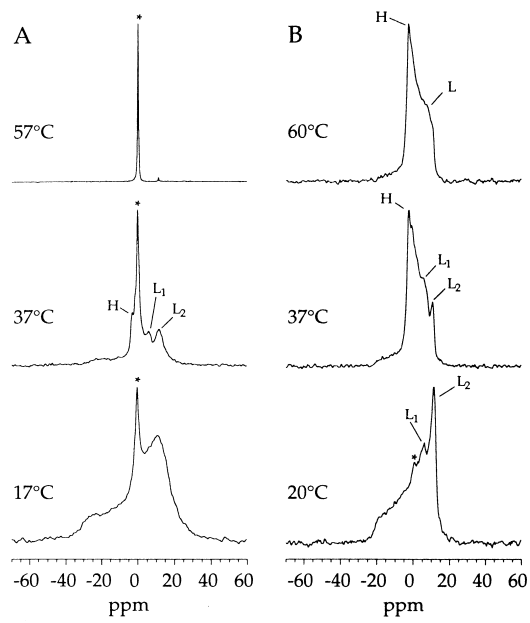


**Fig. 2** Influence of melittin on the solid state $^{31}$P-NMR spectra of the negatively charged DMPA system (pH = 4.2) in the absence and presence of 30 mol% cholesterol, at selected temperatures. Isotropic spectral compounds are labeled with an asterisk, whereas the hexagonal and lamellar phases are labeled by *H* and *L*, respectively. *Left side:* Absence of cholesterol, $R_i = 15$. *Right side:* Presence of cholsterol, $R_i = 10$

The effects of melittin on the negatively charged DMPA systems (pH = 4.2) in the absence of 30 mol% cholesterol are shown in Fig. 2. For the pure DMPA system at high temperature the peptide induces the formation of a phase which shows fast isotropic reorientation (Fig. 2 A, asterisk). For temperatures below the phase transition temperature, $T_C \approx 42$ °C, the presence of this phase is still noticeable, but its amount is drastically reduced (37 °C≈25%; 17 °C≈10%). At 37 °C one remarks further the presence of three powder patterns, one of them (Fig. 2 A, H) with a negative CSA making up as much as ≈19% of the total spectral area. At 17 °C only a superposition of the gel pattern and the isotropic line is obtained.

The cholesterol-containing DMPA system exhibits, at high temperatures (60 °C), a spectrum that consists of powder patterns with positive as well as negative values of the chemical shift anisotropy, $\Delta\sigma$ (Fig. 2 B, L and H, respectively). At intermediate temperature two subspectra with positive $\Delta\sigma$ are observed. The subspectrum with a negative $\Delta\sigma$ is still detected and does not disappear until the low temperature region is reached (20 °C). In this temperature region only powder patterns with positive values of the CSA and a very small amount of isotropic line (≤2%) are observed (Fig. 2 B).

## Discussion

Both DPPC-based systems are already well characterized. In the $L_{\alpha}$-phase of pure DPPC multilayers melittin induces the formation of large unilamellar vesicles, LUV, (diameter ≈4000Å (Dufourc et al. 1986)). Indeed, the observed broad isotropic line in the $^{31}$P-NMR spectrum (Fig. 1 B) corresponds to such objects (Burnell et al. 1980). In the gel phase the peptide transforms the membrane into small discs (diameter 200–400 Å, thickness of one bilayer (Dufourc et al. 1986)), corresponding to a narrow isotropic line in the $^{31}$P-spectra. These gel-phase discs are metastable on the time scale of several days and are usually not observed

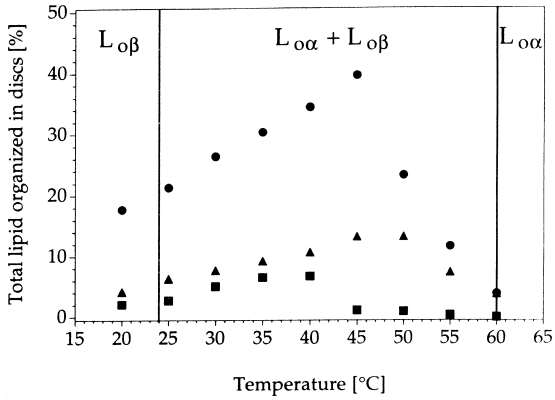


**Fig. 3** Percentages of total lipid (phospholipid and cholesterol) in the form of discs as a function of temperature for the DPPC/30 mol% cholesterol system for three melittin concentrations; *squares:* $R_i = 100$; *triangles:* $R_i = 50$; *circles:* $R_i = 20$. The different phases of the system in the absence of the peptide are indicated according to McMullen and McElhaney (1995)

when incubation with melittin in the fluid phase is forgone (see Fig. 1 C, 20 °C) (Faucon et al. 1995). However, it has been pointed out recently that entering the $P_{\beta'}$-phase is sufficient for disc formation (Monette et al. 1993; Pott and Dufourc 1995), which is demonstrated herein by the presence of a narrow isotropic line in the spectrum at 35 °C. In the presence of cholesterol the toxin-triggered LUV formation is totally inhibited and the coexistence of discs and large vesicles is observed (Pott and Dufourc 1995). Figure 3 shows the total amount of lipid organized in the discs as a function of temperature for different peptide concentrations ($R_i = 100$, 50 and 20). Phase boundaries are indicated according to the newly published DPPC/cholesterol phase diagram (McMullen and McElhaney 1995). It is clearly seen that disc formation is maximal at the center domain where the $L_{o\alpha}$- and $L_{o\beta}$-phases coexist, i. e., where maximal cluster formation and thus defects occur. This defect-theory applies also for the sterol-free system where the $P_{\beta'}$-phase is known to bear the most defects (Alecio et al. 1985). Another presupposition for disc formation is a certain rigidity of the bilayer (Pott and Dufourc 1995). In the case of the cholesterol-containing membrane this is reflected by the occurrence of discs in the $L_{o\beta}$-phase (gel-like), and their progressive disappearance when the $L_{o\alpha}$-phase (fluid-like) become predominant (see Fig. 1 D and Fig. 3).

In the $L_\alpha$-phase of the sterol-free DMPA-system (Fig. 2 A, 57 °C), the melittin induced $^{31}$P-NMR isotropic line corresponds to inverted micelles (Pott et al., submitted). A decrease in temperature leads to a substantial decrease of inverted micelles and an increase in the newly formed hexagonal phase (powder pattern with negative CSA of about minus one half of that of the lamellar phase; Fig. 2 A, H). This transition takes place in the proximity of $T_C$ and is apparently associated with the main transition and the accompanying increase in rigidity. In the presence of cholesterol the micellar phase is not present; instead, the peptide-triggered formation of an inverted hexagonal phase can be seen at high and intermediate temperatures. For both systems the inverted hexagonal phase is in coexistence with a lamellar phase. A further decrease in temperature leads to the disappearance of the hexagonal phase for both systems (Fig. 2, bottom). In the sterol-free system a certain amount of a phase undergoing isotropic tumbling persists.

From a macroscopic point of view it is obvious that the sterol-free membrane/peptide system is only comparable to the cholesterol-containing ones for temperatures $\leq T_C$. It thus appears that an increase in rigidity occurring in the vicinity of $T_C$ is required to pass from inverted micelles to an inverted hexagonal phase, hence approaching the behaviour of the cholesterol-containing system. A further contribution to such a behavior may be found in the assumption that in the proximity of the phase transition temperature defect formation occurs, in spite of the absence of a $P_{\beta'}$-phase for DMPA bilayers at acidic pH. In this context one may also suppose that the DMPA/cholesterol membrane exhibits inhomogenities similar to those found in DPPC/cholesterol bilayers.

Although melittin has pronounced effects on $L_\alpha$-phase bilayers (DPPC: LUV-formation; DMPA: inverted micelles), we have unambiguously shown that these peptide-induced morphological changes are totally different when compared to 30 mol% cholesterol-containing model membranes. In the presence of the sterol the melittin-induced phases (DPPC: disc-formation; DMPA: inverted hexagonal phase) are stabilized over a wide temperature range. The same phases in the cholesterol-free systems are only observed in a quite narrow temperature range and their formation requires that the lipids are in a gel-like organization exhibiting local defects. As already mentioned, herein we report only the melittin-induced macroscopic phase changes of the different systems, but it would be interesting to extend this study to other melittin-triggered phenomena, such as the lysis of vesicles. With reference to the latter, it has been reported that almost complete lysis of fluid phase DPPC vesicles is obtained for $R_i \approx 1000$ (Otoda et al. 1992), whereas $R_i \approx 18$ has to be reached to obtain a similar efflux when gel-phase DPPC vesicles are investigated (Yianni et al., 1986). Unfortunately, there are no data concerning DPPC/30 mol% cholesterol vesicles, but Portlock and coworkers mentioned cholesterol as a compound that confers resistance to vesicles towards the lytic action of melittin (Portlock et al. 1990). It may thus be possible that phenomena other than peptide-induced macroscopic phase changes are affected in a similar way by membrane rigidity and membrane defects. This would be of particular importance with regard to biological membranes where the coexistence of domains is known to occur (Thompson et al. 1992; Tocanne 1992) and where the presence of cholesterol regulates membrane fluidity.

It can be assumed that such defect lines, as well as a certain degree of rigidity, change the insertion/association of a peptide with a membrane, as has been pointed out for the incorporation of glycophorin (Rüppel et al. 1982). Consequently the *biological* action of a peptide may be deter-

mined by these factors. It is clear that a homogeneous $L_\alpha$-model membrane offers none of these. Hence, when trying to match biological conditions with systems of minimalistic simplicity one has to take this into consideration.

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