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Membrane structure and dynamics by ^2H - and ^{31}P -NMR. Effects of amphipatic peptidic toxins on phospholipid and biological membranes

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Summary — The actions of bee venom melittin and delta-lysin from *Staphylococcus aureus* on membranes have been monitored by solid-state deuterium and phosphorus NMR and shown to differ depending on temperature and on the lipid-to-peptide molar ratio R_i .

In the gel phase of phosphatidylcholine model membranes, for lipid-to-peptide ratios $R_i > 15$, melittin induces isotropic lines interpreted as reflecting the presence of small discoidal structures, whereas delta-lysin does not. These small objects are metastable, that is, within a time-scale of hours they return to large lipid bilayers. The kinetics of this process depend on the lecithin chain length.

In the fluid phases, at temperatures greater than that of the gel-to-fluid transition T_c , analysis of the quadruplar splittings in terms of chain ordering indicates that both melittin and delta-lysin similarly disorder the membrane. At temperatures above but close to T_c , melittin preferentially orders the center of the bilayer, while delta-lysin promotes ordering throughout the entire bilayer thickness. These effects are interpreted as reflecting different locations of the peptides with respect to the membrane surface.

The addition of greater amounts of toxins, $R_i = 4$, on phosphatidylcholine model membranes induces very small structures irrespective of the temperature in the case of melittin, but only above T_c for delta-lysin.

NMR spectral features similar to those characterizing the small fast-tumbling objects with phosphatidylcholine are also observed with egg phosphatidylethanolamine and erythrocyte membranes. The formation of small structures is thus inferred as a general process which reflects membrane supramolecular reorganization. This is proposed as a possible step for direct membrane lysis, on both phospholipid bilayers and biological membranes.

^2H -, ^{31}P -NMR / model membranes / melittin / delta hemolysin / erythrocyte ghosts

Introduction

Melittin and delta-lysin are two peptidic toxins of 26 amino-acids, derived from bee venom and secreted by *Staphylococcus aureus*, respectively. Although originating from different species, these amphipatic peptides promote very similar effects on biological membranes, *i.e.* they lyse a wide variety of cells or subcellular compartments

[1,2]. However, they have different efficiencies: for example, melittin is more active than delta-lysin in the release of hemoglobin on sheep and cod erythrocytes [3].

Both toxins bind to phospholipids and are able to induce the release of molecules trapped inside vesicles [2,4,5]. They also have been demonstrated to restructure lipid bilayers, by fragmentation and fusion for melittin [6,7] and

*Abbreviations: NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine; EPE, egg phosphatidylethanolamine; R_i , lipid-to-peptide molar ratio; T_c , temperature of lamellar gel-to-fluid transition.

mainly by fusion for delta-lysin [8].

Here the effects of these two toxins on synthetic model membranes are followed and compared by means of deuterium (^2H) and phosphorus (^{31}P) nuclear magnetic resonance (NMR). ^{31}P -NMR of natural membranes is shown to be very useful in extending the conclusions reached with model membranes.

Materials and methods

Synthetic phospholipids were purchased from Sigma Chemical Co. (U.S.A.); egg yolk phosphatidylethanolamine, grade 1, was provided by Lipid Products (U.K.). Chain deuterated lecithins ($[\text{sn-2-}^2\text{H}_{31}]$ DPPC) were synthesized according to procedures already published [9]. Perdeuterated palmitic acid was a generous gift from Dr B. Perly (CEN Saclay, France). Deuterium-depleted water was purchased from Aldrich Chemical Co. (U.S.A.). Melittin was bought from Bachem A.G. (Switzerland), and delta-lysin was kindly provided by Professors J.H. Freer and T.H. Birkbeck (University of Glasgow, U.K.).

Model membranes (multilamellar dispersions) were obtained by hydrating lipids with a buffered solution (20 mM Tris acetate, 2 mM EDTA, 100 mM NaCl, pH 7.5) on a vortex. Erythrocyte membranes were prepared according to known procedures [10] and resuspended in the same buffer.

Toxins were dissolved in the same buffer solution and added to the membranes at the desired lipid-to-peptide ratio (R_i). Except as otherwise mentioned, samples were incubated for at least 30 min above T_c (the gel-to-fluid phase transition temperature of the pure lipids) before the experiments were performed [11]. For temperature variations, the samples were allowed to equilibrate for at least 30 min before the NMR signal was acquired; the temperature was regulated to $\pm 1^\circ\text{C}$.

NMR signals were acquired on Bruker spectrometers, either a WH-270 adapted for solid-state studies or a MSL200, with quadrature detection and by means of quadrupolar and Hahn-echo pulse sequences, respectively for ^2H and ^{31}P nuclei [12, 13]. Gated broad-band proton decoupling was utilized in the case of ^{31}P -NMR. Data treatment was accomplished on a VAX/VMS 8600 computer.

Results and Discussion

Changes induced on DPPC by the toxins as followed by deuterium NMR

Fig. 1 shows ^2H -NMR powder spectra of $[\text{sn-2-}^2\text{H}_{31}]$ DPPC dispersions in the presence of melittin ($R_i = 20$), as a function of temperature. For this experiment, the toxin was added at

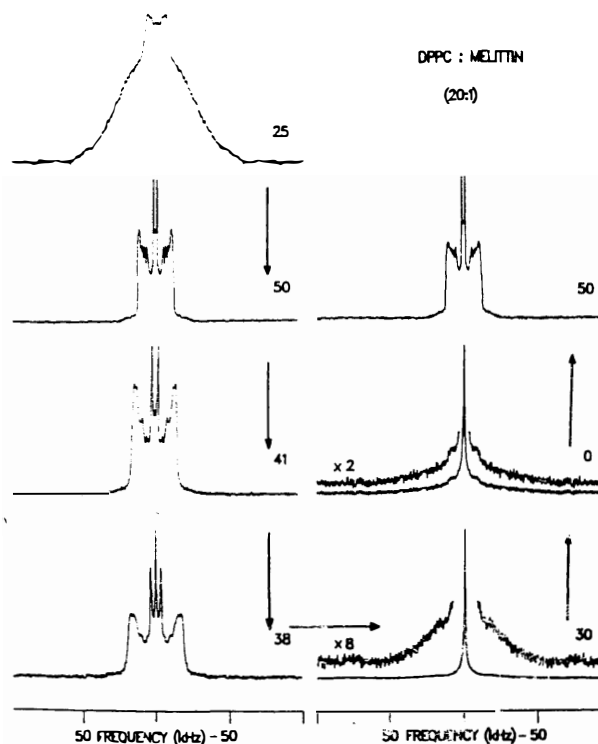


Fig. 1. Temperature variation of ^2H -NMR powder spectra of aqueous dispersions of $[\text{sn-2-}^2\text{H}_{31}]$ DPPC in the presence of melittin ($R_i = 20$). Temperatures ($^\circ\text{C}$) are indicated on the right side of each spectrum. The arrows show the direction of temperature variation during the experiment, after toxin addition at 25°C . Typical experimental parameters: spectral window, 250 kHz; 90° pulse width, $5\mu\text{s}$; delay between two 90° pulses, $40\mu\text{s}$; recycling time, 1.0-1.5 s; 1,800 scans accumulated.

25°C , *i.e.* in the gel phase, without any incubation above T_c (T_c of DPPC is 41°C). The spectrum observed at 25°C indicates that no change occurs upon melittin addition compared to the pure lipid spectrum at the same temperature. This is in contrast to the effect obtained in the same temperature range but after incubation above T_c (Fig. 1, $T = 38^\circ, 30^\circ$, and 0°C); in all these cases an isotropic line is observed. This line is superimposed on the broad spectrum, which represents gel-phase lipids unperturbed by melittin. The presence of this sharp line has to be correlated with the existence of very small melittin-lipid complexes already characterized by freeze-fracture electron microscopy, quasi-elastic light scattering, and gel filtration [6, 7, 14]. When the system is brought above T_c , this

sharp line disappears, and only the powder spectra are detected. This again correlates with the presence under these conditions of large vesicular structures clearly seen by other techniques [6, 7, 14].

When the amount of toxin in the system is increased ($Ri = 4$), spectra are only composed of isotropic lines both below and above T_c . This indicates that the bilayers have been quantitatively converted into very small structures which are fast-tumbling and already described by other direct methods [6, 7].

The changes induced by delta-lysin on the same DPPC system are much subtler (Fig. 2). For the same lipid-to-peptide molar ratio, $Ri = 20$, no isotropic line is detected in the gel phase. At $T < T_c$ spectra show few perturbations, whereas at $T > T_c$ the spectra are markedly modified. For high amounts of toxin in the system, $Ri = 4$, isotropic lines are observed but only in the fluid phase at $T \gg T_c$ [15].

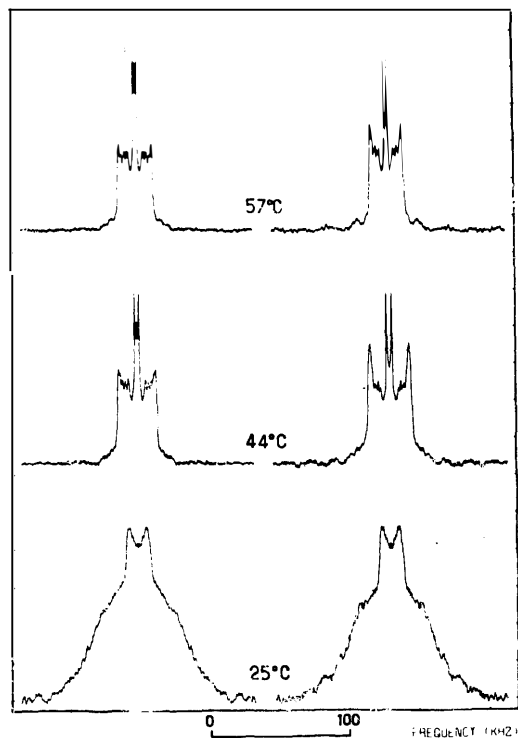


Fig. 2. Temperature variation of ^2H -NMR powder spectra of aqueous dispersions of $[\text{sn}-2-^2\text{H}_{31}]\text{DPPC}$ in the presence of delta-lysin ($Ri = 20$, right-side spectra) and in its absence (left-side spectra). Experimental parameters: spectral window, 166 kHz; 90° pulse, $5\mu\text{s}$; delay between two 90° pulses, $40\mu\text{s}$; recycling time, 2 s; 3000 scans are accumulated.

Such results, interpreted as reflecting changes in the supramolecular morphology of the systems, indicate that both toxins induce comparable effects, as detected by NMR, only at high amounts of toxin and at high temperatures with respect to T_c . Although it has not been demonstrated by other techniques that delta-lysin can induce the formation of small lipid-toxin complexes in the case of DPPC, as it does on lipid mixtures [2], then it is reasonable to think that as with melittin, the isotropic lines in NMR are associated with fast-tumbling small species. Thus, delta-lysin could also be able to fragment DPPC bilayers but only for high doses and at high temperatures with fluid-phase lipids. Melittin promotes similar effects at much lower doses and even preferentially with lipids in their gel phase.

Changes induced by the toxins in lipid ordering in the fluid phase

Deuterium NMR spectra of fluid-phase $[\text{sn}-2-^2\text{H}_{31}]\text{DPPC}$ lipids allows estimation, *via* measurements of quadrupolar splittings, the orientational ordering for each labeled position along the sn-2 acyl chain [16]. The ordering of the membrane may thus be represented as a function of bilayer depth, *i.e.* on going from positions C2-C3, close to the glycerol backbone, to the methyl terminal position at the center of the bilayer [17].

Figs. 3 and 4 illustrate such a variation and show how the ordering is modified by the presence of the toxins. Data are obtained from systems containing moderate amounts of toxins, *i.e.* situations where powder spectra are still observed in the fluid state and where, for melittin, the systems have been proved to be homogeneous and constituted of large unilamellar vesicles [7]. One remarks that both toxins induce a decrease in quadrupolar splitting, *i.e.* decrease the local orientational order, for all labeled positions, at high temperatures (65°C).

Near T_c , Fig. 3 shows that melittin preferentially orders the positions near the center of the bilayer ($T = 41^\circ\text{C}$) whereas delta-lysin promotes an increase in the ordering for all the positions (Fig. 4, $T = 45^\circ\text{C}$). When these results are considered together with the behaviour of lipid-toxin systems below T_c , it is proposed that both toxins partially penetrate into the bilayer at $T \gg T_c$ and act by a wedge effect, but their location with respect to the membrane differs at $T = T_c$. Such a variable position of the peptides compared to the lipid interface has already been proposed

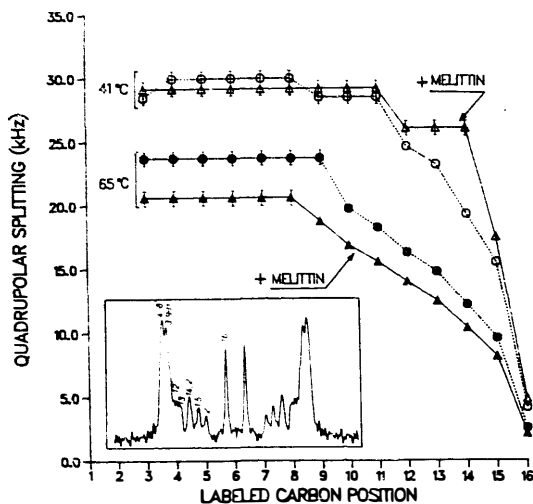


Fig. 3. Quadrupolar splittings from de-Paked spectra (*see insert*) of $[sn-2-^2H_{31}]DPPC$ in the presence ($Ri = 20$) and in the absence of melittin at 41°C and 65°C, as a function of the labeled carbon position along the sn-2-acyl chain. Prime numbers in the de-Paked spectrum (*insert*) represent the assignment of labeled positions.

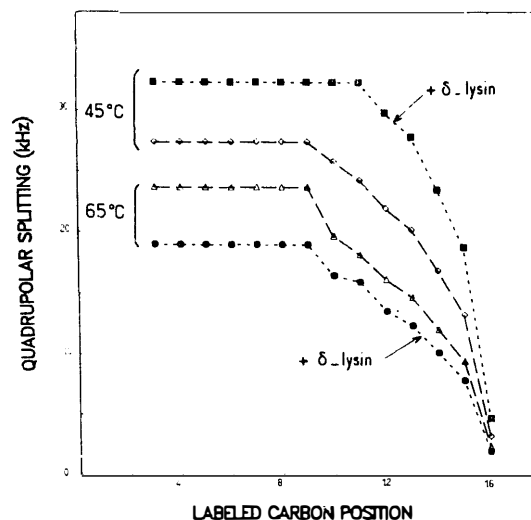


Fig. 4. Quadrupolar splittings from de-Paked spectra of $[sn-2-^2H_{31}]DPPC$ in the presence of delta-lysin ($Ri = 10$) and in its absence at 45°C and 65°C, as a function of the labeled carbon position along the sn-2 acyl chain.

ed very recently, when the charge of lipids varies [18]. Near T_c , the bee venom toxin will penetrate the membrane core [17] and it aggregates [19]. This will result in the formation of small domains that separate from the bulk lipids on cooling below T_c .

On the other hand, the bacterial toxin would stay at the bilayer top, capping the polar head groups. As already mentioned, this toxin would then be capable of bilayer fragmentation only at high doses for highly fluid lipids. Hence, the physical state of the lipids (fluid or gel) acts as a discriminating limiting factor in the ability of the studied toxins to change the morphology of lecithin bilayers.

Stability of melittin-lecithin discoidal complexes followed by ^{31}P -NMR

Passage through the transition temperature, T_c , of melittin-lecithin complexes at $Ri > 15$ results in reversible shape and size changes from large unilamellar vesicles to discoidal particles in the case of DPPC, which has already been demonstrated by several techniques [14]. Moreover, such discoidal complexes can be isolated by gel filtration and characterized [7]. However, depending on the experimental conditions, the long time stability of such species has been questioned, and

their evolution towards large bilayers proposed from Raman study [20].

The stability of these small species can be monitored by ^{31}P -NMR since they give a narrow isotropic line which is easy to detect. The occurrence of and kinetics for the evolution of this sharp line have been followed when the lipid chain length has been increased from dimyristoyl- to diarachidonyl-phosphatidylcholines, *i.e.* from C14 to C20.

In all cases, the isotropic line (*i.e.* small structures) are promoted by moderate amounts of melittin at temperatures below that of the transition of pure lipids. However, the stability of these small objects appears to depend on the lipid chain length. Fig. 5 illustrates the metastability of such small structures with the system DAPC-melittin, $Ri = 15$, at $T-T_c = -11^\circ C$. The first ^{31}P -NMR spectrum of the present series was obtained after quenching the system from the fluid phase. The spectrum changes as function of time and the isotropic line amplitude decreases and finally disappears, while concomitantly the powder pattern characteristic of large structures increases. The isotropic line has disappeared after *ca.* 150 h with DAPC. In the case of shorter chains, the isotropic line persists, in the same conditions, for several hundred hours [21].

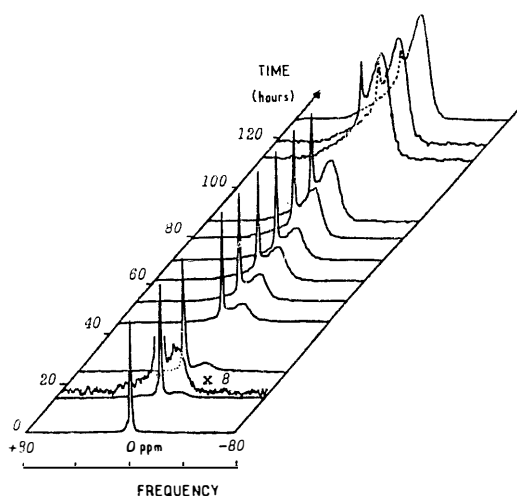


Fig. 5. Kinetics of changes of the ^{31}P -NMR spectra of aqueous dispersions of DAPC-melittin systems at $R_i = 15$, and $T-T_c = -11^\circ\text{C}$. The first spectrum was obtained after quenching from 77°C . Experimental parameters: spectral width, 50 kHz; 90° pulse, $7.5 \mu\text{s}$ delay between 90° and 180° pulses, $40 \mu\text{s}$; recycling time, 4s; 250 accumulations; gated high-power proton decoupling.

Thus it appears that for phosphatidylcholine lipids, the longer the chains, the faster the system returns from small structures to large bilayer sheets.

Effect of melittin on the thermotropism of natural phosphatidylethanolamine detected by ^{31}P -NMR

The influence of the head group nature has already been investigated by NMR using charged lipids [17, 22]. Here we investigated the effect of melittin on ethanolamine phospholipids by following the thermal evolution of ^{31}P -NMR spectra versus temperature [23]. Fig. 6 shows that again for $R_i = 15$ an isotropic line can be detected below T_c (natural EPE has a lamellar gel-to-fluid transition at about 0°C). This line disappears in the lamellar fluid phase. For temperatures above 30°C , at which a lamellar-to-hexagonal II phase transition occurs for the lipids alone [23], an isotropic line instead of the reversed powder pattern can again be detected, indicative of the hexagonal phases. Thus, as opposed to what has been demonstrated for cardiolipin, melittin probably prevents the formation of the hexagonal II phase instead of promoting it, as with the charged lipids [22]. This last effect has also recently been found by other workers [24].

Evolution of ^{31}P -NMR spectra of erythrocyte ghosts upon addition of melittin

Since the presence of isotropic NMR lines appears to occur rather generally with both synthetic and natural lipid membranes, the next logical step was to study melittin-real cellular membrane systems. Fig. 7 reports on the action of bee venom toxin on human erythrocyte ghosts, as detected by ^{31}P -NMR at 30°C . It can clearly be seen that the more toxin is added to the system, the more isotropic line is detected. This effect can be detected at rather low melittin content ($R_i = 50$), but it is not complete even at $R_i = 2$.

These results thus differ in comparison to those already shown with gel-phase phospholipid model membranes, and compared to fluid phase, as well. In any case, these data may be interpreted similarly to the manner outlined above for model membranes by the presence of small, fast-tumbling, lipid-toxin complexes. However, erythrocyte ghosts represent a system much more complex than model membranes. Such real membranes contain various lipid classes and proteins that possess very different dynamic characteristics. Although a strict parallel cannot be drawn between models, it might be imagined that melittin preferentially forms complexes with certain membrane components, charged and of low dynamics, *i.e.* gel-like domains. This

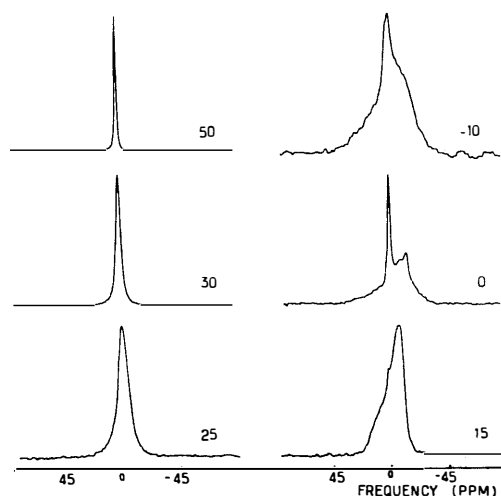


Fig. 6. Temperature variation of ^{31}P -NMR spectra of EPE dispersions in the presence of melittin ($R_i = 15$). Numbers on the right side indicate temperature ($^\circ\text{C}$); experimental conditions similar to those in Figure 5.

would account for the occurrence of the isotropic NMR lines in fluid heterogeneous membranes based on pure lipid systems.

However, such a proposal must be viewed with caution since it does not fully compare to what is already known about the effects of melittin on erythrocytes. First, with regard to cell lysis, the release of hemoglobin occurs at very low toxin concentrations, in the micromolar range, but in an almost similar domain of R_i values. The LD_{50} ranges up to about $R_i = 20$ [25,26], whereas 50% of isotropic line is only obtained at $R_i = 2$. Quantitative membrane fragmentation is thus probably a very late event in the process, with the first being an increase in ionic permeability [26]. Second, melittin has been shown to solubilize membrane components from the erythrocyte membrane, but according to the precision of the technique used, without solubilizing significant amounts of phospholipids unless some phospholipase activity has developed [27].

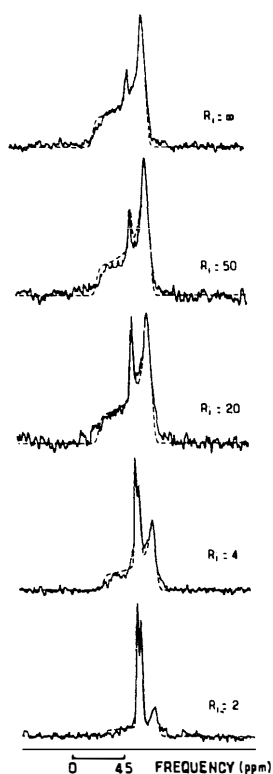


Fig. 7. Evolution of ^{31}P -NMR spectra of human erythrocyte membranes as a function of melittin addition, at 30°C . The experimental conditions are as in Fig. 5. Dashed lines represent simulated spectra.

Conclusions

Despite their similarities, melittin and delta-lysin can affect differently the structure and dynamics of model membranes. From the changes in the lipid order parameters, a positioning of the toxins is proposed. It depends on the physical state of the lipids and can differ for the 2 toxins. This is more striking for ordered gel-phase lipids where melittin, which is the more hydrophobic, appears to be able to penetrate deeper [17] than the bacterial hemolysin and to induce bilayer fragmentation into small species.

The stability of the small melittin-lecithin discoidal complexes in the gel phase appears to be related to the acyl chain length. The longer the chain, the quicker the small structures return to large lipid layers.

By contrast, in the fluid phase, at $T \gg T_c$ both toxins are probably very similarly bound to the bilayer and act as a wedge. At high doses they promote the appearance of very small complexes, fast tumbling on the NMR time scale. They are probably similar to the co-micelles already shown to exist with egg lecithin [7].

The formation of these small objects upon the addition of melittin and delta-lysin to bilayers provides the basis for a proposed mechanism of membrane lysis of erythrocytes.

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