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The lytic activity of the bee venom peptide melittin is strongly reduced by the presence of negatively charged phospholipids or chloroplast galactolipids in the membranes of phosphatidylcholine large unilamellar vesicles

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Abstract

We have investigated the dependence of the lytic activity of the bee venom peptide melittin on the lipid composition of its target membrane. The lysis of large unilamellar liposomes, measured as loss of the fluorescent dye carboxyfluorescein, in the presence of melittin was strongly reduced when the negatively charged lipids phosphatidylglycerol (PG) or phosphatidylserine (PS), or the plant chloroplast lipids monogalactosyldiacylglycerol (MGDG) or digalactosyldiacylglycerol (DGDG) were incorporated into egg phosphatidylcholine (EPC) membranes. This reduction was evident at concentrations below 10 wt% of the additional lipids. It was not due to reduced binding of melittin to the vesicles. It was also not related to a reduced insertion depth of the peptide into the bilayer, as shown by quenching of the intrinsic tryptophan fluorescence of the peptide by the aqueous quencher sodium nitrate. Fourier transform infrared spectroscopy (FTIR) revealed specific interactions of the peptide with the headgroups of the inhibitory lipids. The phosphate peak in PG was shifted by two wavenumbers after the addition of melittin. There was no shift in EPC or PS. Instead, in PS the COO^- peak was strongly distorted in the presence of melittin. These data indicate ionic interactions between the basic peptide and the negative charges on the membrane surface. The galactolipids are uncharged. Here the evidence points to hydrogen bonding between melittin and OH-groups of the sugar headgroups. Liposomes containing DGDG were the only case where we found evidence for changes in fatty acyl chain motion due to the presence of melittin, from the CH_2-scissoring peaks.

Keywords: FTIR; Galactolipid; Liposome; Melittin; Peptide–lipid interaction

1. Introduction

Melittin is a hemolytic toxin from bee venom [1]. It is a peptide of 26 amino acids which form an amphiphilic α-helix [2]. The peptide carries five positive charges from lysine and arginine residues. Melittin binds spontaneously to lipid bilayers, leading to rapid hemolysis of red blood cells [3–5] and the loss of internal markers from pure lipid vesicles ([3]; for a comprehensive review, see [6]). A number of studies have used phosphatidylcholine liposomes to elucidate the mechanisms underlying the lytic action of melittin. At high *R*-values release of internal solutes from the vesicles is observed, while at low *R*-values (20 or less) the vesicles are completely disrupted into micellar discs [7,8].

Melittin shows some structural and functional similarities to mitochondrial signal peptides. These peptides are aminoterminal extensions of nuclear encoded proteins which direct the transport of the protein across the mitochondrial outer membrane and into the target compartment [9]. Signal peptides form amphiphilic α-helices and insert into lipid bilayers [10]. It has been shown that the interaction of these peptides with liposomes can lead to the loss of soluble markers from the vesicle lumen and the induc-

Abbreviations: CF, carboxyfluorescein; DGDG, digalactosyldiacylglycerol; EPC, egg phosphatidylcholine; FTIR, Fourier-transform infrared; MGDG, monogalactosyldiacylglycerol; POPG, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol; PS, phosphatidylserine; *R*, lipid/peptide molar ratio.

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tion of localized non-bilayer structures [11–13]. Therefore, melittin can be used as simple and already well characterized model to study lipid–protein interactions that are of functional significance also for other biological systems.

An interesting and still unresolved question in this regard is how nuclear encoded proteins find their correct cellular compartment after synthesis on cytoplasmic ribosomes. Some recent papers suggest that both mitochondrial and plastidic presequences may have an inherent affinity for lipids that are specific for the membranes of their respective target organelles [14–18]. However, nothing is known about the structural features in these peptides that determine their specificity or about the molecular interactions between the peptides and specific lipid headgroups during binding of the peptides to the membranes.

Similarly, there is some evidence in the literature that the interaction of melittin with liposomes is influenced by the lipid composition of the membranes. Specifically, it has been reported that lipids with negatively charged headgroups and cholesterol inhibit the lytic activity of melittin. Most of these studies, however, were conducted with membranes made exclusively with the charged lipid [19–21] or at very low lipid/peptide molar ratios (R-values) [22–24] and were therefore only of limited relevance to a biological system.

In the present study we have used large unilamellar vesicles made from EPC and a lipid-to-peptide ratio of 250. The effects of negative charges in the membrane were assessed by substituting various fractions of EPC by either POPG or PS. In addition, we have used different fractions of the plant chloroplast lipids MGDG and DGDG in EPC vesicles and found that both lipids inhibit the lytic activity of melittin. Possible mechanisms of interaction between the peptide and the different lipids were investigated by FTIR spectroscopy.

2. Materials and methods

2.1. Lipids

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Galactolipids were purified from fresh spinach (*Spinacia oleracea* L.) leaves obtained from a local market in Davis, CA following published procedures [25,26]. Briefly, thylakoids were isolated from 400 g of spinach leaves and washed twice by centrifugation and resuspension in fresh buffer to remove contaminations from other cellular membranes. Lipids were extracted in chloroform/methanol (1:2, v/v) and aqueous 100 mM NaCl was added to facilitate the partitioning of the lipids into the organic phase. This phase was concentrated by rotary evaporation and applied to a column filled with 80 g of silicic acid (SIL-LC, Sigma) pretreated with petroleum ether and equilibrated in chloroform. Pigments were eluted with chloroform and MGDG with chloroform/acetone (1:1, v/v). The remaining lipids were eluted from the column with chloroform/methanol (1:1, v/v), dried under vacuum and redissolved in chloroform. This fraction was applied to a smaller column (30 g) of the same material and DGDG was eluted with acetone. The purity of MGDG and DGDG was checked by TLC and was found to be greater than 95%.

2.2. Preparation of liposomes

All liposomes were prepared from 0.5 ml of hydrated lipids using a hand-held extruder ([27]; Avestin, Ottawa, Canada) containing two layers of polycarbonate membranes (Poretics, Livermore, CA) with 100 nm pores. The different lipids were mixed in chloroform, dried under a stream of N_2 and stored under vacuum over night to remove traces of solvent. Mixtures of different lipids were made by weight and are expressed as % (w/w). For leakage measurements 5 mg of lipid were hydrated in 0.5 ml of 100 mM CF (Molecular Probes, Eugene, OR; purified according to [28]), 10 mM Tes, 5 mM EDTA (pH 7.4). After extrusion, the vesicles were passed through a column (0.5 × 10 cm) of Sephadex G-50 (Pharmacia) equilibrated in 10 mM Tes, 5 mM EDTA, 50 mM NaCl (pH 7.4) to remove the CF not entrapped by the vesicles. For tryptophan fluorescence measurements 10 mg of the different lipid mixtures were dried from chloroform as above, hydrated in 0.5 ml of 10 mM Tes, 5 mM EDTA, 50 mM NaCl (pH 7.4) and then used in the extruder. For FTIR spectroscopy 25 mg of lipid were dried, hydrated in 0.5 ml of distilled water and extruded.

2.3. Melittin

Melittin (sequencing grade, HPLC purified) was purchased from Sigma and used as supplied. It was solubilized in 10 mM Tes, 5 mM EDTA, 50 mM NaCl (pH 7.4) at 0.1 mg/ml for leakage experiments, at 1 mg/ml for tryptophan fluorescence measurements, and at 2.8 mg/ml in distilled water for FTIR spectroscopy. Except for the data shown in Fig. 1, the lipid/peptide molar ratio (R) was kept at 250 in all experiments.

2.4. Leakage experiments

The time-course of leakage of CF from liposomes was measured over 10 min in a Perkin–Elmer LS-5 fluorometer equipped with a thermostated cuvette holder attached to a circulating water bath and a magnetic stirrer. The temperature in the cuvette was maintained at 15°C and was constantly monitored with a thermocouple and a digital thermometer. Measurements were made at an excitation wavelength of 460 nm and an emission wavelength of 550 nm. The cuvette was filled with 2 ml of melittin in buffer. The experiment was started by adding 10 μl of the liposome suspension to the stirred cuvette. The first fluo-

rescence reading was taken after 15 s. After 10 min 50 μl of a 1% (v/v) solution of Triton X-100 was added to the cuvette to obtain a 100% leakage value. All experiments were performed at least in triplicate (see figure legends for details). The lines were fitted to the data by an interpolation routine provided with the Macintosh Cricket Graph software used to draw the figures.

2.5. Tryptophan fluorescence quenching

Melittin contains one tryptophan residue which can be used as an intrinsic fluorescence probe [29]. Tryptophan fluorescence was excited at 280 nm and emission was measured at 340 nm. All other conditions were identical to those for the leakage experiments, except that both the peptide and lipid concentrations in the cuvette were 10-fold higher. After the addition of liposomes to the melittin solution in the cuvette the fluorescence emission increased due to the exposure of the tryptophan residues to the more hydrophobic environment of the membrane. The relative depth of penetration of the peptide into the hydrocarbon core region of the membranes was estimated by titration of the fluorescence with the hydrophilic quencher $NaNO_3$ [29]. Aliquots of 5 μl of a 5 M $NaNO_3$ solution were added to the cuvette and the fluorescence values recorded up to a final concentration of 125 mM $NaNO_3$. The data were analyzed using a Stern–Vollmer plot of $(F_0/F) - 1$ vs. $NaNO_3$ concentration (F_0 = fluorescence in the absence of quencher, F = fluorescence at different quencher concentrations). From the slopes of the linear curves obtained from these plots the quenching constants (K_{SV}) were calculated (for a review, see [30]) to allow a quantitative comparison of the relative insertion depths of melittin into membranes of different lipid composition.

2.6. FTIR spectroscopy

Spectra from liposomes in the absence and presence of melittin were obtained with a Perkin–Elmer 1750 Fourier-transform infrared spectrometer assisted by a personal computer with the Perkin–Elmer IRDM software as described in detail before [31]. The figures show the normalized and smoothed absorbance spectra.

3. Results

We have used the leakage of the fluorescent dye carboxyfluorescein to quantify the lytic activity of the bee venom peptide melittin for large unilamellar vesicles. Fig. 1A shows that under our experimental conditions the vesicles were very stable in the absence of a lytic agent but leaked dye at a R-value as high as 1000. Leakage over the first two minutes was rapid and then leveled off. The curves shown in Fig. 1A could be transformed into linear curves using a double-reciprocal plot (Fig. 1B), formally

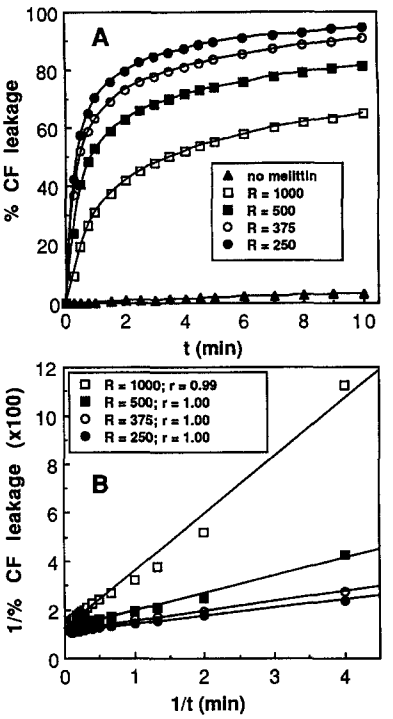

Fig. 1. (A) Leakage of carboxyfluorescein (CF) from large unilamellar vesicles made from EPC as a function of time. The vesicles were added from a concentrated stock solution to the fluorimeter cuvette containing different concentrations of melittin as indicated in the panel. The lipid-to-peptide ratio (R) of 250 was used in all other experiments in this study. The points shown are the means of three measurements. The mean standard deviation of all points in this figure was 1.7±0.8%. (B) Double-reciprocal plot of the data presented in (A). The straight lines were fitted by linear regression analysis. The correlation coefficients (r) are shown in the panel. Extrapolation of the lines to an infinite incubation time ($1/t = 0$) yields theoretical 'maximum CF leakage' values.

analogous to a Lineweaver–Burke plot. The linear fit of the curves was excellent, as judged from the correlation coefficients which were always between 0.99 and 1.00. By extrapolating the straight lines to $1/t = 0$ it was possible to calculate the percentage of CF released after a hypothetical infinite incubation time. For the different melittin concentrations at a constant lipid concentration as shown in Fig. 1A, these 'maximum CF leakage' values were: 82.0% at $R = 1000$ (0.125 μg/ml); 84.8% at $R = 500$ (0.25 μg/ml); 89.3% at $R = 375$ (0.375 μg/ml); and 94.3% at $R = 250$ (0.5 μg/ml). Maximum leakage values and melittin concentration were linearly correlated ($r = 0.99$) over this concentration range. For all further experiments $R = 250$ was used.

When an increasing fraction of the EPC in the vesicle membranes was replaced by a negatively charged phospholipid, the lytic activity of melittin was reduced (Fig. 2A and B) at concentrations of 5% or more of POPG or PS. The largest decrease in CF leakage occurred between 5 and 10% POPG (Fig. 2A) and between 5 and 15% PS (Fig. 2B). Higher concentrations of the negatively charged lipids in the membranes only had small additional effects. Both

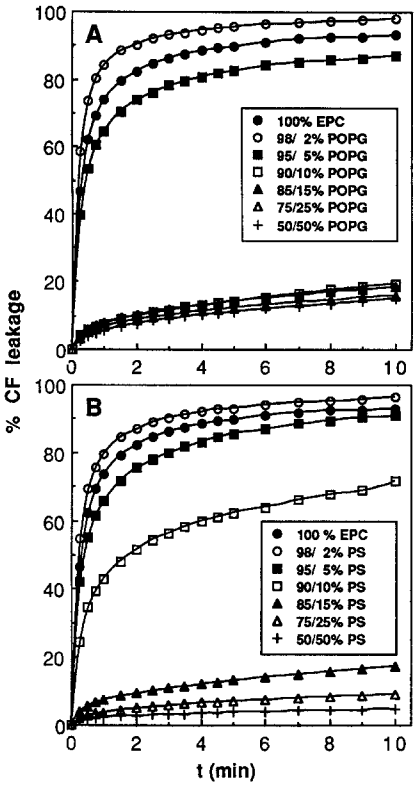

Fig. 2. CF leakage from large unilamellar vesicles as a function of incubation time at an L/P ratio R of 250 (compare Fig. 1). The vesicles were composed of different proportions of EPC and either POPG (A) or PS (B). The points are the means of three or four measurements. The mean standard deviations were $1.5 \pm 1.0\%$ in (A) and $2.8 \pm 1.8\%$ in (B).

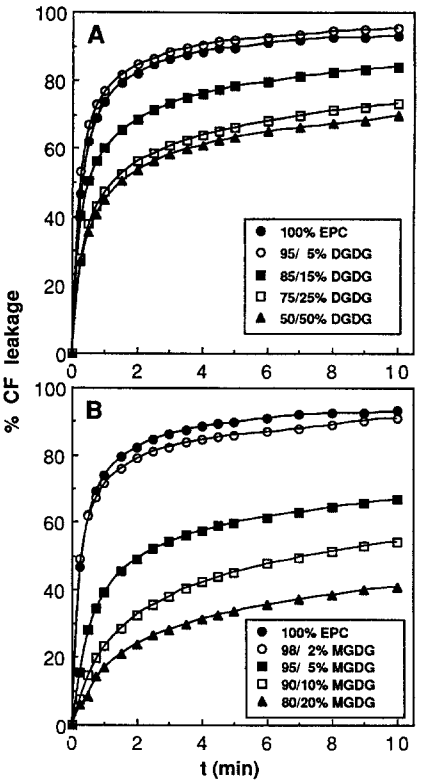

Fig. 3. CF leakage from large unilamellar vesicles in the presence of melittin as a function of incubation time (compare Fig. 1). The vesicles were composed of different proportions of EPC and either DGDG (A) or MGDG (B). The points are the means of 3–5 measurements. The mean standard deviations were $4.2 \pm 1.6\%$ in (A) and $4.6 \pm 1.7\%$ in (B).

POPG and PS at very low concentrations (2%) led to an increase in CF leakage induced by melittin. The increase was small but occurred over the whole measurement time and was highly reproducible with both lipids, and with different melittin and vesicle preparations.

While a reduction in the lytic activity of melittin in the presence of negatively charged lipids could be expected from the results previously reported in other studies, under different experimental conditions (see Section 4 for details), the inhibitory effect of uncharged chloroplast galactolipids is a completely novel finding of our study (Fig. 3A and B). Both plant lipids led to a reduction of melittin induced CF leakage. The monogalactolipid (MGDG) was more effective than the digalactolipid (DGDG). Unlike the negatively charged lipids, the galactolipids induced no increase in leakage at low concentrations.

For a direct, quantitative comparison, all data in Figs. 2 and 3 were plotted in double-reciprocal plots (not shown, compare Fig. 1B) and from the linear regression lines maximum CF leakage values were calculated (Fig. 4). From the fact that the regression coefficients remained between 0.99 and 1.00 (compare Fig. 1B) it can be concluded that the kinetics of CF leakage induced by melittin were not changed by the different lipids. However, the maximum leakage values were greatly reduced in the presence of POPG or PS. MGDG showed a very similar concentration dependence, while DGDG was clearly less effective. Unfortunately, experiments with higher concentrations than 20% of MGDG were not possible, since MGDG is a non-bilayer forming lipid [32] that leads to rapid CF leakage from the vesicles at higher concentrations already in the absence of melittin (data not shown).

It has been proposed recently that the lytic activity of melittin for phospholipid vesicles was reduced in the pres-

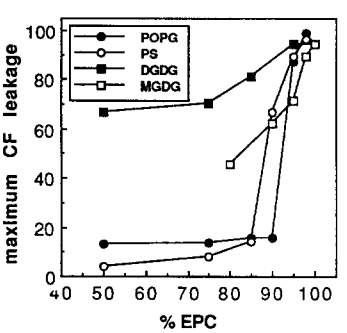

Fig. 4. Maximum CF leakage calculated from the regression lines of double-reciprocal plots (compare Fig. 1B) of the data shown in Figs. 2 and 3 as a function of the percentage of EPC in the vesicle membranes. From right to left the fraction of POPG, PS, DGDG, or MGDG was increased in the membranes as the fraction of EPC decreased.

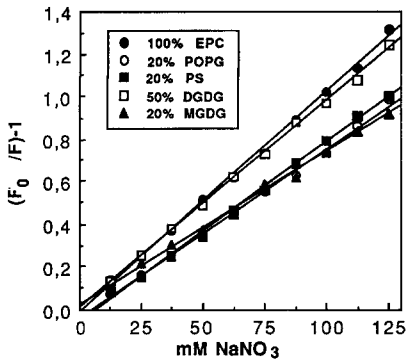

Fig. 5. Stern–Vollmer plot of the quenching of the tryptophan fluorescence of melittin by $NaNO_3$. Fluorescence emission at 340 nm was measured in the presence of liposomes of different lipid composition. Small aliquots of $NaNO_3$ were added from a 5 M stock solution and the fluorescence was recorded at each step. F_0 = fluorescence emission in the absence of $NaNO_3$, F = fluorescence emission after addition of quencher.

ence of negatively charged lipids because of ionic interactions between the positive charges in the peptide and the negative charges on the membrane surface [23,33]. These interactions were thought to anchor the peptide on the surface and thus inhibit its penetration into the membrane core region, which would lead to membrane destabilization. In order to test this hypothesis we used the single tryptophan residue in melittin as an intrinsic fluorescence probe. The tryptophan fluorescence of melittin is enhanced when the peptide inserts into the hydrophobic core of a membrane. This was true to the same degree for all lipid compositions tested (data not shown), indicating that melittin inserts into the bilayers, even when it shows no lytic activity and that reduced lysis was in no case the result of reduced binding of the peptide to the membranes. The relative depth of insertion can be estimated by titrating the tryptophan fluorescence with the aqueous quencher $NaNO_3$ (Fig. 5). From the slopes of the linear Stern–Vollmer plots shown in Fig. 5, the quenching constants (K_{SV}) could be calculated. An increase in K_{SV} would indicate that the tryptophan fluorescence of melittin was more efficiently quenched by the nitrate ions and thus that the peptide was

Table 1
Quenching constants (K_{SV}) of $NaNO_3$ for the fluorescence emission of the single tryptophan residue of melittin in liposomes of different composition

Lipid composition	K_{SV}	Significantly different from 100% EPC
100% EPC	10.3	
20% POPG/80% EPC	8.0	yes ($P < 0.05$)
20% PS/80% EPC	8.5	no($P < 0.1$)
50% DGDG/50% EPC	9.7	no($P < 0.5$)
20% MGDG/80% EPC	7.2	yes ($P < 0.025$)

The values are calculated from Stern–Vollmer plots (see Fig. 5). The means from three titrations are shown. Differences between the samples containing negatively charged lipids or galactolipids, and control vesicles containing only EPC were evaluated using an unpaired Student's t-test.

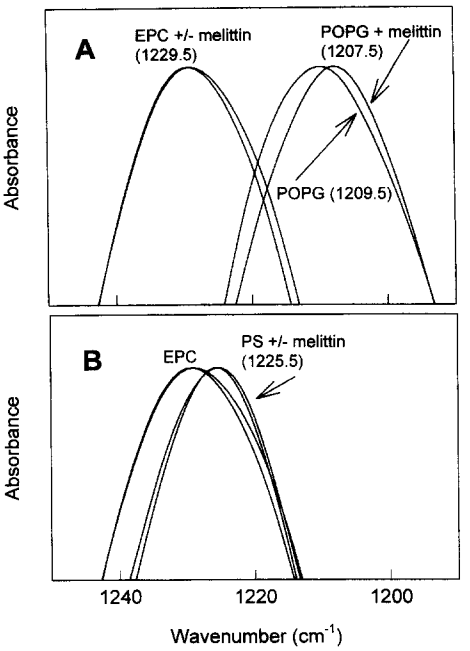

Fig. 6. FTIR spectra showing the P = O peaks in EPC and POPG (A) and EPC and PS vesicles (B) measured in the absence or presence of melittin. The numbers shown in parenthesis are the peak wavenumbers of the respective spectra.

less deeply buried in the membrane. From Table 1 it is evident that this was not the case. The presence of inhibitory concentrations of POPG, PS, DGDG, or MGDG led to either no significant changes in K_{SV} or to a reduction, indicating that the peptide may be even more tightly bound to the membranes.

In order to investigate possible interactions of the different lipid headgroups with melittin in more detail, we used FTIR spectroscopy. In PC, the negative charge of the headgroup phosphate is neutralized by the positive charge of the choline nitrogen. In PG, the choline is replaced by the uncharged glycerol moiety, leading to a free negative charge, while in PS the serine headgroup contributes a second negative charge through the amino acid COO^-. All

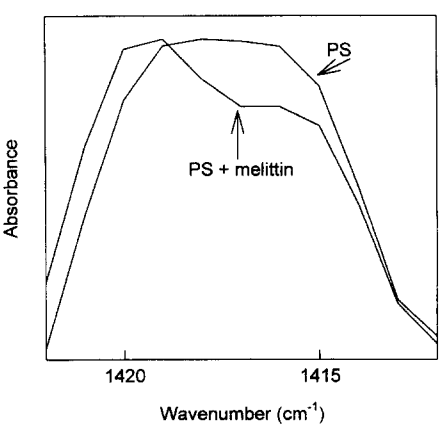

Fig. 7. FTIR spectra showing the COO^- peak in PS vesicles in the absence or presence of melittin.

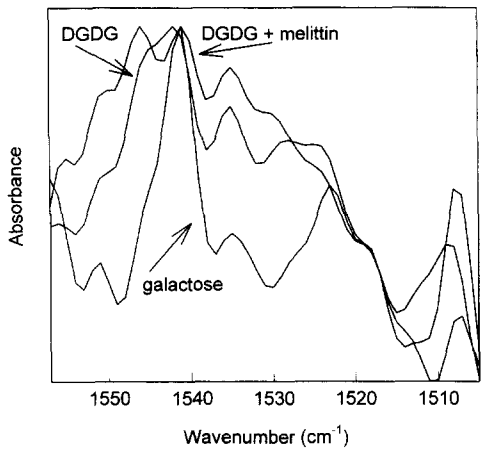

Fig. 8. FTIR spectra showing the influence of melittin on a series of peaks associated with the galactose in the DGDG headgroup. The spectrum of free galactose is shown for comparison.

these negative charges could possibly interact with the positive charges from the arginines and lysines in melittin. Fig. 6A provides evidence that the phosphate in POPG interacts with melittin, leading to a shift of the absorbance peak by two wavenumbers. The peaks in EPC and PS (Fig. 6B), on the other hand, are not influenced by the presence of the peptide. In PS, the main interaction between melittin and the lipid headgroup is mediated through the amino acid carboxyl (Fig. 7). The COO^- peak at 1419 cm^{-1} is strongly distorted by the peptide, a finding that we suggest is consistent with an interaction between the polar headgroup and melittin.

In the plant galactolipids, the headgroups contain no negative charges. Therefore, ionic interactions between the peptide and the lipids can be excluded as an explanation for the effect of MGDG and DGDG on the lytic activity of melittin. However, these lipids contain several OH-groups in their headgroups that could form hydrogen bonds with the hydroxylated amino acids and the amide groups of the glutamines in the peptide and thereby influence its activity. Fig. 8 presents evidence for this hypothesis. The FTIR spectrum of DGDG shows several peaks between 1555 and 1505 cm^{-1}, which coincide with peaks measured in a saturated solution of free galactose (Fig. 8 and Table 2). Although we have no direct evidence for this, it seems reasonable to suggest that this series of peaks is related to the sugar OH-groups. The presence of melittin has a strong

Table 2
Wavenumbers of the peaks associated with free galactose and DGDG in Fig. 8

Sample	Wavenumber				
	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
Galactose	1551	1541	1535	1523	1507
DGDG	no peak	1542	1535	1526	1509
DGDG with melittin	1550 (shoulder)	1541, 1546	1535	no peak	1508

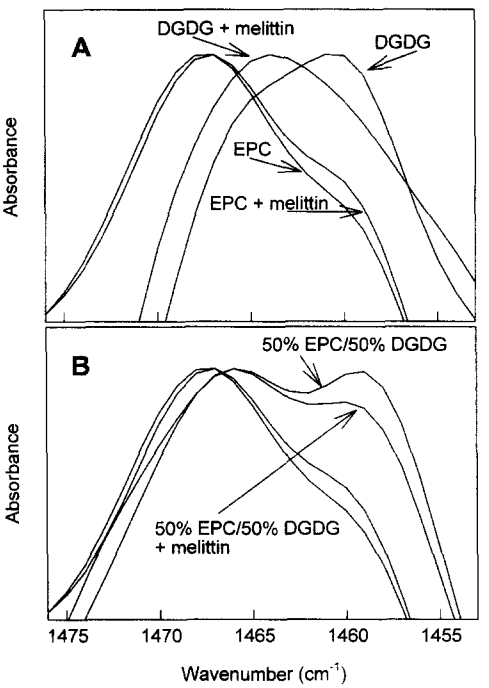

Fig. 9. FTIR spectra of the CH_2 scissoring peaks in EPC and DGDG (A) and EPC and 50% EPC/50% DGDG vesicles (B) measured in the absence or presence of melittin.

influence on some, but not all, of these peaks. The peak at 1535 cm^{-1} is constant in galactose and in DGDG with or without the peptide. The main peak in DGDG at 1542 cm^{-1} corresponds to the peak at 1541 cm^{-1} in the free sugar while it is split into two peaks (1546 and 1541 cm^{-1}) in DGDG in the presence of melittin. Peaks around 1551, 1523, and 1507 cm^{-1} are also more or less strongly affected by the presence of melittin (Fig. 8 and Table 2), indicating a possible interaction between the peptide and several hydroxyl groups in the sugar headgroup of the lipid.

This interaction also has some influence on the packing of the fatty acyl chains of the DGDG molecules, as seen from the FTIR peaks associated with the CH_2 scissoring mode (Fig. 9 and Table 3). In pure EPC membranes the main peak at 1467.5 cm^{-1} is not influenced by the peptide, while the shoulder at 1460 cm^{-1} is slightly increased. In membranes containing only DGDG, only a single peak was apparent which shifted from 1461 cm^{-1} in the absence to 1463.5 cm^{-1} in the presence of melittin. Mem-

Table 3
Wavenumbers of the peaks associated with the CH_2-scissoring mode in EPC and DGDG (compare Fig. 9 A and B)

Lipid	Wavenumber (cm^{-1})	
	no melittin	with melittin
EPC	1467.5	1467.5
50% EPC/50% DGDG	1466, 1459	1466, 1460
DGDG	1461	1463.5

branes containing 50% EPC and 50% DGDG showed intermediate behavior (Fig. 9B), with two peaks, of which only the one at the lower wavenumber was influenced by the peptide.

Unfortunately, similar measurements with MGDG, which was much more effective in reducing the lytic activity of melittin, were not possible. At 20% MGDG in EPC membranes the FTIR spectra were not sufficiently clear to reach unambiguous conclusions and at higher concentrations MGDG separates into domains forming either hexagonal II or cubic phases [34], which make a clear interpretation of the spectra impossible.

4. Discussion

The fact that melittin is not only able to lyse red blood cells but also pure lipid vesicles has been known for many years [3]. In a recent study [33] that used an experimental approach very similar to ours, it was found that 15% phosphatidic acid or 10% POPG led to strongly reduced lysis of POPC liposomes. Although lower fractions of negatively charged lipids were not assayed in that study, their data together with our results shown in Fig. 2, indicate that negatively charged lipids in general are able to inhibit the lytic activity of melittin, even when they only constitute a small fraction of the total lipids.

Since melittin contains five basic amino acids, an ionic interaction between positive charges on the peptide and negative charges on the membrane surface is an obvious possibility. To date, however, no direct evidence for such an interaction has been reported in the literature. Figs. 6 and 7 suggest that such ionic interactions take place and that they are specific for different lipids. While in POPG the headgroup phosphate was involved in peptide binding (Fig. 6A), the corresponding FTIR peak in PS was not influenced by melittin (Fig. 6B). Rather, in PS the amino acid carboxyl seemed to be responsible for the inhibitory action of this lipid (Fig. 7).

A surprising finding of our study was that also uncharged chloroplast galactolipids were able to inhibit the lytic activity of melittin (Figs. 3 and 4). Melittin has been shown to inhibit photosynthetic electron transport in isolated spinach chloroplast thylakoid membranes [35], and to induce release of the lumenal electron transport protein plastocyanin from thylakoids [36]. It was noted, however, that this loss of an internal soluble protein from thylakoids proceeded at a rate that was several hundredfold slower than hemolysis of erythrocytes under comparable conditions. The fact that MGDG and DGDG, which together make up about 75% of the total thylakoid lipids [32], strongly inhibit the lytic activity of melittin provide an explanation for these different lysis rates.

Since plant galactolipids contain no charged groups, ionic interactions can be excluded as a possible reason for the inhibitory effects of these lipids on the peptide. However, melittin contains three hydroxylated amino acids (1 serine, 2 threonines), and two glutamines. The OH- and NH_2-groups of these residues could form hydrogen bonds with the numerous OH-groups of the galactoses that make up the galactolipid headgroups. Evidence for this hypothesis is provided from FTIR spectra (Fig. 8; Table 2) which show a series of peaks that are thought to result from OH vibrations in galactose. It should be stressed that these were the only differences in the FTIR spectra from samples with and without melittin that we were able to find. The addition of melittin to the membranes has distinct effects on some, but not all, of the peaks, suggesting well-defined interactions between the lipid headgroups and the peptide.

Such interaction may also be the basis for the lipid specificity observed for both mitochondrial and plastidic signal peptides (see Section 1 for references). It can be assumed that the sequence and secondary structure of the peptides determines their preferential interactions with specific lipid headgroups and the subsequent orientation of the peptide in the membrane. To date, however, no systematic analysis of such structure–function relationships has been published.

Our results obtained with different lipids also allow some new insights into the still controversial question of the mechanism underlying the lytic activity of melittin (for a review, see [6]). Two main mechanisms have been proposed so far. One is that the peptide binds parallel to the membrane surface and that lysis occurs when several peptide molecules reorient perpendicular to the membrane plane, thereby forming a hydrophilic pore through the membrane bilayer. This pore formation could either lead to direct efflux of small molecules such as fluorescent dyes or to colloid osmotic lysis in the case of red blood cells [37–40].

Alternatively, melittin is thought to be oriented parallel to the membrane surface, with the hydrophobic face of the α-helix buried in the membrane. This 'wedge' would then lead to a destabilization of the membrane that results in lysis [33,41–44]. Proponents of both models, however, have come to the conclusion that for a given vesicle lysis is an 'all-or-none' event [33,39].

In our experiments we found that the presence of negatively charged lipids or galactolipids had the same effect on vesicle lysis as a reduction in melittin concentration (i.e. an increase in R; compare Fig. 1A with Figs. 2 and 3). The kinetics of lysis were unaltered, but the maximum percentage of lysis was reduced (Fig. 1B and Fig. 4). In terms of the 'wedge' hypothesis, the effect of negatively charged lipids was explained with the assumption that the negative charges on the membrane surface would immobilize the peptide in the lipid headgroup region and thus prevent it from entering the hydrophobic part of the bilayer. The results from the tryptophan fluorescence quenching experiments shown in Fig. 5 and Table 1 clearly disagree with this assumption. The K_{SV} value

measured in our experiments with EPC (10.3 M^{-1}) is very close to values reported in the literature for the quencher $NaNO_3$ (13.8 M^{-1} [29]; 12.8 M^{-1} [20]).

Although the data give no clear support for the conclusion that the peptide is buried significantly deeper in the membrane, there is certainly no evidence for an increase in K_{SV} in the presence of the inhibitory lipids. This is in agreement with a similar quenching study where the interaction of melittin with the negatively charged mitochondrial lipid cardiolipin was investigated [20]. It has also recently been shown that lysis of liposomes only occurs when a significant part of the melittin molecule is inserted in the membrane [44]. Taken together, these data suggest that melittin induced lysis of lipid vesicles may depend on a reorientation of the peptide perpendicular to the membrane plane and that this is prevented by the lipids investigated in our study.

It should be emphasized that the FTIR spectra we measured with different lipid compositions showed no evidence that melittin had any effects on lipid acyl chain motion (CH_2 stretch, CH_2 scissoring, C = O; data not shown) at this high R-value, even under lytic conditions. This means that even complete lysis of the vesicles involved no gross disturbance of the bilayers. Also, the immobilisation of melittin by the negatively charged lipids had no effect on their acyl chain motions. The only exception were DGDG vesicles, where the addition of melittin led to an increase of 2.5 cm^{-1} in the CH_2 scissoring peak (Fig. 9; Table 3). This indicates reduced hydrocarbon chain fluidity, as has also been observed after lowering the temperature [45]. A similar result has been reported with electron paramagnetic resonance spectroscopy after binding of melittin to sarcoplasmic reticulum membranes [46]. This reduced fluidity could be a further barrier for the reorientation of melittin in the bilayer. It has been shown that melittin induced lysis is reduced in PC vesicles as the degree of unsaturation is reduced in the lipid acyl chains [47].

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