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Headgroup structure and fatty acid chain length of the acidic phospholipids modulate the interaction of membrane mimetic vesicles with the antimicrobial peptide protegrin-1¶

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Abstract: The interaction of protegrin-1 (PG-1), a small β-sheet antimicrobial peptide with acidic phospholipid model membranes was investigated by differential scanning calorimetry. We found that PG-1 can distinguish between liposomes of the anionic phospholipids DPPG, DPPS and DPPA, eventhough the headgroups of these phospholipids all have the same net charge and they carry the same hydrocarbon chains. Specifically, PG-1 had only a minor effect on the thermotropic phase behavior of DPPA liposomes, while it interacted preferentially with the fluid phase of DPPS. Furthermore, PG-1 could induce a phase separation in DPPG liposomes resulting in the formation of peptide-rich domains even at low concentrations of the peptide. However, this peptide-rich domain was not evident when the fatty acyl chains were longer or shorter by two carbon atoms. In addition, PG-1 can also form peptide-rich domains in DPPS vesicles but only at high concentrations of the peptide. These results suggest that in addition to an overall negative charge, the structural features of the phospholipid headgroups, lipid packing and thus membrane fluidity will influence the interaction with PG-1, thereby modulating its biological activity.

Keywords: antimicrobial peptide; membrane mimetics; lipid headgroup; peptide-lipid interactions; differential scanning calorimetry

INTRODUCTION

Pathogenic bacteria are increasingly becoming resistant to commonly used antibiotics and the emergence of so-called 'super bugs' has created a need for novel strategies to fight bacterial infections. Also the recent HIV epidemic and harmful infections caused by the West-Nile and SARS viruses reveal a need for novel antiviral agents. Antimicrobial peptides, which form the first line of defense in the innate immune system of animals, insects and plants, offer hope as a potential alternative for antibiotics. Many of these cationic peptides are capable of killing bacteria, viruses and even cancer cells by recognizing negatively charged membrane surfaces [1–3].

Protegrin-1 (PG-1), is an antimicrobial peptide that was originally isolated from porcine leukocytes [4]. It is a small cysteine- and arginine-rich peptide with a molecular weight of 2000 Da. It shows potent antimicrobial activity *in vitro* against many Gram-negative and Gram-positive bacteria, including *Escherichia coli* [4], *Listeria monocytogenes* [4,5] and *Neisseria gonorrhoeae* [6,7]. In addition, it has been shown to have antifungal properties against the fungus *Candida albicans* [8] as well as antiviral properties against HIV-1 [9]. Although PG-1 also displays appreciable cytotoxicity toward eukaryotic host cells [10], its overall properties make it an interesting lead compound for designing analogs that could be useful therapeutics [11].

Native PG-1 (RGGRLCYCRRRFCVCVGR-NH_2) contains 18 amino acid residues and has no negative charges because the carboxy-terminal end is amidated. Its overall charge is +7, with six positive charges contributed by the Arg side-chains and one by the unmodified *N*-terminus. Investigations by solution NMR spectroscopy [12,13] showed that PG-1 possesses two intramolecular disulfide bonds which stabilize a β-sheet structure comprised of two antiparallel strands linked by a distorted β-turn. It has been found that the two disulfide bonds are crucial for its antimicrobial activity in media containing physiological salt concentrations [5,11].

Although the mode of action of PG-1 is not fully understood, there is evidence that the microbicidal activity of PG-1 stems from its ability to disrupt the cell membranes of microorganisms. In particular, a

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synthetic all-D amino acid enantiomer of PG-1 exhibits both identical activity to the all-L native peptide counterpart [14] and the same effect on membrane model systems [15]. Similarly, all-D magainin, cecropins and melittin showed comparable antibacterial and hemolytic activity to their naturally occurring L forms [16,17], although this is certainly not the case for all L- and D-forms of antimicrobial peptides [18]. This indicates that the action of PG-1, like several other antimicrobial peptides, does not involve stereo-specific receptors; rather, the action is the result of direct interactions with the lipid matrix of the plasma membrane. Therefore, to elucidate the mechanism of action of PG-1, it is crucial to study the interactions of PG-1 with the lipids found in cell membranes. PG-1 has been shown to cause ion leakage in planar lipid bilayers [19] and form stable pores in fully hydrated fluid membranes [20]. Extensive solid-state NMR studies have shown that PG-1 inserts at an angle into the membrane and it can induce an isotropic phase in anionic bilayers containing phosphatidylglycerol (PG) while only causing orientational disorder in zwitterionic phosphatidylcholine (PC) bilayers [21–24]. In addition, PG-1 was also found to insert readily into an anionic PG monolayer, but significantly less so into a PC film [25]. Using DSC (differential scanning calorimetry), we have studied the effect of PG-1 on various model membrane systems emphasizing the role of the different membrane architecture of bacterial and erythrocyte membranes. We found that the peptide had no effect on the phase behavior of neutral dipalmitoyl-phosphatidylcholine (DPPC) and sphingomyelin, which are among the main components of mammalian plasma membranes (data not shown, see also Ref. 25). However, as described before, significant effects were observed for the negatively charged phospholipid DPPG, which is a main component of many bacterial cytoplasmic membranes. Clearly, electrostatic interactions between PG-1 and negatively charged bacterial cell membranes are important for its antimicrobial activity. However, other negatively charged phospholipids can be found in mammalian cell membranes, e.g. PS and PA, which would give similar long-range electrostatic interactions. It should be noted that in mammalian cells PS is normally located almost exclusively in the inner leaflet of the bilayer but increased amounts of this lipid accumulate in the outer leaflet in certain cancer cells [26]. It is unclear whether PG-1 can discriminate between these three acidic phospholipids. So far, little attention has been paid to this point for PG-1 and related antimicrobial peptides.

In this study, we have systematically compared the impact of the different headgroup structure of acidic phospholipids on the interaction with the antimicrobial peptide PG-1. We have also analyzed the effects of different saturated fatty acid groups on these interactions for phospholipids having a PG headgroup and found that the membrane-perturbing action of PG-1 strongly depended on the headgroup structure of the lipid and in addition on the length of the saturated fatty acid chains.

MATERIALS AND METHODS

Lipids and peptide

1,2-Dimyristoyl phosphatidylglycerol (DMPG), 1,2-dipalmitoyl phosphatidylglycerol (DPPG), 1,2-distearoyl phosphatidylglycerol (DSPG), 1,2-dipalmitoyl phosphatidylserine (DPPS) and 1,2-dipalmitoyl phosphatidic acid (DPPA) were purchased as the sodium-salts from Avanti Polar Lipids, Inc. (purity >99%) and were used without further purification. Stock solutions of DPPA were prepared in chloroform/methanol (1 : 2 v/v), DPPS and DPPG were dissolved in chloroform containing 10 vol% methanol. Before and after the experiments, degradation of the phospholipids was checked by thin layer chromatography, which showed only one spot using $CHCl_3/CH_3OH/NH_3$ (75 : 25 : 6, by vol.) as a solvent. PG-1 was synthesized using standard Fmoc chemistry as described elsewhere [13]. The stock solution of the peptide was prepared in 20 mM phosphate buffer (pH 7.4) with 0.01% acetic acid.

Preparation of liposomes

Appropriate amounts of the phospholipid stock solutions were dried under a stream of nitrogen and stored in vacuum overnight to totally remove the organic solvent. The lipid film was then dispersed in excess buffer (20 mM sodium phosphate, pH 7.4) prepared from doubly distilled H_2O containing a known volume of the PG-1 stock solution to yield the desired lipid-to-peptide molar ratio. The mixture was then hydrated at temperatures 10 °C above the liquid crystalline phase for 1 h with intermittent, vigorous vortex mixing. Then the samples were kept at room temperature for 2 h before measurements were made.

Differential scanning calorimetry

Calorimetric experiments were performed using a VP-DSC differential scanning calorimeter (Microcal, Northampton, MA). Prior to scanning, the samples were degassed for 10 min. For all samples, a scan rate of 30 °C/h was used. Samples were measured at least twice to ensure reproducibility. The total lipid concentration was 1.0 mg/ml. Data acquisition and analysis was performed using Microcal's Origin software. The enthalpy change of the phase transition, ΔH, was obtained from the area under the peak after normalization of the heat capacity functions to scan rate and mass of phospholipid in each sample. The phase transition temperatures are defined as the temperature at the peak maximum.

EXPERIMENTAL RESULTS

DSC heating scans illustrating the effects of PG-1 on the thermotropic phase behavior of DPPA liposomes are presented in Figure 1. Aqueous dispersions of pure DPPA exhibited a single, relatively energetic L_β to

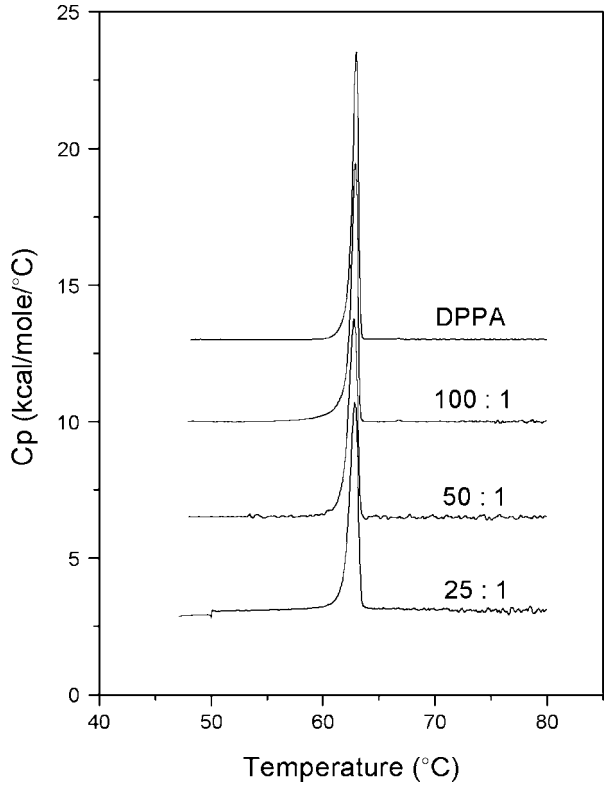

Figure 1 DSC heating scans of DPPA liposomes with and without PG-1 in 20 mM sodium phosphate, pH 7.4, scan rate 30 °C/h; lipid-to-peptide molar ratios indicated in the figure.

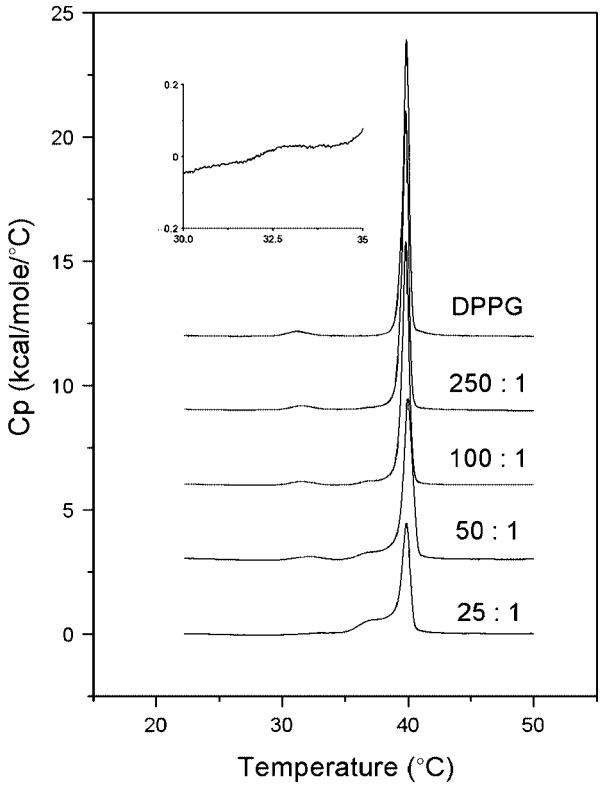

Figure 2 DSC heating scans of DPPG liposomes with and without PG-1 in 20 mM sodium phosphate, pH 7.4, scan rate 30 °C/h; lipid-to-peptide molar ratios are indicated in the figure. The inset shows the pretransition range for the lipid-to-peptide molar ratio of 25 : 1.

L_α phase transition near 63 °C. The addition of PG-1 has only minor effects on the phase behavior of DPPA liposomes, even at high concentrations of the peptide. PG-1 did not alter the temperature of the main transition. However, the presence of the peptide had a minor broadening effect on the half-width of the main transition, which is a measure for the cooperativity of the phase transition.

DSC heating scans illustrating the effects of PG-1 on the thermotropic phase behavior of DPPG liposomes are presented in Figure 2. Aqueous dispersions of DPPG exhibited two endothermic events upon heating, a pretransition at 31.3 °C and a main transition or chain melting phase transition centered at 39.9 °C. The pretransition arises from the conversion of the $L_{\beta'}$ to the $P_{\beta'}$ phase and the main transition from the conversion of the $P_{\beta'}$ to the L_α phase [27]. The incorporation of PG-1 significantly altered the thermotropic phase behavior of DPPG (Figure 2). The pretransition was detectable in the presence of PG-1 at all lipid-to-peptide molar ratios investigated. While its enthalpy decreased with increasing peptide concentration, the pretransition temperature increased gradually from 31.3 to 32.9 °C (Table 1). Most remarkably, in addition to the main transition at 39.9 °C an additional transition at 36.9 °C was observed even at low concentrations of PG-1 (lipid-to-peptide molar ratio of 100 : 1) and this transition markedly increased upon increasing the concentration of the peptide. This transition reflects the fraction of lipid domains affected by PG-1 as has been proposed earlier for other antimicrobial peptides [28,29]. The main transition temperature at 39.9 °C remained unaffected by the presence of PG-1 while the half-width of the main transition increased along with the peptide concentration. Interestingly, the total enthalpy of the chain melting transition, i.e. the sum of the enthalpy of the peptide-rich and poor domains for the lipid-to-peptide molar ratios of 100 : 1 to 50 : 1 is comparable to the value of pure DPPG. However, at the lipid-to-peptide molar ratio of 25 : 1, there was a significant drop in enthalpy and a marked increase in the half-width of the peak (Table 1).

DSC heating scans illustrating the effects of PG-1 on the thermotropic phase behavior of DPPS liposomes are presented in Figure 3. Aqueous dispersions of DPPS exhibited only one endothermic event, a main transition at 51.4 °C, which is because of the conversion of the L_β to the L_α phase [30]. A minor transition is detected at the high temperature side that decreases upon rescans and particularly upon addition of PG-1. This suggests that this transition may be due to incomplete hydration of the pure lipid. Further, at lipid-to-peptide molar ratios of 100 : 1 to 50 : 1, PG-1 had slight effects on the main transition of DPPS liposomes with a moderate

Table 1 Pretransition and main transition temperatures (T_{pre}, T_m) and enthalpies (ΔH_{pre}, ΔH_m) of different liposomes in the presence and absence of protegrin-1. $\Delta T_{1/2}$ is the half-width of the peak of the main phase transition

Lipid : peptide mol : mol	T_{pre} (°C)	ΔH_{pre} (kcal/mol)	T_m (°C)	ΔH_m (kcal/mol)	$\Delta T_{1/2}$ (°C)
DPPA			62.9	8.7	0.67
100 : 1			62.9	8.4	0.70
50 : 1			62.9	8.3	0.72
25 : 1			62.9	8.1	0.73
DPPS			51.4	8.9	0.43
100 : 1			51.3	8.4	0.33
50 : 1			51.3	8.2	0.49
25 : 1			51.1	7.4	1.54
DPPS (cooling)			50.4/51.1[a]	8.9	0.43
100 : 1			50.4/51.2/48.8[a]	8.8	0.39
50 : 1			50.5/51.1/48.8[a]	8.5	0.38
25 : 1			50.5/51.0/48.9[a]	6.9	1.19
DPPG	31.3	0.42	39.9	8.5	0.53
100 : 1	31.6	0.37	39.9/37.1[b]	8.5/0.71[c]	0.56
50 : 1	32.2	0.30	39.9/36.9[b]	8.1/1.15[c]	0.60
25 : 1	32.9	0.18	39.9/37.1[b]	4.56/2.04[c]	0.78
DMPG	10.9	0.57	21.2/24.2[d]	6.2	0.27
50 : 1	10.2	0.42	21.0/23.4[d]	5.5	0.34
25 : 1	9.0	0.17	20.7/22.1[d]	4.1	0.40
DSPG			53.5	9.7	0.56
50 : 1			54.0	9.5	0.43
25 : 1			54.3	9.4	0.45

[a] Additional transitions observed upon cooling.
[b] $T_{m,\ domain}$.
[c] $\Delta H_{,\ domain}$.
[d] $T_{posttransition}$.

reduction in enthalpy (Table 1). However, at a lipid-to-peptide molar ratio of 25 : 1, the cooperativity of the main phase transition decreased strongly and the enthalpy was reduced significantly as compared to the pure lipid (Table 1). It should be noted that in cooling scans, additional peaks were observed in the excess heat capacity profiles. While the peak at the high temperature side was also detected for pure DPPS being better resolved for the peptide samples, a peak at the low temperature side was only be seen in the presence of PG-1 (see arrow in Figure 4). This peak increased by increasing the concentration of PG-1 on expense of the major peak. Owing to the strong overlap of these transitions, it was not possible to obtain a reasonable fit in order to calculate the exact enthalpies of the individual transitions. However, this shoulder indicated the potential formation of peptide-rich and peptide-poor domains in PS. Nevertheless, the effect is kinetically limited as it is only seen on cooling scans and in addition the effect is weaker compared to the DPPG matrix. Since PG-1 exhibited the strongest effect on DPPG as compared to DPPS and DPPA, we further investigated the impact of the acyl chain architecture of the lipid on the peptide interactions using DMPG and DSPG. These experiments were performed at higher peptide concentrations, i.e. a lipid-to-peptide molar ratio of 50 : 1 and 25 : 1, respectively.

DSC heating scans illustrating the effects of PG-1 on the thermotropic phase behavior of DMPG liposomes are presented in Figure 5. Aqueous dispersions of DMPG exhibited a complex heat capacity profile, a pretransition near 10.9 °C, a main transition at 21.2 °C and a 'posttransition' around 24.5 °C together called the broad main transition region [31]. The pretransition arises from the conversion of the $L_{\beta'}$ gel to the $P_{\beta'}$ gel phase. The broad main transition had been found by DSC [32], light scattering [33], as well as ESR [34] and was shown to be highly dependent on the solution ionic strength. Using electron microscopy combined with calorimetry and viscometry, Heimburg [35] proposed that the occurrence of the broad main transition is due to the formation of a high-viscosity extended three-dimensional network of lipid membranes. On the other hand, Riske *et al.* [36] suggested that for salt concentrations below 100 mM the melting of the hydrocarbon chains occurs through the formation of an

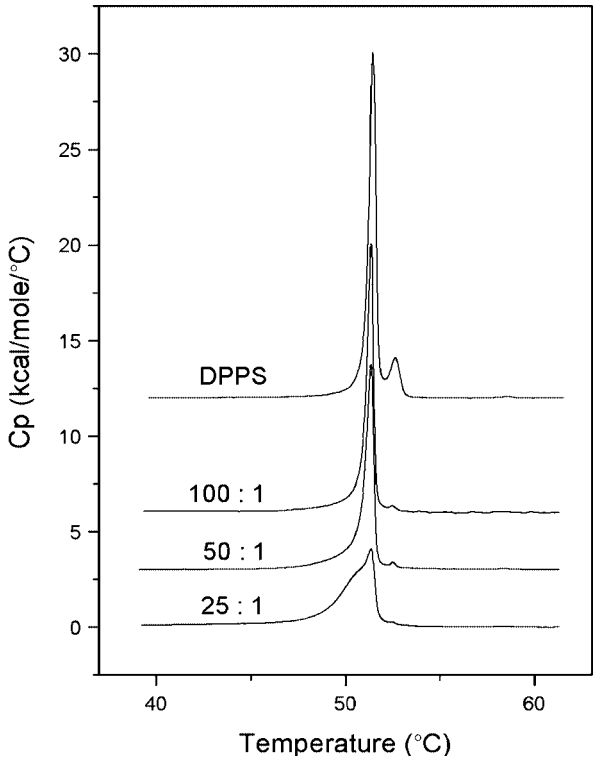

Figure 3 DSC heating scan of DPPS liposomes with and without PG-1 in 20 mM sodium phosphate, pH 7.4, scan rate 30 °C/h; lipid-to-peptide molar ratios are indicated in the figure.

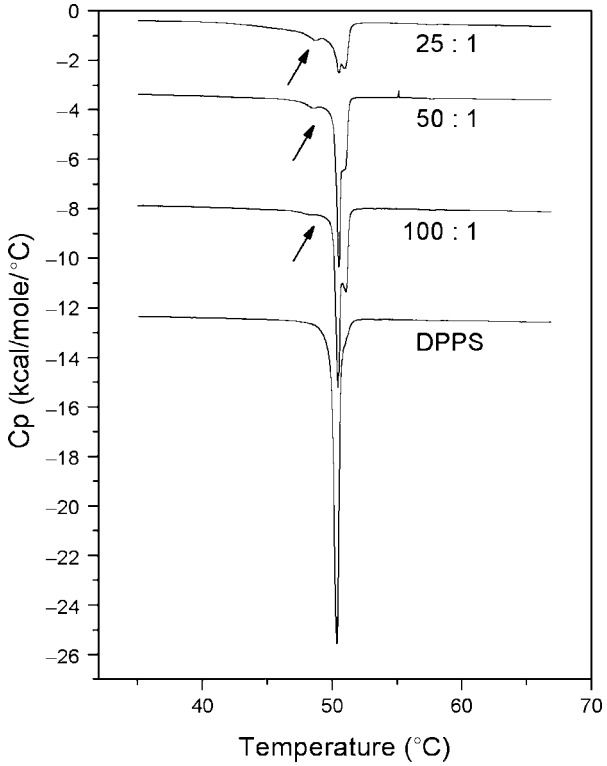

Figure 4 Cooling scan of DPPS liposomes with and without PG-1 in 20 mM sodium phosphate, pH 7.4, scan rate 30 °C/h; lipid-to-peptide molar ratios are indicated in the figure.

intermediate defect-rich phase. Although the physical basis of this behavior is not fully understood, both thermal events in the broad main transition region are involved in chain melting. The interaction of PG-1 with DMPG clearly alters the thermotropic phase behavior of DMPG liposomes. From the data presented in Figure 5 and Table 1, it is found that the temperature of both pretransition and main transition decreased significantly upon increasing the concentration of the peptide. Furthermore, the 'posttransition' became less and less pronounced by increasing the concentration of the peptide, whereby its temperature decreased strongly from 24.2 to 22.1 °C. Moreover, the enthalpy of both pre- and main phase transition was reduced rather markedly even at low concentrations of the peptide (molar ratio 50 : 1). In particular, a significant drop of the enthalpies is observed at the lipid-to-peptide molar ratio of 25 : 1 that is reduced by ~70% for the pretransition and ~35% for the main transition compared with the value of the pure lipid.

The heat capacity functions obtained for the effect of PG-1 on the thermotropic phase behavior of DSPG liposomes are presented in Figure 6. Under low ionic strength and lipid concentration, aqueous dispersions of pure DSPG only show a very cooperative main phase transition at 53.5 °C which arises from the conversion of an L_β phase to the L_α phase [27]. The presence of PG-1 increased the temperature of the main phase transition, from 53.5 to 54.3 °C. Furthermore, the enthalpy was not markedly affected and the cooperativity of the main phase transition only decreased slightly.

DISCUSSION

An intriguing feature of antimicrobial peptides is their ability to distinguish between prokaryotic and eukaryotic cells. Most researchers believe that this is because the lipid composition of the prokaryotic cell membrane is different from that of a eukaryotic cell. The outer leaflet of mammalian cell membranes is mainly comprised of zwitterionic PC, sphingomyelin and cholesterol. In contrast, bacterial membranes contain substantial amounts of negatively charged phospholipids such as PG and cardiolipin [1]. Therefore, vesicles made of PG lipids are widely used to mimic bacterial cell membranes. However, some antimicrobial peptides have been found to have anticancer activity [37–39]. It has been shown that cancer cells include varying proportions of negatively charged phosphatidylserine in the outer leaflet of their membranes compared to normal healthy cells [26], and it has been suggested that the small difference in the amount of PS might be sufficient for recognition by antimicrobial peptides [39]. Can antimicrobial peptides distinguish between these negatively charged phospholipids?

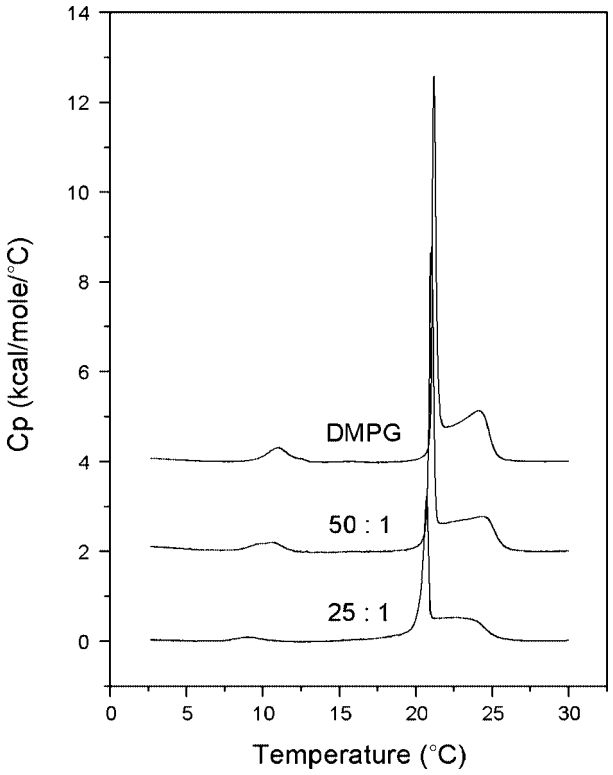

Figure 5 DSC heating scans of DMPG liposomes with and without PG-1 in 20 mM sodium phosphate, pH 7.4, scan rate 30 °C/h; lipid-to-peptide molar ratios are indicated in the figure.

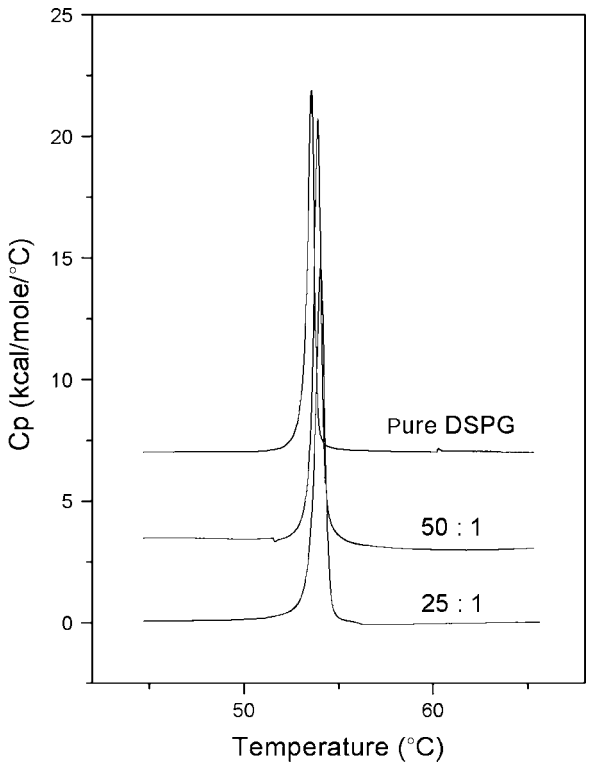

Figure 6 DSC heating scans of DSPG liposomes with and without PG-1 in 20 mM sodium phosphate, pH 7.4, scan rate 30 °C/min; lipid-to-peptide molar ratios are indicated in the figure.

In this work, we found that the antimicrobial peptide PG-1 interacts with membranes in a manner that depended not only on the lipid headgroup structure but also on the lipid acyl chain length. In particular, PG-1 had a very minor effect on the thermotropic phase behavior of DPPA bilayers. The temperature and the enthalpy of the main phase transition were only slightly affected. Moreover, the presence of the peptide did not cause large changes in the half-width of the main transition, which is a measure for the cooperativity of the phase transition. Therefore, we conclude that PG-1 only slightly perturbs the structure of the DPPA bilayer.

On the other hand, PG-1 had a significant effect on the phase behavior of DPPG bilayers. In addition to the unperturbed lipid fraction, a new transition was found at a lower temperature even with very small amounts of PG-1. The existence of two phases can be explained by phase separation within the liposomes resulting in clusters of pure lipid and peptide-rich lipid domains [40]. This kind of lipid-peptide domain has also been found when other antimicrobial peptides interact with DPPG, such as human neutrophil defensin HNP-2 [28], PGLa [29], magainin [41] and rhesus theta defensin 1 (RTD-1) [42]. The physical basis of the peptide-lipid domain is not completely understood. ^{31}P solid-state NMR studies have shown that PG-1 can induce the formation of an isotropic phase in POPC/POPG (1-palmitoyl-2-oleoyl phosphatidylcholine/1-palmitoyl-2-oleyl phosphatidylglycerol) vesicles (3 : 1 molar ratio) [21]. It has been shown previously that the surface charge density can alter membrane properties and charge neutralization can induce nonlamellar phase formation [43]. Furthermore, it was also reported for melittin that the surface charge density can modulate the peptide effect on membranes [44]. However, on the basis of the calorimetric data we cannot deduce the aggregation form of the lipids, which needs further structural investigations.

At the lipid-to-peptide molar ratios from 100 : 1 to 50 : 1, the enthalpy of the whole transition was comparable to the value of pure DPPG. However, at a lipid-to-peptide molar ratio of 25 : 1, the enthalpy of the main transition, i.e. of the peptide-rich and pure lipid domains, markedly decreased (Table 1). This result indicates that PG-1 binds in a concentration-dependent manner to DPPG. Peptides or proteins interacting with the hydrophobic regions of membranes often exhibit a large reduction in the transition enthalpy [45,46]. Therefore the strong decrease in the enthalpy at the high peptide-to-lipid molar ratio may be explained by a state change of the peptide from a surface associated state to a state in the hydrophobic region of the membranes, as also suggested by Huang and coworkers [47]. Similarly, Shai [48] also proposed that the peptides

initially bind onto the surface of a membrane in a carpet-like manner and that permeation of the membrane occurs only after a threshold concentration of the peptide has been reached, which may result in deeper insertion of the peptides into the hydrophobic core as well as transient pore formation. The addition of PG-1 to DMPG liposomes also caused a significant reduction of the enthalpy, while PG-1 had no effect on the main transition enthalpy of DSPG. However, the main phase transition temperature of DSPG increased which may be because of the screening of the negative headgroup charge upon binding of the peptide as reported earlier for the binding of cations to negatively charged lipids [49]. These observations suggest that PG-1 penetrates into the hydrophobic region of both DMPG and DPPG, but not into DSPG under our experimental conditions.

These differences may be explained in a way that for a series of phospholipids with identical headgroups, the van der Waals interactions between the hydrocarbon chains increase with chain length and will control the physical properties of the lipids [50]. It is possible that the hydrocarbon chains of DSPG pack rather tightly in comparison to DPPG and DMPG. This in turn may result in a less dense interface for the shorter chain analogs, thereby reducing the energy barrier for the peptide to penetrate. Moreover as mentioned above, DMPG exhibits a peculiar chain melting behavior that is accompanied by networking [35] and/or a defect-rich phase [36]. Both conditions would facilitate the interaction with amphipathic cationic peptides, resulting in a deeper insertion of PG-1 and perturbation of the hydrocarbon chain. This is in accordance with the strong decrease of the main transition temperature and enthalpy in the case of DMPG and the formation of peptide-enriched lipid domains at lower temperature in the case of DPPG.

During heating scans, PG-1 has a minor effect on the thermotropic phase behavior of DPPS bilayers at lipid-to-peptide molar ratios from 100 : 1 to 50 : 1. In this concentration range the cooperativity of the main transition is not strongly affected by the presence of PG-1, which indicates that the peptide does not perturb the bilayer structure. This may be explained again by a tighter packing of DPPS as compared to DPPG that can be inferred from the higher chain melting transition of DPPS. However, at high peptide concentration (lipid-to-peptide molar ratio of 25 : 1), both enthalpy and cooperativity of the chain melting transition decreased significantly, which indicates that PG-1 now inserts into the hydrophobic region of DPPS bilayers. By analogy to the DPPG/PG-1 system, we can assume that PG-1 also has a concentration-dependent mode of interaction when binding to DPPS liposomes. PG-1 has a marked effect on the phase behavior of DPPS during the DSC cooling scans indicating that PG-1 can preferentially interact with the fluid phase of DPPS. In the fluid membranes, PG-1 may be inserted into the hydrophobic region, thus disrupting the lipid organization. Furthermore, rescans showed complete reversibility of this behavior, which implied that PG-1 could be excluded from the hydrophobic region of the bilayer, when DPPS adopts the gel phase upon cooling. Preferential interaction with the fluid lipid phase has also been shown for the cyclic beta-sheet antimicrobial peptide gramicidin S [51].

Both phospholipids, PG and PS, possess a net negative charge of 1. While PG, the main component of bacterial cell membranes, has only one negative charge (the phosphodiester group), PS, which is the major acidic lipid in mammalian membranes, possesses two negative charges and one positive charge at neutral pH. Still it is not clear why there are such significant differences in the interaction of PG and PS with antimicrobial peptides. This in part might be related to a different hydrogen bonding potential of both headgroups resulting in different packing densities. While it was proposed that the condensation of PS suggests intermolecular hydrogen bonding between their headgroups [52], a molecular dynamics simulation showed that intermolecular hydrogen bonding between PG headgroups are rare [53]. Studies on model membranes have demonstrated that PG binds monovalent and divalent cations less efficiently than PS. This is believed to be due to the presence of the carboxyl group of the PS headgroup, which is easily accessible from the aqueous phase. Moreover, the carboxyl groups have a weaker effect on the orientation of the water dipoles than the phosphate groups [54,55]. The cytotoxic peptide melittin was indeed found to bind and internalize more efficiently when the bilayer contained PS rather than PG [56]. In contrast, the α-helical antimicrobial peptide magainin-2 is more effective at inducing leakage in liposomes composed of PG than in the PS liposomes, suggesting that the membrane charge is not the only factor determining the rate of leakage [41]. These results are in agreement with the outcome of our experiments. One factor to consider is the membrane curvature. PS, PA and CL are known to form the inverse hexagonal (H_{II}) phase under conditions of reduced interlipid electrostatic repulsion [57,58]. The binding of PG-1 would locally reduce electrostatic repulsion between the surrounding PS or PA, thus imposing negative curvature strain on the membrane. However, our DSC results indicate that PS and PA still behave differently, suggesting that the effects of the antimicrobial peptide on membrane curvature is also not sufficient to predict the potency of this peptide. However, in this context it should be mentioned that PG-1 may induce membrane curvature strain under certain conditions. For example, a hydrophobic mismatch between dilauryl-PC bilayer and PG-1 length resulted in local membrane thinning, which in turn could be the precursor to toroidal pores that implies positive membrane curvature [22].

In all, we can conclude that the bilayer interface as determined by the structure of the headgroups is an important parameter in controlling selective interactions of antimicrobial peptides with membranes. Moreover, this interaction can be further fine-tuned through the nature of the hydrophobic fatty acid groups of the phospholipids.

In closing, it is interesting to speculate on how the formation of PG-1-DPPG lipid domains may potentially contribute to the antimicrobial activity of the peptide. For those bacteria whose cytoplasmic membranes are mostly composed of a mixture of PG and PE lipids, such peptide-PG domains could simply lead to unmixing of the two lipids, leaving patches of the bilayer of PE, which are known to form a nonlamellar phase [38]. Indeed, phase separation in PG/PE mixtures resulting in PG-enriched domains has been observed for HNP-2 [28], RTD-1 [42] and PGLa [59]. Further investigations of this phenomenon are currently in progress in our laboratories. Another implication of this work is that bacteria could in principle protect themselves against membrane-active antimicrobial peptides by altering the lengthening of the fatty acids in their membranes. Indeed it is well known that the presence of various membrane-perturbing toxic compounds can lead to changes in the fatty acid composition [60,61] and sometimes even the head group composition [62] of bacterial membranes. Nevertheless, this is a relatively slow response, and since cationic antimicrobial peptides typically have a fast action, such an adaptation may be too slow to protect exposed bacteria.

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