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Amphipathic Helical Cationic Antimicrobial Peptides Promote Rapid Formation of Crystalline States in the Presence of Phosphatidylglycerol: Lipid Clustering in Anionic Membranes

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ABSTRACT Five AHCAPs exhibiting a broad-spectrum of antimicrobial activity, were examined with regard to their action in lipid mixtures with two anionic lipids, PG and CL. We find that all of the peptides studied were capable of promoting the formation of crystalline phases of DMPG in mixtures of DMPG and CL, without prior incubation at low temperatures. This property is indicative of the ability of these peptides to cluster CL away from DMPG. In contrast, the well studied antimicrobial cationic peptide magainin 2 does not cluster anionic lipids. We ascribe the lower anionic lipid clustering ability of magainin to its low density of positive charges compared with the five other AHCAPs used in this work. The peptide MSI-1254 was particularly potent in segregating these two anionic lipids. Consequently, clusters enriched in DMPG appear in a lipid mixture with CL. These can rapidly form higher temperature crystalline phases because of the increased permeability of the bilayer caused by the AHCAPs. The polyaminoacids, poly-L-Lysine and poly-L-arginine are also very effective in causing this segregation. Thus, the clustering of anionic lipids by AHCAPs is not confined only to mixtures of anionic with zwitterionic lipids, but it extends to mixtures containing different anionic headgroups. The resulting effects, however, have different consequences to the biological activity. This finding broadens the scope for which an AHCAP agent will cluster lipids in a membrane.

INTRODUCTION

Large numbers of AHCAPs have been characterized, having great variability in structure and in antimicrobial activity against a wide range of infectious agents. These AHCAPs have evolved to combat pathogens as a component of the innate defense system of the host. The two common elements they exhibit are a net cationic charge and an amphipathic structure. The short linear α-helical peptides are one of the most abundant class of antimicrobial peptides and have been studied widely (1). AHCAPs are generally unstructured in solution but due to their cationic charge, they are electrostatically attracted to the anionic bacterial membranes where they tend to become helical. This initial first interaction of the AHCAP with the outer leaflet of the cytoplasmic bacterial membrane is a crucial determining step in the events that lead to their bacteriostatic or bactericidal activity. Hence, the importance of understanding how this interaction affects rearrangements in the membrane resulting from the segregation of one kind of lipid from another, vis a vis the formation of defects and permeabilizing efficiency. PG and CL are the two most abundant anionic lipids in bacterial membranes, for both Gram positive as well as Gram negative bacteria. Understanding their differential interaction with AHCAPs then is essential to a deeper understanding of membrane disruption, a question that has occupied scientists in the field for many years.

DMPG has been one of the most studied negatively charged phospholipids due to its ability to exhibit low temperature phase polymorphism. In addition to the gel phase, there are two more highly ordered, less hydrated low temperature phases. One is the subgel phase that forms rapidly at low ionic strength (2 mM NaCl) below 0°C (3 min at −10°C) (2). In the presence of high ionic strength, covering the range of physiological salt concentrations (>100 mM NaCl), prolonged low temperature incubation (several days at 0°C) leads to the gradual formation of a highly crystalline gel phase (HC phase), which is more ordered than the subgel phase (3–5). This highly ordered crystalline HC phase exhibits hysteresis and is transformed directly into the L_α phase at 42°C, with a transition enthalpy of 14 kcal/mol and without the intermediate formation of the L_β phase (4). The HC phase is not immediately reformed on cooling, but again requires exposure to low temperature for days to reform. The melting of the HC phase is accompanied by large morphological changes leading to vesiculation (4). Recently Kinoshita et al. (6) found that the HC phase could be formed more rapidly at low temperature if the DMPG was first completely hydrated by incubation for 2 h at 65°C. The conversion to the HC phase was only partial and the rate depended on the lipid concentration and the ionic

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Abbreviations used: AHCAPs, amphipathic helical cationic antimicrobial peptides; CL, cardiolipin; DMPG, dimyristoyl phosphatidylglycerol; DPPG, dipalmitoyl phosphatidylglycerol; DSC, differential scanning calorimetry; HC phase, highly crystalline gel phase; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PG, phosphatidylglycerol; PLL, poly-L-Lysine; PLA, poly-L-arginine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; SUVs, small unilamellar vesicles; TOCL, tetraoleoyl cardiolipin.

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TABLE 1 Sequence of peptides studied and comparison with magainin 2

Peptide name	Sequence	Overall charge at neutral pH	Overall charge/residue at neutral pH
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	+3.5	0.15
MSI-78 (Pexiganan)	GIGKFLKKAKKFGKAFVKILKK-NH_2	+10	0.45
MSI-103	KIAGKIAKIAGKIAKIAGKIA-NH_2	+7	0.33
MSI-469	Octyl-KIAGKIAKIAGKIAKIAGKIA-NH_2	+6	0.29
MSI-843	Octyl-OOLLOOLOOL-NH_2	+6	0.6
MSI-1254	Octyl-XXLLXXLXXL-NH_2	+6	0.6

O, ornithine; X, 2,4 diaminobutyric acid (Dab).

strength. In contrast, without low temperature incubation an L_β phase is formed that reversibly transforms into the L_α phase at 23°C, with an enthalpy of 5 kcal/mol in 150 mM NaCl (4).

Recently we had undertaken a study of the group of five AHCAPs used in this study (MSI-78, MSI-103, MSI-469, MSI-843, and MSI-1254) but in lipid systems mimicking the outer and inner membranes of Gram-negative bacteria (7). In that study we reported on the ability of these peptides to cluster anionic lipids away from zwitterionic ones. We also determined their antimicrobial potency, their ability to penetrate the inner and outer membranes of Gram-negative bacteria, as well as their interactions with lipopolysaccharides and lipoteichoic acid.

In this study we report on the unique property of these peptides of promoting the rapid conversion of DMPG bilayers to higher melting phases. This phenomenon is indicative of the fact that the five MSI peptides, but not the naturally-occurring magainin 2, are capable of inducing the separation of PG and CL in a membrane. Because PG and CL are the dominant lipids in most bacterial membranes, our results provide insights into the function of potent AHCAPs and will be important in the design of novel peptide antibiotics. To our knowledge, this is the first observation in the literature of the rapid promotion of crystalline phases in PG bilayers by AHCAPs.

The amino acid sequences and number of charges of the peptides used in this work are presented in Table 1. A comparison is also made with the well-studied antimicrobial peptide, magainin 2.

EXPERIMENTAL PROCEDURES

Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Poly-L-lysine hydrobromide (molecular weight (MW) = 68,300), and poly-L-arginine hydrochloride (MW = 40,000) were purchased from the Sigma Chemical Co (Ontario, Canada).

Preparation of phospholipid vesicles

Lipid films were made by dissolving appropriate amounts of lipids in chloroform/methanol 2:1 (v/v) followed by solvent evaporation under a stream of nitrogen to deposit the lipid as a film on the walls of a tube. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap for 3–4 h. Dried films were kept under argon gas at −20°C until used. Films were hydrated with buffer or with an appropriate amount of peptide solution to obtain a specific lipid to peptide ratio and vortexed extensively to make MLVs. To obtain LUVs, MLVs were subjected to five cycles of freezing and thawing and further processed with 10 passes through two stacked 100 nm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA) in a high pressure barrel extruder (Lipex Biomembranes, Vancouver, BC). LUVs were kept on ice and used within a few hours of preparation. Lipid phosphorus was determined by the method of Ames (8).

DSC

Measurements were made using a Nano II Differential Scanning Calorimeter (Calorimetry Sciences, Linden, UT). The method was adapted from that reported by Epand et al. (9). MLVs were prepared by extensive vortexing at room temperature to hydrate the films with 20 mM PIPES buffer pH 7.4, 1 mM EDTA, 140 mM NaCl. Peptides were incorporated into the lipid by hydrating the lipid films with a solution of the peptide in buffer. The lipid suspension was placed in the calorimeter cell, brought to 0°C and scanned for several cycles of heating and cooling at 1°C/min. Curves were plotted with the program Origin v.7.0 and analyzed with the fitting program DA-2 provided by MicroCal (Northampton, MA). Buffer was placed in the reference cell and buffer versus buffer baselines, conducted at the same scan rate, were always subtracted from the sample scans.

RESULTS

DSC with DMPG/TOCL 75:25 mixtures

This lipid composition was chosen because it is a good mimic of the cytoplasmic membrane of Gram positive bacteria and the individual components appear well mixed before the addition of peptide, with a single transition temperature at 14–15°C (Fig. 1). Pure DMPG has a gel to liquid crystalline phase transition at 25°C and with pure TOCL this transition is below 0°C. Bacterial membranes are fluid and in the range of temperatures at which regular bacterial viability assays are carried out, 25–37°C, this lipid mixture is in the liquid crystalline state. The AHCAPs were added to the lipid mixture at room temperature, that is, at a temperature at which the binary mixture is in the liquid crystalline state. An optimum lipid to peptide ratio of 20 was chosen, to avoid clumping or micellization at higher peptide content and the dilution of the effects of the peptide at much higher lipid content. The concentration of peptide at the membrane in bacteria is much higher than that of the bulk solution and disruption of membranes occurs generally at low lipid to peptide ratios, in the range of those chosen for this study.

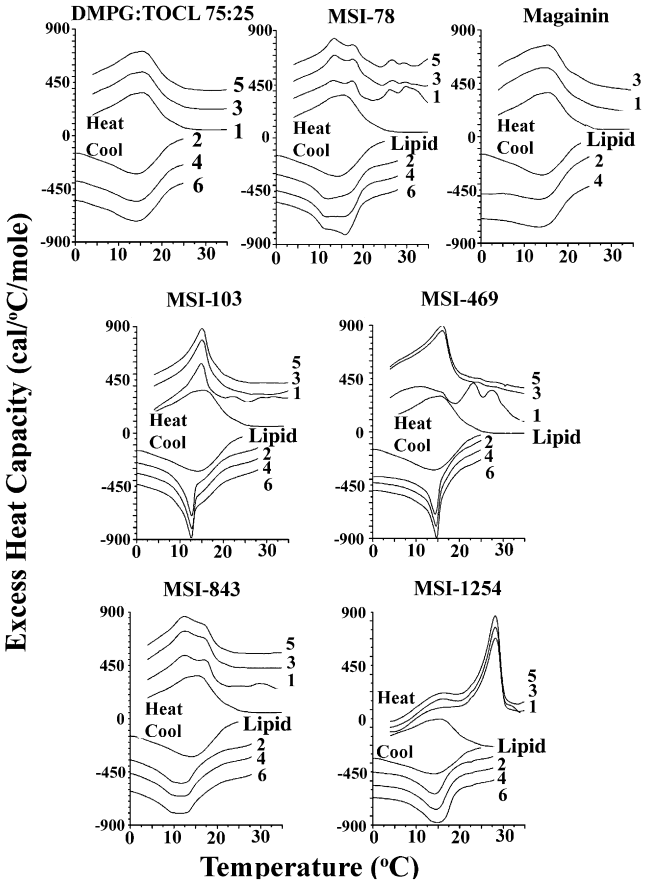

FIGURE 1 DSC in DMPG/TOCL 75:25 in absence and presence of peptides: Lipid films were hydrated at room temperature, with 20 mM PIPES, 1 mM EDTA, 140 mM NaCl, pH 7.4 or with peptide solutions in this buffer at a lipid to peptide ratio of 20. The scan rate was 1°C/min and the temperature range from 0 to 35°C. Odd numbers are heating scans and even numbers are cooling scans. When peptide is present, the first heating and cooling cycle in each figure corresponds to that of the lipid mixture without peptide, followed by two or three cycles containing the corresponding peptide.

The low melting temperature of the mixture allows heating to only 35°C, so denaturation of peptides in the presence of anionic lipid is less likely. The mixture itself has a gel to liquid crystalline transition at the same temperature as that of POPE/TOCL 75:25, a mixture used to study clustering in zwitterionic-anionic systems, that mimic the Gram negative cytoplasmic membrane (10,11). Although the phase transition is somewhat broader with the binary anionic mixture, it is still adequate to study the influence of AHCAPs on the phase transition. In this study, we deal with two lipids with different negatively charged headgroups as well as different acyl chain lengths, to obtain a mixture mimicking the Gram positive bacterial membrane, but with low enough gel to liquid crystalline phase transition temperature as well as high enough miscibility to be suitable for clustering studies by DSC. We have also investigated other mixtures of two anionic lipids but found in general that these exhibited poor miscibility even as a pure lipid mixture before peptide was added (see Supporting Material).

Pure DMPG was shown to be able to form a rapidly reversible L_β gel phase that converts to the L_α phase at 24°C. When this L_β phase is incubated for several days at 0°C in 140 mM NaCl the HC phase is formed, which is more ordered than the L_β phase and has a higher calorimetric ΔH at 40.6°C for conversion to the L_α phase of 14 kcal/mol, as opposed to 4.8 kcal/mol for the rapidly formed L_β phase of DMPG (4). This transition to the HC phase was found to be initiated by changes in the headgroup of the bilayer, most likely via the formation of interlipid hydrogen bonds. A stable high temperature gel phase does not appear in mixtures of DMPG with CL in the absence of peptide (Fig. 1, *upper left panel*) or in the presence of magainin 2 at L/P = 20 (Fig. 1, *upper right panel*), where the main transition temperature of the mixture remains at 14–15°C in successive cycles of heating and cooling.

A rapid transformation of the lipid mixture DMPG/TOCL 75:25 to higher transition temperatures is promoted by AHCAPs (Fig. 1). The peptides were added to lipid at room temperature and immediately placed in a cooled calorimeter. The temperature was equilibrated to 0°C and the scans were started immediately at a rate of 1°C/min. The DSC scans shown in Fig. 1 were done only up to 35°C to avoid thermal denaturation of some of the peptides. The samples spent a minimal time at 0°C. These scans were repeated by heating up to 45°C and the same results were obtained.

Unlike the transitions of the pure lipid mixture or the lipid mixture in the presence of magainin 2, the samples with the other AHCAPs exhibited a small hysteresis between heating and cooling curves. This hysteresis did not diminished by scanning at slower scan rates. In addition to the kinetics of the transition and instrumental factors, time and temperature dependent thermal denaturation of the peptide can contribute to the lack of complete reversibility. There are other additional factors including the time dependence of peptide aggregation; the time dependence of peptide binding to lipid; the slow conversion to lipid to the crystalline phase, all of which will be time and temperature dependent. Nevertheless, the lack of reversibility means that the system is not in true thermodynamic equilibrium and the transitions must be considered, at least in part, to be kinetically limited.

In the following paragraphs we discuss the results obtained with each of the individual AHCAPs studied.

MSI-78

In the presence of MSI-78 phase transitions occur above 25°C, suggesting that the peptide rapidly transforms some of the DMPG/TOCL 75:25 into intermediate stages of formation of the HC phase of DMPG. These new intermediate phases are metastable, they are not observed in cooling scans and are only partially reformed in subsequent heating scans because of kinetic effects. This behavior is consistent with

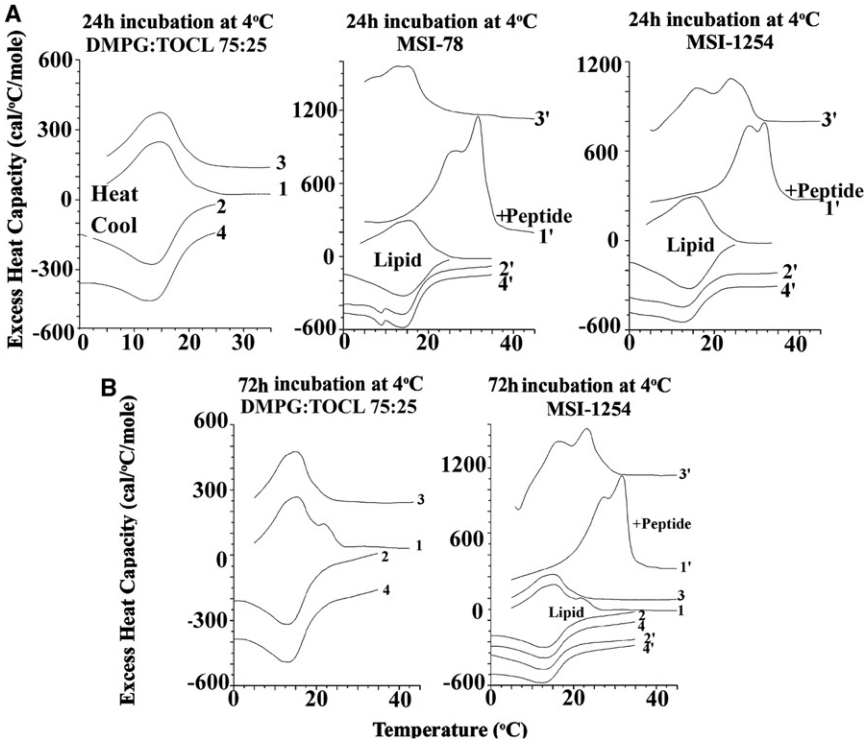

FIGURE 2 DSC in DMPG/TOCL 75:25 after incubation at 4°C in absence and presence of peptides. (*A*) 24 h incubation. (*B*) 72 h incubation. Lipid films were hydrated with 20 mM PIPES, 1 mM EDTA, 140 mM NaCl, pH 7.4 or with peptide solutions in this buffer at a lipid to peptide ratio of 20. The scan rate was 1°C/min, and the temperature range from 0 to 45°C. Odd numbers are heating scans and even numbers are cooling scans. Primed numbers correspond to lipid with peptide. When peptide is present, the first heating and cooling cycle in each figure corresponds to that of the lipid mixture without peptide, followed by two or three cycles containing the corresponding peptide.

the formation of metastable HC phases and not with the reversible binding of DMPG with the peptide.

MSI-103 and MSI-469

These peptides also produce higher temperature intermediate stages for conversion into the HC phase in DMPG in the first heating scan, which disappear in subsequent cooling. The main transition of the mixture itself becomes sharper, particularly in the cooling curve and the temperature of this transition is only slightly lowered compared with that for the pure lipid mixture. We suggest that there is formation of intermediate stages for conversion into the HC phase in DMPG; these intermediate states are metastable and are not observed after the first heating scan. It is interesting to note that there is almost no difference in the conversion to intermediate states between the peptide carrying an octyl chain (MSI-469) and the one without it (MSI-103).

MSI-843 and MSI-1254

These two short octyl-chain lipopeptides behaved quite differently from each other. MSI-843 exhibited similar behavior in the 1st heating scan as MSI-78 did, with the appearance of intermediate stages of the HC gel phase transition at higher temperatures, which disappear when cooling from the liquid crystalline state.

A remarkable dramatic effect is seen with MSI-1254, where a transition appears as a single peak at 28.2°C in each successive heating scans but it is absent in cooling scans. In the heating scans, this transition at 28.2°C coexists with the transition of the mixture at 14-15°C. The very marked hysteresis in this system can be explained by the rapid formation of the higher melting metastable semi-crystalline phase. The formation of this phase is rapid as it is observed in every subsequent heating scan, but it requires low temperature exposure for its formation, because it is not observed in cooling scans. The temperature of this transition with MSI-1254 is not as high as would be expected for a pure crystalline phase of DMPG, indicating that it is not as ordered as the HC phase and that there is only a partial transformation, possibly because it is mixed with some TOCL.

Although MSI-843 and MSI-1254 have similar chemical structures, MSI-1254 is much more effective in inducing changes in the phase behavior. It should be pointed out that each residue of 2,4-diaminobutyric acid in MSI-843 is substituted for ornithine in MSI-1254. This represents an addition of two methylene groups per residue. There are six of these residues in each of the two peptides. Hence MSI-1254 differs overall from MSI-843 by having an additional $C_{12}H_{24}$ that likely contributes to the greater potency of MSI-1254.

To confirm the presence of intermediate phases of the HC phase, we incubated MSI-78 and MSI-1254 for 24 h (Fig. 2 *A*) and MSI-1254 also for 72 h with DMPG/TOCL 75:25 (Fig. 2 *B*), using the lipid mixture in the absence of peptide as control. No change took place in the pure lipid mixture after 24 h incubation in 140 mM NaCl containing buffer (Fig. 2 *A*) and only a small change was observed after 72 h (Fig. 2 *B*). However, in the presence of either MSI-78 or MSI-1254 there was a further marked increase

TABLE 2 Values for the high temperature phase transition(s) in DMPG and DMPG/TOCL

Lipid*	T (°C)
DMPG incubated several days	41
DMPG + MSI-1254, fresh sample	32
DMPG/TOCL 75:25 + MSI-1254, fresh sample	28
DMPG/TOCL 75:25 + MSI-1254 incubated 24 h	27
	32
DMPG/TOCL 75:25 + MSI-1254 incubated 72 h	24
	32

*All measurements were done at 140 mM NaCl and pH 7.4, with 2.5 mg/mL total lipid. All incubations were carried out at 4°C. Lipid to peptide ratios were maintained at 20. Calculations were carried out on the first heating scan only.

in the temperature of the transition observed in the first heating scan (Table 2). This temperature was higher than that observed without preincubation (Fig. 1). Incubating the sample with MSI-1254 an additional 48 h did not result in further significant changes. These higher temperature transitions observed after incubating the lipid mixture with the two peptides were not completely reversible on cooling and reheating. There are thus several stable intermediate gel phases whose formation is dependent on the presence of peptide and on the time of incubation at low temperature.

The ability of these peptides to rapidly induce the formation of these higher temperature phases is of particular interest because it suggests that the peptides can induce the separation of two anionic lipids. It has been shown that DMPG alone can form high temperature melting phases but it requires incubation of the lipid for these crystalline phases to form (4). In this study, however, we show that mixtures of DMPG and TOCL in the absence of peptide can form higher melting phases only to a very limited extent after 72 h of incubation at 4°C (Fig. 2 *B*). In contrast, with these AHCAPs there is formation of intermediate higher melting phases in a matter of minutes (Fig. 1) that progress further to more thermally stable forms on prolonged incubation at low temperature (Fig. 2). Higher order phases are not formed by lipids having unsaturated acyl chains, such as TOCL, even after prolonged incubation. Therefore the higher melting phases must be enriched with DMPG, showing the segregation of DMPG from TOCL by the peptides.

Cationic polymers

Cationic polymers like PLL and PLA also produce higher melting phases without incubation in DMPG/TOCL 75:25 mixtures (Fig. 3, *upper panels*). A major peak appears at 37°C that is not recovered after the first heating cycle. The transition temperature of this higher melting peak is close to what is found with the HC phase of pure DMPG that forms after long incubation: 41–42°C for DMPG, 38°C with PLA and 36°C with PLL. These results indicate that both cationic polyaminoacids induce the phase separation of DMPG from TOCL and promote the rapid formation of the HC phase of DMPG.

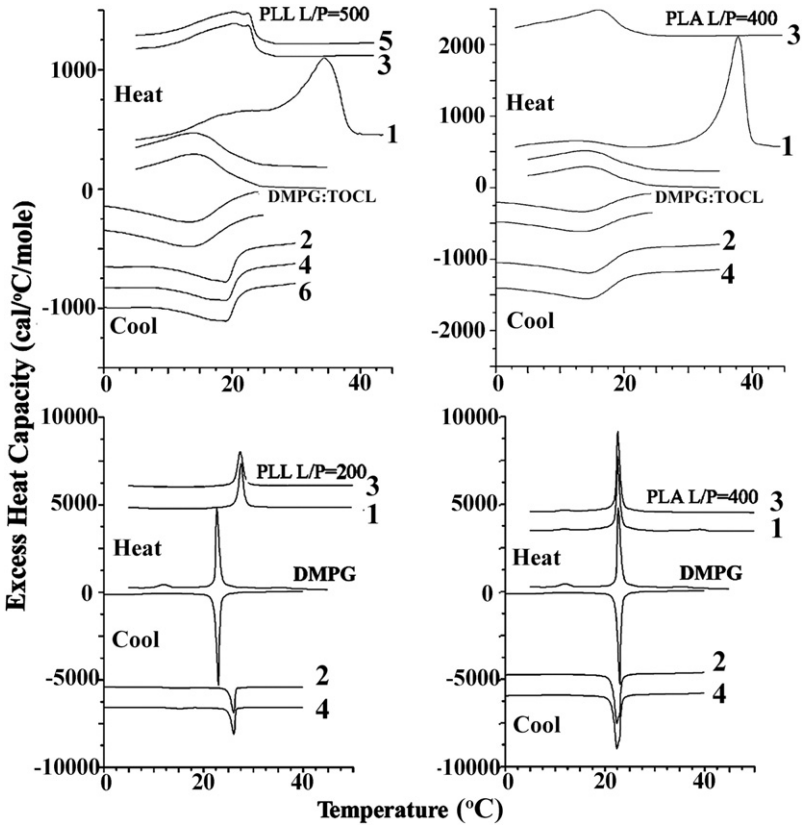

FIGURE 3 DSC of PLL (*left panels*) and PLA (*right panels*) in DMPG/TOCL 75:25 (*upper panels*) or in DMPG (*lower panels*). Lipid films were hydrated at room temperature with 20 mM PIPES, 1 mM EDTA, 140 mM NaCl, pH 7.4 or with polymer solution in this buffer at a lipid to polymer ratio of 500 for PLL and 400 for PLA in the lipid mixture and at lipid to polymer ratio of 200 for PLL and 400 for PLA in pure DMPG. The scan rate was 1°C/min and the temperature range was from 0 to 35°C. Odd numbers are heating scans and even numbers are cooling scans. When polymer is present, the first heating and cooling cycle in each figure corresponds to that of the lipid mixture without peptide, followed by three or two cycles in the presence of the corresponding polymer.

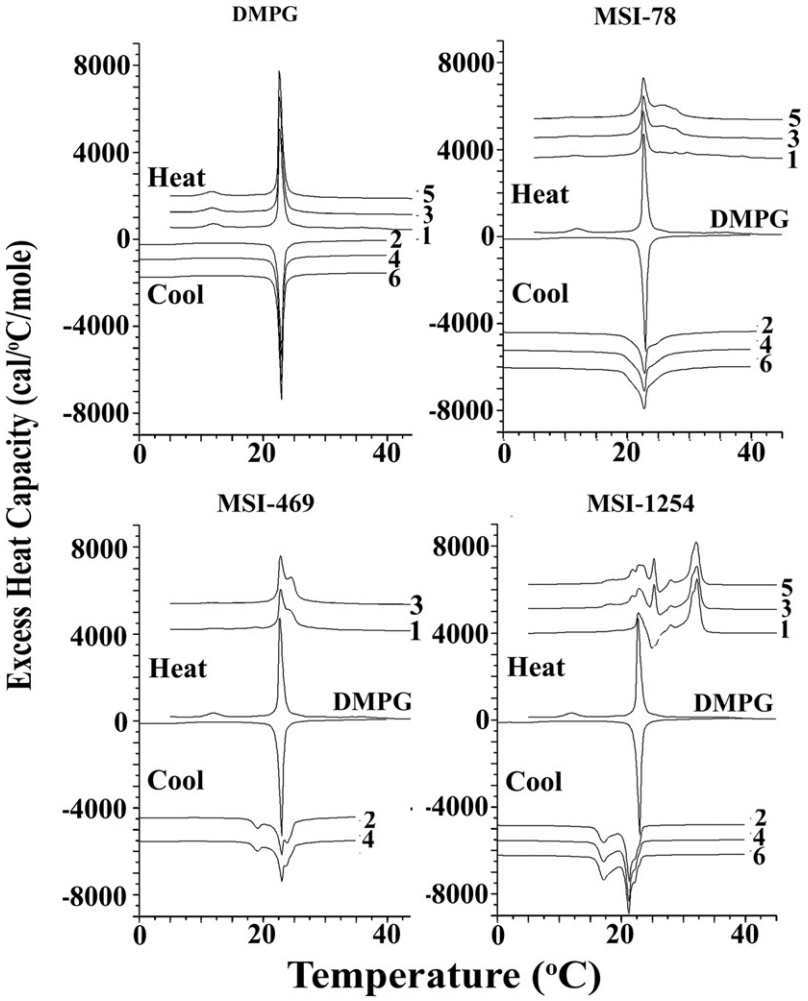

FIGURE 4 DSC in pure DMPG in absence and presence of peptides. Lipid films were hydrated at room temperature with 20 mM PIPES, 1 mM EDTA, 140 mM NaCl, pH 7.4 or with peptide solutions in this buffer, at a lipid to peptide ratio of 20. The scan rate was 1°/min, and the temperature range from 0 to 50°C. Odd numbers are heating scans and even numbers are cooling scans. When peptide is present, the first heating and cooling cycle in each figure corresponds to that of the lipid mixture without peptide, followed by two or three cycles containing the corresponding peptide.

Because the cationic polymers acquire helical structure in the presence of anionic lipids, CD spectra of these polymers was measured in the presence of SUVs of DMPG/TOCL 75:25 at 222 nm, as a function of temperature. Between 35 and 40°C there was no loss of secondary structure by the polymers (not shown), confirming that a change in conformation was not the cause of the calorimetric transitions observed.

Neither PLL nor PLA promote the formation of high temperature transitions with pure DMPG (Fig. 3, *bottom panels*), indicating that TOCL plays a role in the transformation. In the case of PLL there is a small shift in the main phase transition to higher temperatures. This change is stable on cooling and reheating. We believe it is a consequence of binding of the cationic polymer to pure DMPG, rather than formation of a stable more ordered crystalline phase because the shift in transition temperature is small and the new transitions are reversible.

DSC of AHCAPs with DMPG

Previous studies with pure PG bilayers have shown that their main phase transition is greatly affected by cationic antimicrobials (9,12). We studied how MSI-78, MSI-469, and MSI-1254 modify the phase transition of DMPG in the absence of TOCL, without prior incubation, at a lipid to peptide ratio of 20, the same ratio used with the lipid mixtures (Fig. 4). The formation of new higher melting phases is small with MSI-78 or with MSI-469, but with MSI-1254 multiple higher melting transitions appear (Fig. 4). There is a clear transformation to a precursor of the HC phase at 32.2°C similar to what was observed with the DMPG/TOCL mixture in the presence of this peptide. This peak exhibits hysteresis when cooling down from the liquid crystalline phase of the mixture, indicating kinetic effects. However, in comparison to the time period of several days to convert DMPG to more highly ordered phases, this process occurs in a matter of minutes in the presence of MSI-1254.

DPPG/TOCL 50:50 mixtures

Mixtures of TOCL with DPPG in place of DMPG were of interest due to the fact that the rate of formation of a crystalline phase is slower for DPPG than for DMPG (5). In addition, whereas DMPG forms a crystalline phase that converts

directly to the L_α at a temperature ~20°C higher than the $P_{\beta'}$-L_α transition temperature, in the case of DPPG it is only a few degrees higher (5,13,14).

Mixtures of DPPG/TOCL at different ratios exhibit multiple overlapping thermal transitions (see Supporting Material). Even poorer miscibility and more complex transitions are observed for mixtures of DPPG and DOPG (see Supporting Material) as well as mixtures of DPPG and DMPG in the middle range compositions, at pH 7 in 100 mM NaCl (15). Poor miscibility is a common feature of mixtures of two lipids with the same anionic headgroup. Binary lipid mixtures containing the same anionic headgroup, i.e., DMPG/DOPG 50:50 and DMPG/DSPG 50:50, also displayed multiple components in DSC (16). This lipid mixture was also used to study the effects of α-helical peptides and penetratin. It was concluded that these peptides interacted preferentially with the lipid in the liquid crystalline state to cause lipid segregation. Selectivity of peptide interaction with one of the lipid components can be a factor when the pure lipid mixture, in the absence of peptide, already exhibits the presence of separate domains, such as the mixture DMPG/DSPG 50:50 (16). In such cases, the peptide is not necessarily inducing lateral phase separation. However, with the mixtures of CL and PG, that are the focus of this study, we have chosen cases in which the two lipids are initially miscible. Hence initially there is no higher or lower melting lipid for the peptide to interact with because the lipids alone are well mixed and there is no separated lower melting lipid component.

We can also compare the effects of the peptides on single component lipids with that of lipid mixtures. This is clearest with the polyaminoacids, PLL and PLA (Fig. 3). With pure DMPG, the effects of the polymers are quite small and result in a small increase in the transition temperature. This would indicate preferential interaction with gel state lipid and not selection of the lower melting component. But the results obtained with these polyaminoacids in a mixture of DMPG and TOCL indicate preferential interaction of the peptides with TOCL, causing the formation of a metastable HC phase in DMPG. This could not be because the polyaminoacids preferentially interact with the liquid crystalline state; that would result in the lowering of the temperature of the pure DMPG phase transition. Therefore these macromolecules interact preferentially with TOCL because of its structure and not its phase. Additionally, the shift of the transition temperature in the first heating scan is much greater with the DMPG/TOCL mixture compared with DMPG alone demonstrating the difference in the two systems. Similar arguments can be made for the three peptides shown in Fig. 4 in comparison with their effects on the DMPG/TOCL lipid mixture (Fig. 1).

Among the compositions of DPPG and TOCL we tested, the equimolar mixture gives the simplest endotherm, largely as one component with a reversible transition at 21°C. This composition is also a good mimic of the cytoplasmic membrane of some Gram positive bacteria. Peptides were added to hydrate a film of DPPG/TOCL 50:50 at a lipid to peptide ratio of 20 and at a temperature above the main transition temperature of the mixture, that is, in the liquid crystalline state. The samples were immediately cooled in the calorimeter and scans were carried out at 1°/min (Fig. 5). MSI-1254 produced a higher temperature transition at 32–33°C without loss of the main transition of the mixture at 21°C. The transitions were reversible on reheating (Fig. 5). MSI-78, MSI-469, and MSI-843 did not greatly affect the thermal transition of DPPG/TOCL 50:50, indicating that they did not interact as strongly with this lipid mixture as MSI-1254 does (not shown). After a 24 h incubation at 4°C of the DPPG/TOCL 50:50 in the presence of MSI-1254, there is relatively little additional change compared with the fresh samples but even after the 24 h incubation at 4°C the shift in the transition temperature of this mixture is greater in the presence of MSI-1254 than in its absence (Fig. 5).

The main transition for pure DPPG ($P_{\beta'} \rightarrow L_\alpha$) is centered at 40.6°C, but the pure lipid also exhibits a transition from the subgel phase to the $L_{\beta'}$ phase at ~14°C, depending on the ionic strength (13), and a premelt transition at 32–33°C ($L_{\beta'} \rightarrow P_{\beta'}$) (Fig. 6). After a 24 h incubation period at 4°C with pure DPPG, we observed a subgel phase transition of pure DPPG at 28°C (Fig. 6). After 96 h incubation at 4°C, the transition in the 32–35°C temperature range becomes even more pronounced. When MSI-1254 is added to pure DPPG at a lipid to peptide ratio of 20, similar changes take place as in the pure lipid in absence of peptide: a transition appears at 25°C, which becomes more prominent with low temperature incubation, shifting to 29°C (Fig. 6). Thus, there are only small differences caused by MSI-1254 in the transitions of pure DPPG both before and after incubation (Fig. 6). This can be compared with the large effects of this peptide on the transitions of DPPG with TOCL (Fig. 5), again showing the stronger interaction of the peptide with TOCL compared with PG.

DISCUSSION

The clustering of anionic lipids in the presence of zwitterionic ones has been demonstrated for several antimicrobial agents (10,11,17–19), including the AHCAPs used in this study (7). We now present results that we believe provides evidence for a novel activity of these peptides in being able to segregate two different anionic lipids and thus accelerating the formation of crystalline phases. Evidence that domains enriched in DMPG are being formed is provided by the observation that AHCAPs promote the rapid formation of higher melting components in the first DSC heating curve for mixtures of DMPG/TOCL 75:25 (Fig. 1). These phases are formed to only a very minor extent and much more slowly in the absence of peptide. That the new phases formed in the presence of peptides are crystalline phases is

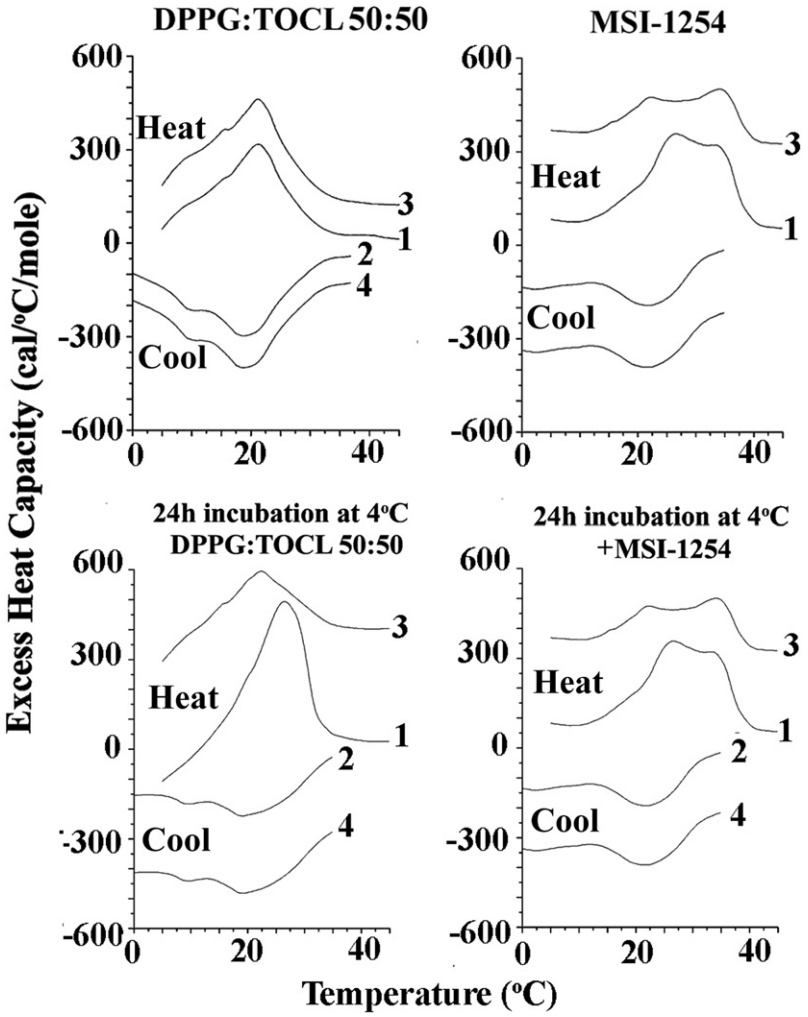

FIGURE 5 DSC of DPPG/TOCL 50:50 in absence and presence of MSI-1254. Lipid films were hydrated at room temperature with 20 mM PIPES, 1 mM EDTA, 140 mM NaCl, pH 7.4 or with a solution of MSI-1254 in this buffer at a lipid to peptide ratio of 20. The scan rate was 1°/min, and the temperature range from 0 to 45°C. Freshly prepared samples or samples incubated for 24 h at 4°C. Odd numbers are heating scans and even numbers are cooling scans.

supported by the observation that the phase is metastable and is not observed in cooling scans. In addition, on incubation at low temperature (Fig. 2) these transitions approach the transition temperatures observed for the crystalline phase of DMPG (4). In some cases the crystalline phase is partly reformed on reheating. We suggest that the more rapid kinetics observed for the formation of this phase, and the fact that the phase already begins to form without prolonged incubation at low temperature, is a consequence of the increased permeability of the bilayer to passage of water and ions caused by the presence of the antimicrobial peptide. There is evidence that the slow equilibration to form the higher ordered phases with pure DMPG is a consequence of the rate-limiting diffusion of ions across bilayer vesicles; when the morphology of DMPG is as flat sheets, then there is a more rapid conversion to the crystalline phases (2,6). The observation that this occurs rapidly in the presence of these peptides indicates that the peptides facilitate the translocation of ions across the bilayer. It has been shown that ion and water equilibration across the membrane is rate limiting for the formation of the crystalline phase in DMPG (6).

Small cationic peptides can also affect PG by binding to the headgroup (20). However, that behavior differs from what we observed in this study involving formation of a metastable state, similar to the HC phase formed on prolonged incubation of pure PG. The metastable phases that are formed rapidly in the presence of peptide from mixtures of PG and CL, mimic the behavior of pure PG after prolonged incubation at low temperature. These transitions thus arise most likely from domains in the membrane that are depleted of both CL and peptide. Furthermore, with pure PG (in the absence of CL) the effects of the peptides are much smaller (Fig. 4 and Fig. 6). This behavior is indicative of the peptide binding preferentially to the lipid headgroup of CL. Thus the peptide by itself is not promoting the formation of crystalline phases with PG but rather induces the segregation of PG into a domain enriched in that lipid component. This is seen even more markedly with the polyaminoacids PLA and PLL (Fig. 3).

Some peptides are more potent than others in promoting the segregation of TOCL and DMPG. The difference among these peptides will be determined by the strength of their

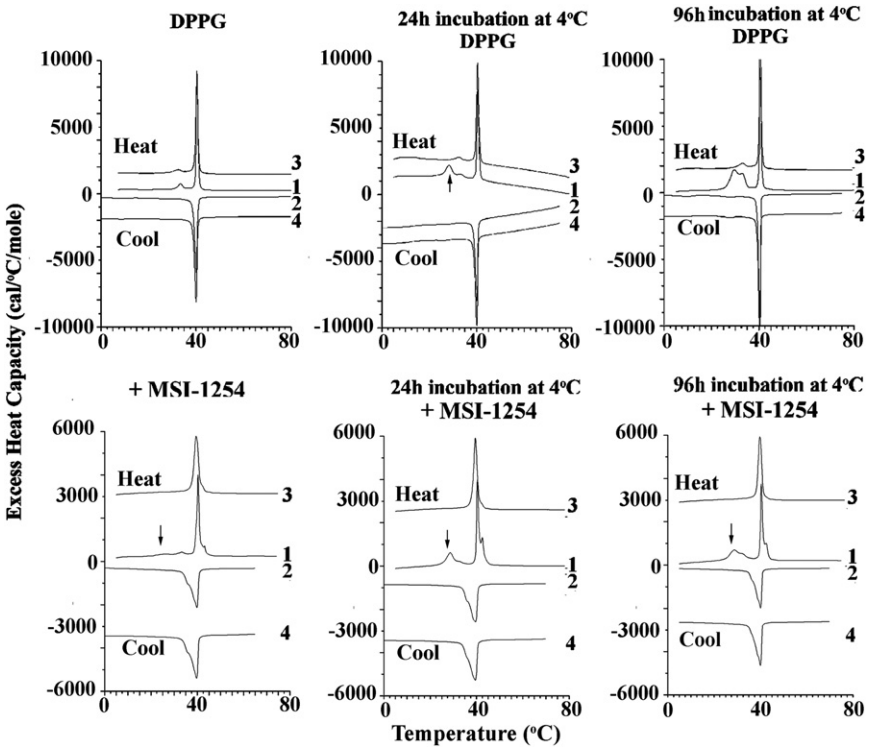

FIGURE 6 DSC of pure DPPG, in the absence and presence of MSI-1254, before and after 24 h and 96 h incubation at 4°C. Lipid films were hydrated with 20 mM PIPES, 1 mM EDTA, 140 mM NaCl, pH 7.4. The scan rate was 1°/min. Odd numbers are heating scans and even numbers are cooling scans. The arrow indicates the conversion of the subgel phase to a higher temperature crystalline phase. Lipid/peptide ratio was 20. Odd numbers are heating scans and even numbers are cooling scans.

interactions with the headgroup of CL, which in turn will depend on the positive charge density of the peptide and the stability of peptide conformations giving the required distance between charges to be able to maximally interact with CL. The importance of charge density on the peptide can be seen by the fact that the peptide with the lowest charge density (Table 1), magainin 2, has no ability to segregate these lipids, whereas the poly aminoacids, PLL and PLA, are the most potent (Fig. 3).

The process of separating two anionic lipids into domains resembles what has been described as anionic lipid clustering for the segregation of zwitterionic-anionic lipids by some cationic antimicrobial agents. That phenomenon allowed for the prediction of which bacterial species would be affected by certain antimicrobial agents (10,11,21,22). All these peptides were able to depolarize the membranes of Gram positive bacteria (data not shown). There are significant differences among the peptides studied in their ability to form crystalline domains of DMPG in mixtures with TOCL (Fig. 1), yet there is little difference in the MICs for these peptides for a Gram positive bacteria (7). Thus, in the case of two different anionic headgroups, separation into domains does not predict the species selectivity of these compounds, as it does in a zwitterionic-anionic lipid mixture. One should take into consideration that the formation of a crystalline phase in the samples tested in this study was used as a tool to demonstrate segregation of PG and CL in the membrane and not to show conversion to crystalline forms *in vivo*. There are several reasons why separation of two anionic lipids into domains is of less predictive value regarding species selectivity of antimicrobial agents compared with segregation of anionic and zwitterionic lipids. In general, antimicrobial agents are not highly specific and most of them use more than one mechanism that contributes to their antimicrobial activity. In the case of toxicity against bacteria whose membranes are composed largely of anionic lipids, like Gram-positive bacteria, the membranes of these species are highly negatively charged and will therefore sequester more cationic antimicrobial peptides. This will allow the surface concentration of these peptides to surpass more easily the threshold required for pore formation (23). Hence pore formation will become the dominant contributing factor in the antimicrobial action and this phenomenon will be insensitive to the segregation of lipids. Gram positive bacteria tend to be more sensitive than Gram negative bacteria because they lack the barrier of an outer membrane and they have little or no periplasmic space, allowing the antimicrobial peptides to accumulate directly on the surface of the outer leaflet of the cytoplasmic membrane, where they can easily reach threshold concentrations for permeabilization. Nevertheless, segregation of two different anionic lipids is likely to be a contributing factor to antimicrobial activity in certain cases and there may even be examples where it is the principal mechanism, such as the action of PLL against most Gram-positive bacteria (24).

This finding broadens the range of systems in which lipid clustering can occur and illustrates a different mechanism for clustering. In addition, it presents a new role for PG bilayers that form crystalline phases as a tool to probe anionic lipid clustering. Thus agents that bind to membranes and have a high density of positive charge will cluster membrane lipids.

In the case of agents that separate anionic from zwitterionic lipids, these will be more toxic to bacteria with a high content of phosphatidylethanolamine, as is present in most Gram negative bacteria. When anionic lipids predominate, as in most Gram positive bacteria, preferential interaction with one headgroup over another will contribute to lipid clustering and consequently to membrane reorganization and disruption. In addition, as a result of anionic lipid clustering the functioning of proteins embedded in the bacterial cytoplasmic membrane will be impaired, particularly those with strong affinity for anionic lipids.

To our knowledge this is the first demonstration of peptides promoting the rapid formation of intermediates of high crystalline phases in PG bilayers as a result of anionic lipid clustering in membranes.

SUPPORTING MATERIAL

One figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00318-8.

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