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Biochimica et Biophysica Acta 1417 (1999) 211–223

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Differential scanning calorimetric study of the effect of the antimicrobial peptide gramicidin S on the thermotropic phase behavior of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol lipid bilayer membranes

Elmar J. Prenner [a], Ruthven N.A.H. Lewis [a], Leslie H. Kondejewski [a,b], Robert S. Hodges [a,b], Ronald N. McElhaney [a,*]

[a] *Department of Biochemistry, University of Alberta, Edmonton, Alta. T6G 2H7, Canada*
[b] *Protein Engineering Network of Centres of Excellence, University of Alberta, Edmonton, Alta. T6G 2H7, Canada*

Received 22 September 1998; received in revised form 21 December 1998; accepted 7 January 1999

Abstract

We have studied the effects of the antimicrobial peptide gramicidin S (GS) on the thermotropic phase behavior of large multilamellar vesicles of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE) and dimyristoyl phosphatidylglycerol (DMPG) by high-sensitivity differential scanning calorimetry. We find that the effect of GS on the lamellar gel to liquid-crystalline phase transition of these phospholipids varies markedly with the structure and charge of their polar headgroups. Specifically, the presence of even large quantities of GS has essentially no effect on the main phase transition of zwitterionic DMPE vesicles, even after repeating cycling through the phase transition, unless these vesicles are exposed to high temperatures, after which a small reduction in the temperature, enthalpy and cooperativity of the gel to liquid-crystalline phase transitions is observed. Similarly, even large amounts of GS produce similar modest decreases in the temperature, enthalpy and cooperativity of the main phase transition of DMPC vesicles, although the pretransition is abolished at low peptide concentrations. However, exposure to high temperatures is not required for these effects of GS on DMPC bilayers to be manifested. In contrast, GS has a much greater effect on the thermotropic phase behavior of anionic DMPG vesicles, substantially reducing the temperature, enthalpy and cooperativity of the main phase transition at higher peptide concentrations, and abolishing the pretransition at lower peptide concentrations as compared to DMPC. Moreover, the relatively larger effects of GS on the thermotropic phase behavior of DMPG vesicles are also manifest without cycling through the phase transition or exposure to high temperatures. Furthermore, the addition of GS to DMPG vesicles protects the phospholipid molecules from the chemical hydrolysis induced by their repeated exposure to high temperatures. These results indicate that GS interacts more strongly with anionic than with zwitterionic phospholipid bilayers, probably because

Abbreviations: GS, gramicidin S; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PS, phosphatidylserine; SpM, sphingomyelin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DSC, differential scanning calorimetry; ESR, electron spin resonance; NMR, nuclear magnetic resonance; IR, infrared; NBD-GS, *N*-4-nitrobenz-2-oxa-1,3-diazole gramicidin S; L_c or L_c', lamellar crystalline phase with untilted or tilted hydrocarbon chains, respectively; L_β or L_β', lamellar gel phase with untilted or tilted hydrocarbon chains, respectively; P_β', lamellar rippled gel phase with tilted hydrocarbon chains; L_α, lamellar liquid-crystalline phase

* Corresponding author. Fax: +1-403-492-0095; E-mail: rmcelhan@gpu.srv.ualberta.ca

PII: S0005-2736(99)00004-8

of the more favorable net attractive electrostatic interactions between the positively charged peptide and the negatively charged polar headgroup in such systems. Moreover, at comparable reduced temperatures, GS appears to interact more strongly with zwitterionic DMPC than with zwitterionic DMPE bilayers, probably because of the more fluid character of the former system. In addition, the general effects of GS on the thermotropic phase behavior of zwitterionic and anionic phospholipids suggest that it is located at the polar/apolar interface of liquid-crystalline bilayers, where it interacts primarily with the polar headgroup and glycerol-backbone regions of the phospholipid molecules and only secondarily with the lipid hydrocarbon chains. Finally, the considerable lipid specificity of GS interactions with phospholipid bilayers may prove useful in the design of peptide analogs with stronger interactions with microbial as opposed to eucaryotic membrane lipids.

1. Introduction

Gramicidin S (GS)[1] is a cyclic decapeptide[cyclo-(Val–Orn–Leu–D–Phe–Pro)$_2$] first isolated from *Bacillus brevis* (see [1]) and is one of a series of antimicrobial peptides produced by this microorganism (see [2]). This peptide exhibits appreciable antibiotic activity against a broad spectrum of both Gram-negative and Gram-positive bacteria as well as against several pathogenic fungi [3,4]. In aqueous solution GS forms an amphiphilic, two-stranded, antiparallel β-sheet structure in which the four hydrophobic Leu and Val residues project from one side of this disk-shaped peptide molecule and the two basic Orn residues project from the other [2,5–8]. In general, the antimicrobial and hemolytic activity of GS analogs increase with the degree of hydrophobicity and amphiphilicity of the peptide up to some optimal value and the presence of two positively charged amino acids are essential for maximal activity [3,9,10]. Although the Orn residues of GS may be replaced by Lys or Arg residues, the presence of two positively charged amino acid residues on the same face of the peptide molecule are also known to be required for maximal antimicrobial activity [2,4,6,11].

Considerable evidence exists that the primary target of GS is the lipid bilayer of cell surface membranes and that this peptide kills cells by destroying the structural integrity of the lipid bilayer by inducing the formation of pores or other localized defects [12–17]. Unfortunately, GS is rather nonspecific in its actions and exhibits appreciable hemolytic as well as antimicrobial activity [18–20]. The therapeutic utilization of GS has therefore been limited to topical applications [19]. A major goal of our current studies on the interaction of GS with model and biological membranes is to provide the fundamental knowledge of the mechanism of action of this peptide on lipid bilayers which is required to design GS analogs with enhanced activity for bacterial membranes and diminished activity against the plasma membranes of human and animal cells.

Although considerable evidence exists that the principle target of GS is the lipid bilayer of cell surface membranes, only a limited number of studies of the interaction of this peptide with lipid bilayer model membranes have been published to date. Moreover, the conclusions reached from these studies do not always agree with one another. Pache et al. [21] reported that even at a low lipid/peptide ratio (10:1), GS had almost no effect on the gel to liquid-crystalline phase transition temperature, enthalpy or cooperativity of DPPC multilamellar vesicles, nor was the organization the DPPC bilayer significantly perturbed by the presence of the peptide, as monitored by NMR or ESR spectroscopy. The only significant effect of GS addition observed was a decrease in the DPPC P–O stretching frequency as monitored by IR spectroscopy. Pache and coworkers thus concluded that GS is bound to the surface of the DPPC bilayer and interacts only with the lipid polar headgroups. A similar conclusion was reached by Datema et al. [22], based on their observations of the very small reduction of the temperature and cooperativity of the main phase transition of DPPC observed by DSC even at low lipid/peptide ratios (6:1), the insensitivity of the motional characteristics of the GS molecule to the DPPC gel to liquid-crystalline phase transition, and the ability of GS to induce an isotropic component in the ^{31}P-NMR spectrum at higher temperatures. However, based on the generally appreciable hydrophobicity of the GS molecule, the sensitivity of the lateral diffusion coefficient of this peptide to the host bilayer chain-melting phase transition, and the ability of cholesterol to induce lateral aggregation of GS in DPPC and particularly in DMPC bilayers, Wu

et al. [23] suggested that GS must penetrate at least partially into the hydrophobic core of the host lipid bilayer. This conclusion was later supported by Zidovetzki et al. [24], who demonstrated by ^{2}H-NMR spectroscopy that GS alters the orientational order parameter profile of the hydrocarbon chains of DMPC, at least at low lipid/protein ratios (5.5:1), and by ^{31}P-NMR spectroscopy, which suggested that the structure of the bilayer is abolished at even lower lipid/peptide ratios (2.7:1). Thus these early studies, which were done exclusively on zwitterionic PC systems, failed to resolve either the issue of the location of GS in lipid model membranes or the nature of its interactions with the host lipid bilayer.

We have recently studied the effect of GS on the thermotropic phase behavior of a variety of synthetic phospholipids using primarily ^{31}P-NMR spectroscopy supplemented by DSC and X-ray diffraction. We found that at physiologically relevant concentrations of GS (lipid/protein ratios of 25:1), GS does not affect the lamellar phase preference of the zwitterionic lipids PC and SpM nor of the anionic lipids DPG and PS, even at high temperatures. However, GS was found to potentiate inverted cubic phase formation in zwitterionic PE and, to a lesser extent, in anionic PG dispersions, as well as in total polar lipid extracts from the glycolipid-based membranes of *Acholeplasma laidlawii* and from the phospholipid-based membranes of *Escherichia coli*. Moreover, the ability of GS to induce nonlamellar phase formation was found to increase with the intrinsic nonlamellar phase-forming propensity of the PE molecular species studied. We suggested that the ability of GS to induce localized regions of high curvature stress in the lipid bilayers of biological membranes may be relevant to the mechanism by which this peptide disrupts cell membranes.

It thus seems likely that interactions between GS and biological membranes will vary markedly with the physical properties, and thus with the lipid composition, of the target membrane. Also, because lipid polar headgroups essentially determine the surface properties of biological membranes, interactions between GS and biological membranes should be particularly sensitive to membrane lipid polar headgroup composition. We are thus studying the nature of the interactions of GS with lipid bilayer model membranes which contain a wide range of polar headgroups. This research is intended to provide basic information on the sensitivity of various membrane lipid classes to the action of GS and on how these are affected by variations in the structure of the peptide molecule. Because the lipid compositions of bacterial and eucaryotic cell membranes are quite different and because antimicrobial and hemolytic activities can be at least partially dissociated in GS analogs [4], such information could potentially be used in the design of more therapeutically useful GS derivatives. In fact, our recent work has shown for the first time that GS does exhibit an appreciable lipid polar headgroup specificity, at least as regards the lamellar/nonlamellar phase behavior of various glycerophospho- and sphingophospholipids [25]. The present high-sensitivity DSC study of the effect of GS on the thermotropic phase behavior of DMPC, DMPE and DMPG bilayer membranes represents a continuation of this research program. Since PCs are virtually absent in bacterial membranes but are generally the most abundant phospholipid in eucaryotic plasma membranes, DMPC can serve as a model for the surface membrane of the cells of higher animals, particularly so since PCs are typically found primarily in the outer monolayer of the lipid bilayer of such membranes. Similarly, PGs are absent in eukaryotic plasma membranes but are ubiquitous and often abundant in bacterial membranes, so that DMPG can serve as a good model for the bacterial membrane. Finally, PEs are also major components of virtually all eukaryotic plasma membranes but are also abundant in most Gram-negative bacteria and in members of the Gram-positive *Bacillus* genus of bacteria as well. However, in eukaryotic cell membranes PEs are usually enriched in the inner monolayer of the lipid bilayer. Thus information gained from a study of DMPE may be applicable to both bacterial and animal plasma membranes, but especially to the former.

2. Materials and methods

DMPC, DMPE and DMPG were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Gramicidin S was obtained from Sigma (St. Louis, MO) and dissolved in pure ethanol and stored at −20°C. The lipid stock

solutions were dissolved in redistilled methanol. Lipid/peptide mixtures were prepared by mixing appropriate amounts of the lipid and peptide stock solutions, drying under N_2, and exposure of the lipid/peptide films to high vacuum overnight. The multilamellar GS-containing vesicles were then prepared by vortexing in excess buffer (10 mM Tris–HCl, 100 mM NaCl and 2 mM EDTA) normally at temperatures above the main phase transition temperature of the phospholipid. All chemicals were purchased from BDH (Toronto, Ont.).

We found that the effects of GS on the thermotropic phase behavior of the phospholipid vesicles studied depended significantly on the method of preparation of these peptide–phospholipid complexes and on their thermal history. In order to mimic the in vivo effects of GS as closely as possible, we initially added peptide to preformed large unilamellar vesicles at temperatures above the phospholipid gel to liquid-crystalline phase transition temperature. However, the chain-melting phase transition of these large unilamellar vesicles was so broad, even in the absence of peptide, that the effects of the addition of smaller quantities of GS on phospholipid thermotropic phase behavior could not be accurately determined. Moreover, the effects of the addition of larger amounts of GS to these vesicles varied considerably with the number of heating and cooling scans performed, presumably because GS can cause vesicular lysis and fusion at higher concentrations, allowing the peptide access to both surfaces of the lipid bilayer. Alternatively, when small amounts of GS are added to preformed large multilamellar vesicles, which exhibit much more cooperative phase transitions in the absence of peptide, only very small effects on phospholipid thermotropic phase behavior were observed, probably because GS was interacting only with the outer monolayer of the single bilayer on the surface of these multilamellar vesicles. Again, when larger amounts of peptide were added to these systems, the characteristic effects of GS on the thermotropic phase behavior of the multilamellar phospholipid vesicles became increasingly more marked with each DSC heating and cooling cycle, presumably because the peptide was progressively gaining access to the surfaces of bilayers which were initially inaccessible. Thus, in order to maximize the observable effects of GS on phospholipid thermotropic phase behavior in a system exhibiting highly cooperative lipid phase transitions and to minimize the thermal history dependence of the system, lipid–peptide complexes were prepared as described above and then these complexes were hydrated to produce large multilamellar vesicles in which the GS was presumably uniformly distributed among the various bilayers. In this way we could accurately measure the effects of very small amounts of GS on phospholipid thermotropic phase behavior while minimizing, although not entirely eliminating, any thermal history dependence of the sample. This latter effect was obviated by performing three heating and cooling cycles in the DSC instrument before collecting data.

The calorimetry was performed on a Microcal MC-2 high-sensitivity Differential Scanning Calorimeter (Microcal, Northampton, MA). For all samples a scan rate of 10°C/h was used. Sample runs were repeated at least three times to ensure reproducibility. Data acquisition and analysis was done using Microcal's DA-2 and Origin software (Microcal). The total lipid concentrations used for the DSC analyses were about 0.5 mg/ml, providing for full hydration for the GS–phospholipid mixtures. Samples containing GS alone, dissolved in buffer at peptide concentrations corresponding to those of the highest peptide/lipid molar ratios studied (1:10), exhibited no thermal events over the temperature range 0–90°C. This indicates that GS does not denature over this temperature range and that the endothermic events observed in this study arise exclusively from phase transitions of the phospholipid vesicles.

3. Results

3.1. Thermotropic phase behavior of GS/DMPC mixtures

DSC heating endotherms illustrating the effects of GS on the thermotropic phase behavior of large, multilamellar vesicles of DMPC alone are presented in Fig. 1. Aqueous dispersions of DMPC alone, when not extensively annealed at low temperatures, exhibit only two endothermic events, a less energetic pretransition near 14°C and a more energetic main transition near 24°C. Under these conditions a sub-

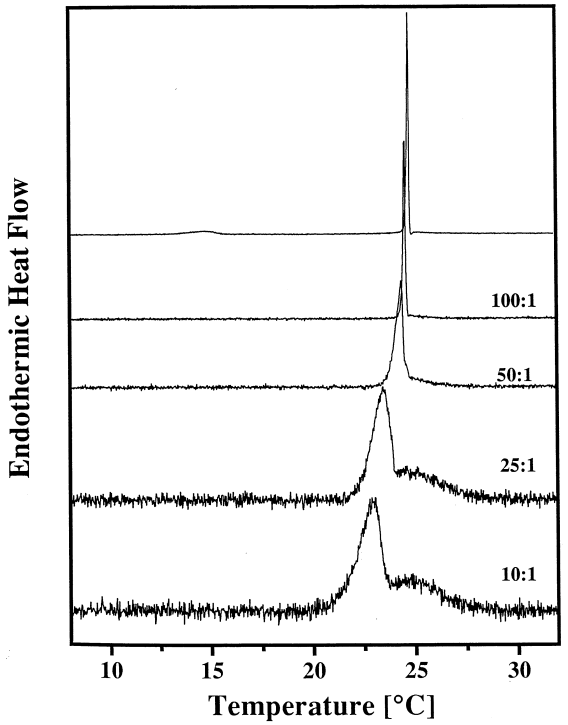

Fig. 1. High-sensitivity DSC heating scans illustrating the effect of the addition of increasing quantities of GS on the thermotropic phase behavior of DMPC multilamellar vesicles. The top scan is of DMPC alone, and the lipid/peptide molar ratios of the lower scans are indicated on the figure itself.

transition centered at 18°C is not observed, since there is insufficient time for the formation of an L_c' phase and this phase was not nucleated by exposure to low temperatures (−20°C). The pretransition arises from the conversion of the L_β' to the P_β' phase and the main or chain-melting phase transition from the conversion of the P_β' to the L_α phase. For a more detailed discussion of the thermotropic phase behavior of DMPC and other members of the homologous series of linear saturated PCs, see Lewis et al. [26].

The interaction of GS with DMPC vesicles clearly alters the thermotropic phase behavior of the latter, as illustrated in Fig. 1. Small amounts of GS decrease the temperature (Fig. 2) and enthalpy (Fig. 3) of the pretransition and the pretransition is abolished entirely in DMPC vesicles having lipid/peptide ratios of 100:1 or less (Fig. 1). Moreover, the presence of increasing quantities of GS also results in the induction of a two-component main phase transition, with a more cooperative, lower temperature endotherm superimposed over a less cooperative, higher

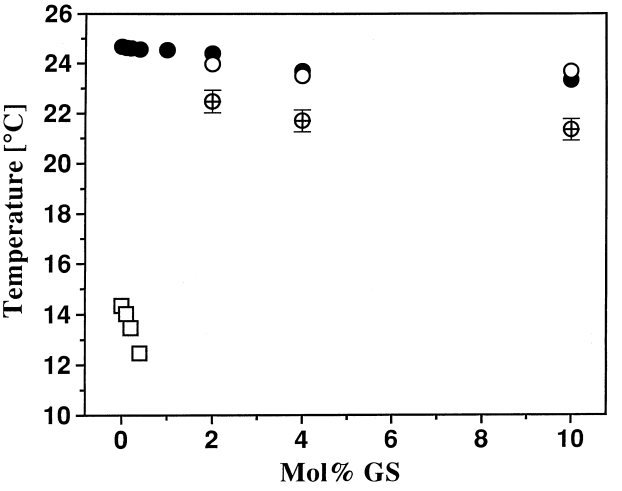

Fig. 2. A plot of variations in the midpoint temperatures for the pretransition and main transition of DMPC multilamellar vesicles as a function of GS concentration. □, pretransition temperature; ●, overall temperature for main phase transition; ⊕, temperature of the sharp component of the main phase transition; ○, temperature of the broad component of the main phase transition.

temperature endotherm. The temperature (Fig. 2), enthalpy (Fig. 3) and cooperativity (Fig. 1) of both components of the chain-melting phase transition also decrease with increases in peptide concentration relative to DMPC alone. However, the GS-induced decreases in the phase transition temperature of the

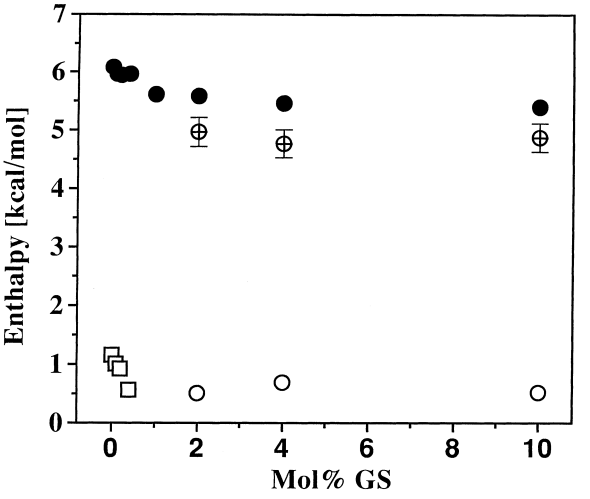

Fig. 3. A plot of the transition enthalpies for the pretransition and main transition of DMPC multilamellar vesicles as a function of GS concentration. The symbols used are the same as for Fig. 2.

two main phase transition components are modest (2–3°C) and the total enthalpy of chain melting is reduced by only 10–15%, even at a phospholipid/peptide ratio of 10:1. These results indicate that the presence of GS produces only a modest destabilization of the gel state of DMPC bilayers, even after maximizing GS–DMPC interactions by multiple cycling through the gel to liquid-crystalline phase transition. Generally similar although less detailed results were reported previously for GS/DMPC and GS/DPPC mixtures studied by low-sensitivity DSC [22,23].

3.2. Thermotropic phase behavior of GS/DMPE mixtures

DSC heating scans illustrating the effects of GS addition on the thermotropic phase behavior of large, multilamellar vesicles of DMPE are presented in Figs. 4 and 5. Aqueous dispersions of DMPE alone, in the absence of extensive incubation at low temperature, exhibit a single, relatively energetic L_β/L_α phase transition near 50°C (see [27], and references therein for a more complete description of the thermotropic phase behavior of DMPE and other members of the homologous series of linear saturated PEs). If GS is added to DMPE vesicles at temperatures above but near to the gel to liquid-crystalline phase transition temperature of DMPE, essentially no effect on the thermotropic phase behavior is observed upon heating (Fig. 4), even at high GS concentrations. However, if GS-containing DMPE vesicles are exposed to temperatures well above the gel to liquid-crystalline phase transition temperature, increasing quantities of GS lower the temperature, enthalpy and cooperativity of the chain-melting phase transition of DMPE slightly when DSC cooling curves are run (Fig. 5). Moreover, upon subsequent reheating, the characteristic effects of the presence of the peptide on the phase behavior of DMPE vesicles are retained (Fig. 5). However, the effects of GS addition on the temperature, enthalpy and cooperativity of the main phase transition observed upon cooling and subsequent reheating are very small, even at the highest peptide–phospholipid ratio tested. These findings indicate that the presence of GS produces only a very slight destabilization of the gel state of DMPE bilayers and then only if the GS-containing vesicles are first exposed to high temperatures. However, the interaction of GS with DMPE

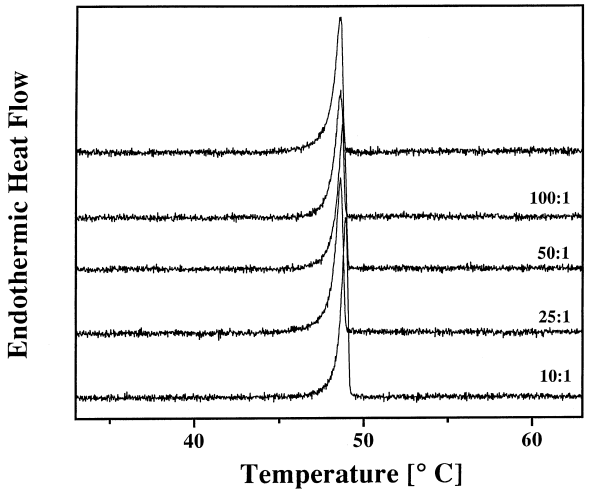

Fig. 4. High-sensitivity DSC heating scans illustrating the effect of the addition of increasing quantities of GS on the thermotropic phase behavior of DMPE multilamellar vesicles not exposed to high temperatures (i.e., temperatures above 65–70°C). The top scan is of DMPE alone, and the lipid/peptide molar ratios of the lower scans are indicated on the figure itself.

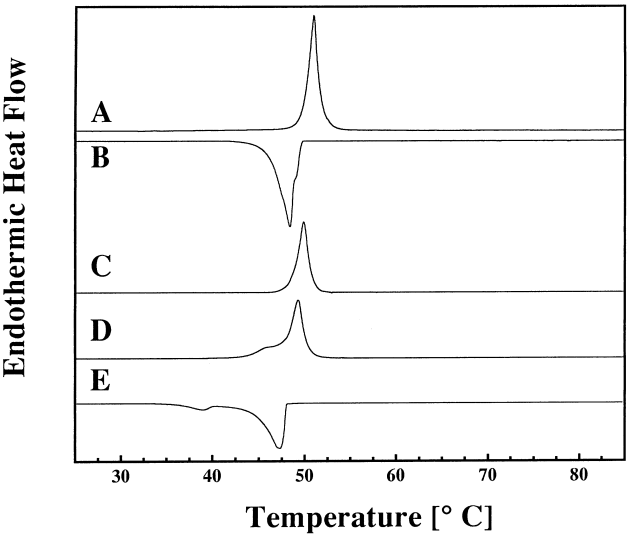

Fig. 5. High-sensitivity DSC heating and cooling scans illustrating the effect of the exposure to high temperature on the thermotropic phase behavior of DMPE multilamellar vesicles having a lipid/peptide molar ratio of 25:1. Heating and cooling thermograms of DMPE alone are illustrated in A and B, respectively, for comparison. The heating scan of GS-containing DMPE vesicles not previously exposed to high temperature is shown in C, while heating and cooling scans of the same vesicles exposed briefly to a temperature of 75°C are shown in D and E, respectively.

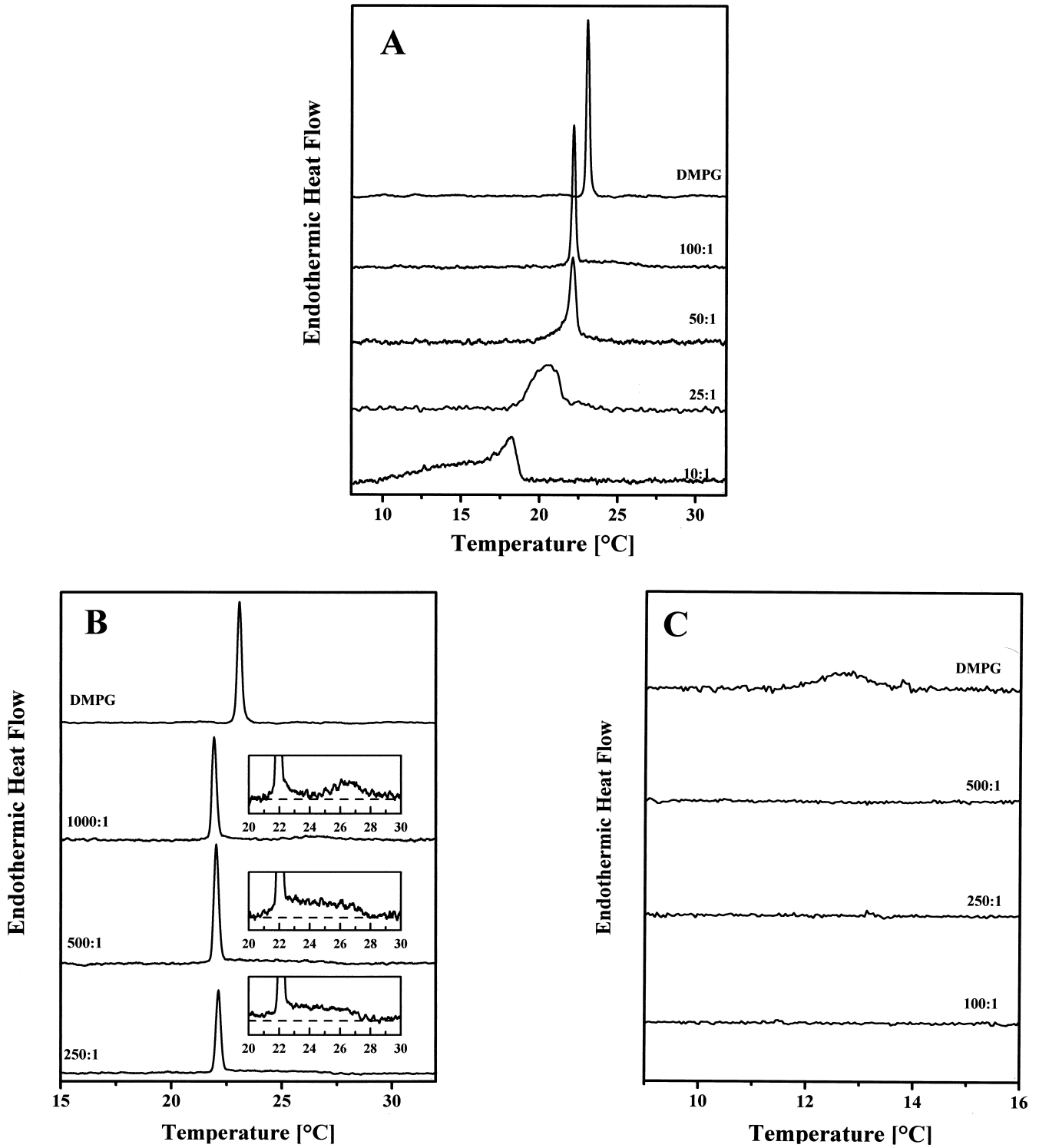

Fig. 6. High-sensitivity DSC heating scans illustrating the effect of the addition of relatively large or relatively small quantities of GS on the main phase transition of DMPG multilamellar vesicles are presented in panels A and B, respectively. In each panel the top scan is of DMPG alone, and the lipid/peptide molar ratios of the lower scans are indicated on the figure panels. The insets of panel B are blow-ups of the DSC scan in the temperature range just above the sharp component of the main phase transition, illustrating the behavior of the minor broad endothermic peak present in these samples. High-sensitivity DSC scans illustrating the effect of the addition of small quantities of GS on the pretransition of DMPG are presented in panel C. The DMPG concentration is threefold that of the samples utilized in panels A and B, and only the pretransition regions of the DSC thermograms are illustrated.

bilayers at high temperatures can also induce the L_α phase of this phospholipid to form an inverted cubic phase [25], indicating that structurally significant interactions between GS and a sufficiently fluid phase of DMPE can occur, and that these interactions do persist after cooling to temperatures below the main phase transition temperature.

3.3. Thermotropic phase behavior of GS/DMPG mixtures

DSC heating scans illustrating the effect of GS addition on the thermotropic phase behavior of large, multilamellar vesicles of DMPG are presented in Fig. 6A,B. Aqueous dispersions of DMPG, not extensively annealed at low temperatures, also exhibit two endothermic events upon heating, a less energetic pretransition near 14°C and a more energetic main near 24°C. Again, under these conditions a subtransition is not observed. The pretransition arises from the conversion of the L'_β gel to the P'_β gel phase and the main transition or chain-melting phase transition from the conversion of the P'_β to the L_α phase. For a more detailed discussion of the thermotropic phase behavior of DMPG and other members of the homologous series of linear saturated PGs, see Zhang et al. [28].

The interaction of GS with DMPG has a major effect on the thermotropic phase behavior of this anionic phospholipid. Specifically, the presence of increasing quantities of GS reduces the temperature and enthalpy of the pretransition, which appears to be abolished entirely at lipid/peptide ratios of 500:1 or less. Moreover, at low concentrations of peptide, two endotherms are clearly present in the DSC heating thermograms, a sharp, relatively energetic endotherm centered at 22°C and a broad, less energetic endotherm centered near 27°C (Fig. 6B). As the GS concentration increases, the initially higher temperature endotherm decreases in temperature and cooperativity but becomes relatively more energetic as compared to the lower temperature, more cooperative endotherm (Fig. 6A). However, at higher peptide concentrations, both endotherms decrease in temperature (Fig. 7) but the less cooperative transition, which becomes increasingly more prominent, is now centered at a lower temperature than the more cooperative transition. Moreover, the total enthalpy associated with both transitions decreases with increasing peptide concentration (Fig. 8), particularly at higher peptide concentrations. If one assumes that the sharp endotherm is due to the chain-melting of DMPG domains relatively poor in peptide and the broad endotherm is due to the presence of domains of DMPG enriched in peptide, then it appears that at lower concentrations GS stabilizes the gel state of DMPG bilayers, as might be expected for a positively charged peptide. However, at higher GS con-

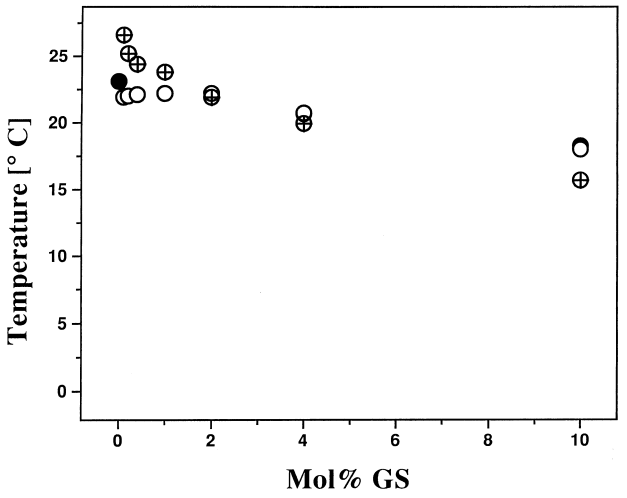

Fig. 7. A plot of variations in the midpoint temperatures of the main phase transition of DMPG multilamellar vesicles as a function of GS concentration. The symbols used are the same as for Fig. 2.

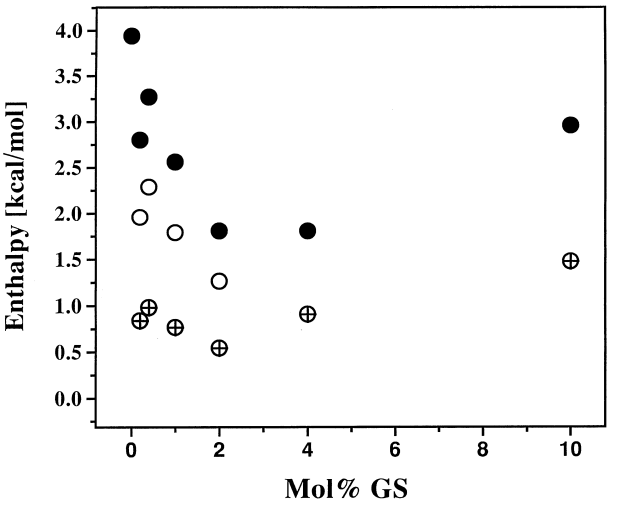

Fig. 8. A plot of the transition enthalpies of the main phase transition of DMPG multilamellar vesicles as a function of GS concentration. The symbols used are the same as for Fig. 2.

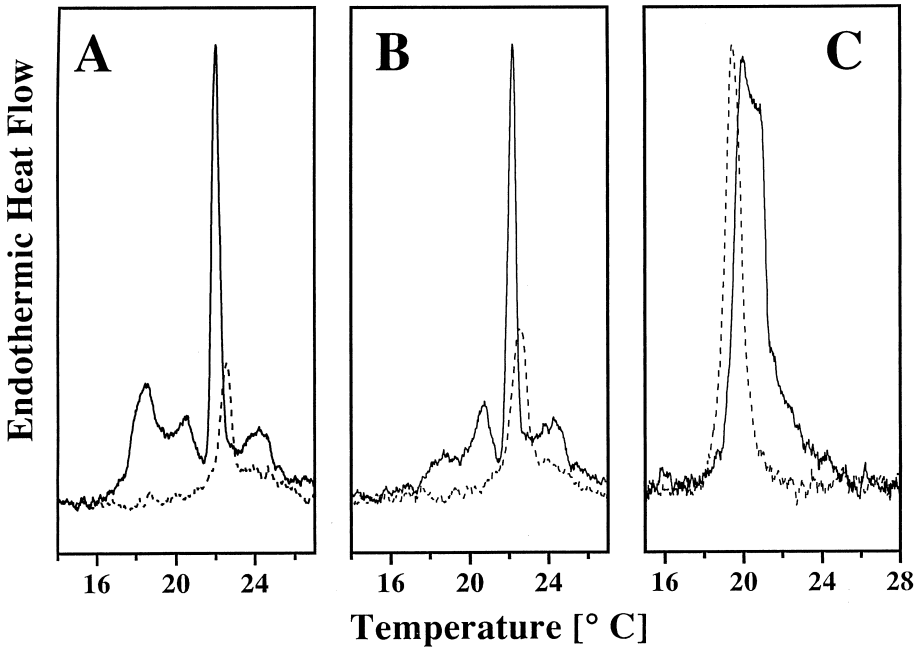

Fig. 9. DSC heating scans illustrating the effect of repeated heating to high temperature on the thermotropic phase behavior of DMPG multilamellar vesicles alone (A), or containing smaller (lipid/peptide molar ratio 250:1) (B) or larger (lipid/peptide molar ratio of 25:1) (C) quantities of GS. The first heating scan terminating at 95°C is shown as the solid line and the fourth such scan is shown as the dotted line.

centrations, the presence of GS appears to destabilize both the relatively peptide-rich and peptide-poor domains of DMPG vesicles. Note that the effects of GS on the temperature, enthalpy and cooperativity of the gel to liquid-crystalline phase transition of DMPG vesicles are much larger than those observed in DMPE or DMPC vesicles at comparable peptide concentrations.

3.4. *Effect of GS on the thermal stability of DMPG bilayers*

The effect of exposure to high temperature on the thermotropic phase behavior of DMPG vesicles, reconstituted with and without GS, is illustrated in Fig. 9. In the absence of peptide, the DMPG vesicles exhibit complex DSC endotherms after repeated heating to 95°C and subsequent cooling. We have shown by TLC chromatographic analysis (data not presented) that the complex thermotropic phase behavior of the DMPG vesicles observed after repeated heating to high temperatures is due to the progressive chemical hydrolysis of these phospholipids and that such hydrolysis is not observed, or at least is much less pronounced, with the DMPE and DMPC vesicles. However, GS-containing DMPG vesicles exhibit thermotropic phase behavior which is much more similar to GS-containing DMPG vesicles not exposed to high temperatures, and in fact the presence of increasing quantities of GS results in a progressively greater inhibition of heat-catalyzed chemical hydrolysis of DMPG vesicles (Fig. 9). These results suggest that the binding of GS to DMPG bilayers at high temperatures is relatively strong and that the phospholipid–peptide complex formed provides protection from heat-catalyzed chemical degradation. Unfortunately, the intrinsically strong resistance of DMPC and DMPE bilayers to hydrolysis upon exposure to high temperatures precluded an analysis of the effect of the presence of GS on the chemically stability of these phospholipids.

4. Discussion

The major new finding of this work is that the effect of GS on the thermotropic phase behavior of

phospholipid bilayers varies markedly with both the structure and charge of the lipid polar headgroup. Specifically, the addition of GS has essentially no detectable effect on the thermotropic phase behavior of zwitterionic DMPE bilayers employed in this study, even at very high peptide levels and even after multiple cycling through the gel to liquid-crystalline phase transition temperature. Only after exposure of GS-containing DMPE vesicles to high temperatures is a slight decrease in the phase transition temperature, enthalpy and cooperativity of this phospholipid observed (see also [25]). Similarly, GS has only a small effect on the thermotropic phase behavior of zwitterionic DMPC bilayers, inducing a small decrease in the temperature and enthalpy, and a moderate decrease in the cooperativity, of the main phase transition of this phospholipid. Moreover, under these conditions the pretransition of DMPC is abolished at moderate GS concentrations. However, GS has a much more pronounced effect on the thermotropic phase behavior of DMPG, substantially reducing the temperature, enthalpy and cooperativity of the main phase transition at higher peptide concentrations and abolishing the pretransition at lower GS concentrations than are required for DMPC. These results indicate that GS interacts much more strongly with anionic rather than zwitterionic lipids, and, in the latter category, more strongly with DMPC than with DMPE at comparable reduced temperatures in the liquid-crystalline state.

The stronger interaction of GS with anionic DMPG bilayers in comparison with zwitterionic DMPC and DMPE bilayers is almost certainly due in part to the more favorable electrostatic interactions between the peptide and lipid, and specifically to the strong attractive interactions between the two positively charged ornithine residues of the peptide and the negatively charged phosphate moieties of the phosphorylglycerol headgroups of the former lipid. Although attractive electrostatic interactions between the positively charged ornithine residues of GS and the negatively charged phosphate moieties of the phosphorylcholine and phosphorylethanolamine polar headgroups probably also exist, the overall strength of the electrostatic interactions between peptide and phospholipid will be markedly attenuated by the presence of the positive charges on the choline and ethanolamine moieties of the DMPC and DMPE bilayers, respectively. However, the shielding of the positively charged quaternary nitrogen of the choline moiety in DMPC by the three methyl groups attached to it may result in a somewhat stronger (but still rather weak) net electrostatic attractive interaction between GS and DMPC as compared to DMPE bilayers, perhaps accounting in part for the slightly stronger interactions of GS with the former lipid observed in the present study. The fact that the presence of two ornithine or other positively charged amino acid residues at specific positions in the GS molecule are required for antimicrobial activity [2,4,11] suggests that electrostatic interactions between this peptide and anionic phospholipids, of the type observed here between GS and DMPG, are of functional significance in vivo.

However, other differences between the physical properties of DMPC and DMPE bilayers may also account for the apparently stronger interactions of GS with DMPC and compared to DMPE vesicles. For example, GS may interact preferentially with liquid-crystalline DMPC rather than DMPE bilayers because of the greater fluidity and decreased packing density of the former phospholipid as compared to the latter (see [27]). This suggestion is supported by our present findings that GS interacts less strongly with gel as compared to liquid-crystalline bilayers of both of these zwitterionic phospholipids and more strongly with DMPC than with DMPE at comparable reduced temperatures in the liquid-crystalline state, and by our previous observation that significant GS–DMPE interactions are only observed after such exposure to high temperatures [25]. This suggestion is also supported by the fact that the incorporation of cholesterol into DMPC vesicles attenuates the effect of GS addition on both their thermotropic phase behavior and permeabilization (unpublished observations from this laboratory). It is also possible that the capacity for hydrogen bond formation between GS and the polar headgroup and interfacial regions of the host phospholipid bilayer may also be important in determining the strength and nature of peptide–phospholipid interactions. Additional studies on a wider variety of lipids employing various spectroscopic techniques will clearly be required to elucidate the nature of GS–phospholipid interactions in greater detail.

The comparative effects of the incorporation of

GS on the thermotropic phase behavior of zwitterionic and anionic phospholipid bilayers can also be used to deduce both the general location of this peptide relative to the lipid bilayer and the nature of the lipid–peptide interactions involved. For example, Papahadjopoulos et al. [29] and McElhaney [30] have proposed that many membrane-associated proteins can be classified into one of three groups with regard to their interactions with phospholipid bilayers. Group I proteins are typically positively charged, water soluble, peripheral membrane proteins that interact much more strongly with anionic than with zwitterionic lipids. The interactions of such proteins with anionic phospholipid bilayers typically increases both the temperature and enthalpy of the main phase transition temperature while decreasing its cooperativity only slightly. Group I proteins are localized on the bilayer surface where they interact only with the phospholipid polar headgroups primarily by electrostatic interactions. Group II proteins are also typically positively charged at neutral pH but are somewhat less water soluble and also interact more strongly with anionic than with zwitterionic phospholipid bilayers. The interactions of these proteins with anionic phospholipid bilayers usually decreases the temperature, enthalpy and cooperativity of the main phase transition moderately, at least at relatively high protein concentrations. Group II proteins are localized at the bilayer interface where they interact primarily with the polar headgroups and glycerol backbone region of the phospholipid molecules by both electrostatic and hydrogen bonding interactions, although some hydrophobic interactions with the region of the hydrocarbon chains near the bilayer interface also occurs. Finally, Group III proteins have a range of charges but are water-insoluble, integral membrane proteins that interact equally well with anionic and zwitterionic phospholipid bilayers. The effect of the incorporation of such proteins into phospholipid bilayers is usually to reduce the temperature only slightly but to decrease the enthalpy and cooperativity of the main phase transition markedly. Group III proteins penetrate into or through phospholipid bilayers and interact with the phospholipid hydrocarbon chains by hydrophobic and van der Waal's interactions, as well as with the phospholipid polar headgroups. The incorporation of Group I proteins also usually has little effect on the permeability of the anionic phospholipid vesicles with which they interact, while the incorporation of Group II and Group III proteins increases vesicle permeability. The results of the present study indicate that overall GS behaves most like a Group II protein. Thus it is probably located in the interfacial region of phospholipid bilayers, where it interacts primarily with the polar headgroup and glycerol-backbone region of the phospholipid molecules by electrostatic and hydrogen-bonding interactions, its modest perturbation of hydrocarbon chain packing in the gel state being a secondary effect of its interactions primarily with the polar region of the phospholipid molecules. We also suggest, however, that the hydrophobic leucine and valine residues of the GS molecule may have limited interactions with the phospholipid hydrocarbon chains near the interfacial region of the bilayer, and the somewhat amphiphilic phenylalanine residues may interact with both the glycerol backbone and hydrocarbon chains at the bilayer polar/nonpolar interface. However, the exact location of GS molecules in liquid-crystalline lipid bilayers may vary somewhat with the charge and structure of the lipid polar headgroup (unpublished data from this laboratory). An interfacial location of the GS molecules would also explain the protection afforded to DMPG bilayers from the thermally induced hydrolysis of the fatty acyl ester linkages of these phospholipid molecules by this peptide.

We believe that our suggestion for the generally interfacial localization of the GS molecule in lipid bilayers is actually compatible with most of the data on GS–PC interactions previously published. For example, the failure of Pache et al. [21] to detect any effect of GS on the thermotropic phase behavior of DPPC bilayers was almost certainly due to the insensitivity of the calorimeter employed, since subsequent workers [22,23] including ourselves ([25], and the present work) have clearly shown a modest but significant effect of GS incorporation on the pretransition and main transition of DPPC and DMPC bilayers. Thus, this piece of calorimetric evidence for an exclusively surface location is not valid. Moreover, a careful inspection of the ESR spectrum published by Pache et al. [21] reveals a small but significant degree of immobilization of the 12-nitroxyl stearate spin-labeled PC molecule in the presence of admittedly high levels of GS, indicating some effect

on the lipid hydrocarbon chains. Moreover, the DSC and lateral diffusion and lateral aggregation measurements of Wu et al. [23], which suggested penetration of the peptide into the bilayer, utilized NBD-GS, a fluorescent derivative in which one or both of the ornithine residues in the GS molecules were chemically modified. Such a modification will reduce the positive charge distribution on the GS molecule as well as altering its amphiphilicity, hydrophilicity and size. Thus the interactions of NBD-GS with PC bilayer may well not accurately reflect the behavior of the underivatized peptide. Moreover, it seems to us that the sensitivity of the lateral diffusion coefficient of NBD-GS to the phase transition of the PC bilayer, and the lateral aggregation of this derivatized peptide induced by the addition of cholesterol, would both be expected from a peptide molecule localized at the bilayer interface as well as one penetrating partially or fully into the lipid bilayer hydrocarbon core. Moreover, the ^{2}H-NMR results of Zidovetzki et al. [24], which show that GS incorporation slightly reduces overall hydrocarbon chain disorder in fluid DMPC bilayers and decreases signal intensity primarily in the methylene segments closest to the glycerol backbone, are fully compatible with the postulated interfacial location of this peptide. The only information which does not seem compatible with a generally interfacial location of the GS molecule in liquid-crystalline PC bilayers is the report of Datema et al. [22], which suggested that GS molecular motion is insensitive to the phase state of DPPC bilayers. However, this result is at any rate at variance with the report of Wu et al. [23], who found that the lateral diffusion coefficient of at least NBD-GS *is* markedly altered by the phase state of DMPC bilayers. Clearly, additional work will be required to resolve this latter discrepancy, but in our view the preponderance of evidence supports a location of GS in fluid lipid bilayers in which the primary interactions are between the peptide molecule and the polar headgroups and the interfacial regions of the phospholipid molecule.

In closing, we note the importance of applying a number of physical techniques to studying various aspects of GS–phospholipid interactions in order to arrive at a more complete understanding of these systems. Thus, in our previous ^{31}P-NMR spectroscopic and X-ray diffraction studies of the effect of GS on phospholipid phase preference at temperatures above the main phase transition temperature [25], we found that the major effect of GS on lamellar/nonlamellar phase preference was on DMPE bilayers, with a weaker effect on DMPG and no effect at all on DMPC bilayers. Moreover, in general GS induced inverted cubic phases more readily in zwitterionic as opposed to anionic phospholipids. In contrast, our present DSC studies on the effect of GS on the main phase transition of these phospholipids indicate that GS interacts more strongly with the anionic DMPG vesicles than with the zwitterionic DMPC and DMPE vesicles, and more strongly with the former than the latter. Moreover, both of these types of effects of GS on phospholipid thermotropic phase behavior may be relevant to the action of GS on natural membranes. For example, anionic lipids such as PG may increase the rate or extent of GS binding to the bilayer surface, thus facilitating the interaction of this peptide with zwitterionic lipids such as PE or nonionic lipids such as monoglycosyl diacylglycerols, which can in turn form localized regions of nonlamellar structure which disrupt the lipid bilayer of the target membrane. We are thus continuing to study the effects of GS on a variety of lipid systems using additional physical techniques.

Acknowledgements

This work was supported by operating and major equipment grants from the Medical Research Council of Canada (R.N.M. and R.S.H.), by major equipment grants from the Alberta Heritage Foundation for Medical Research (R.N.M. and R.S.H.), and by the Protein Engineering Network of Centres of Excellence (R.S.H.). E.J.P. and L.H.K. were supported by postdoctoral fellowships from the Alberta Heritage Foundation for Medical Research and the Protein Engineering Network of Centres of Excellence, respectively.

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