Zwitterionic Phospholipids and Sterols Modulate Antimicrobial Peptide-Induced Membrane Destabilization

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ABSTRACT Cationic amphipathic α-helical peptides preferentially disrupt anionic lipids in mixed model membranes, potentially causing a catastrophic release of the cell contents or attenuation of the membrane potential. The effective role of such peptides requires considerable discrimination between target and host cells, which is likely to occur at the level of the cell membrane. Here, we explore the roles of a variety of common membrane constituents in mediating the interaction between the antimicrobial peptide pleurocidin and model membranes. We employ intrinsic tryptophan fluorescence and circular dichroism to observe the effect of increasing concentrations of sterol in the membrane on peptide binding, using ^{2}H solid-state NMR of chain deuterated lipids simultaneously to probe the effective chain disruption of the anionic phospholipid component of the membrane. We show that the degree of ordering of the lipid acyl chains in the membrane is dependent on the nature of the zwitterionic phospholipid headgroup in mixed anionic membranes. Furthermore, the presence of cholesterol and ergosterol increases acyl chain order in the liquid crystalline model membranes, but to differing degrees. Our results show how sterols can protect even negatively charged membranes from the disruptive effects of antimicrobial peptides, thereby providing a molecular view of the differences in sensitivity of various target membranes to linear cationic antibiotic peptides where bacteria (no sterols) are most susceptible, lower eukaryotes including fungi (containing ergosterol) exhibit an intermediate degree of sensitivity, and higher organisms (containing cholesterol) are largely resistant to antimicrobial peptides.

INTRODUCTION

Cationic α-helical peptides can have important antimicrobial (1,2) or gene transfer capabilities (3,4). For each of these roles, the peptide should have a high degree of selectivity to avoid host toxicity. Since it is thought that these peptides interact with the cell membrane and not a specific chiral cell membrane receptor, there is a significant risk that an effective antimicrobial peptide may also prove toxic to its eukaryote host or a vector peptide may damage a eukaryote cell membrane while delivering its nucleic acid cargo.

Understanding why certain peptides are particularly effective as antimicrobials, yet remain relatively well supported by eukaryote cells, requires detailed knowledge of a number of factors, including the volume, structure, and oligomeric state in solution of the peptide (5), synergistic effects (6), and the key peptide-lipid interactions that determine the activity of the peptide within its target membrane. When considering the peptide-lipid interactions, the selective activity of cationic antimicrobial peptides has been primarily ascribed to the presence of negatively charged lipids in bacterial membranes (7), and indeed, a litany of previous studies have shown that cationic antimicrobial peptides associate far more readily with model membranes containing such lipids (e.g., (7–16)). The relationship between the activity and toxicity of antimicrobial peptides was therefore proposed to be related to the surface charge of the cell and then to the hydrophobicity of the peptide (7). As a consequence, the role of other lipids, such as other phospholipids and sterols, in target membranes was considered to be secondary (7) and has been studied in much less detail, as it has been supposed that antimicrobial peptides would encounter neither negatively charged membranes containing cholesterol nor neutral membranes lacking cholesterol in vivo. However, in fact, such situations can be commonplace in vivo, such as when eukaryotic membrane asymmetry is disrupted during tumorigenesis, where negatively charged lipids are presented at the cell surface (17); or in the membranes of parasites such as the eukaryote *Plasmodium spp*., whose neutral membranes contain much lower levels of cholesterol (18); or in the membranes of enveloped viruses, where the membrane is enriched in both negatively charged phosphatidylserine and cholesterol (19). Cationic peptides with a suitable level of hydrophobicity do interact with membranes with a neutral surface (e.g., (20,21)), and indeed, *in silico* studies show that they are capable of forming disordered toroidal pores in such membranes (22), they have been designed to attack tumor cells (23), and they also seem active against *Plasmodium falciparum* even within an erythrocyte cell host (24). Hence, revealing the subtlety of the peptide-lipid interactions in membranes mimicking these in vivo situations may yield a greater understanding of how antimicrobial peptides function in nature and may aid our design of membrane interacting peptides, be they vector peptides or antimicrobial or antitumor therapeutics.

Here, we illustrate our point by studying the interaction of pleurocidin with anionic membranes containing phosphatidylglycerol (PG) as the anionic component and either

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phosphatidylcholine (PC) or phosphatidylethanolamine (PE) as zwitterionic lipids with and without either cholesterol or ergosterol as sterol components. Pleurocidin is a cationic amphipathic helical (25) peptide antibiotic found in the skin (26) and intestine (27) of the winter flounder *Pleuronectes americanus*. Pleurocidin inserts into membranes and adopts a surface orientation (28), is capable of causing dye leakage from liposomes (16) and translocating across model membranes (16), and demonstrates pore-forming activity in planar lipid bilayers (29). These properties are greatly enhanced when the membranes are partly composed of anionic lipids.

We use optical spectroscopy methods to show that the presence of sterol reduces the insertion of pleurocidin into anionic mixed membranes, whereas the peptide maintains a mostly α-helical secondary structure. ^{2}H NMR results show that both sterols increase the lipid chain order of the labeled anionic lipid model membranes, but in PE/PG membranes, which most closely mimic the bacterial target membrane, an apparent threshold is reached above which the membrane order is increased by cholesterol but not by ergosterol. Similar NMR measurements performed in the presence of pleurocidin reveal how effective elevated levels of cholesterol are at attenuating the membrane disruptive activity of pleurocidin when compared with ergosterol, providing a possible explanation for how certain natural membranes are more resistant than others to the action of antimicrobial peptides.

MATERIALS AND METHODS

Peptide and lipids

Pleurocidin amide (GWGSFFKKAAHVGKHVGKAALTHYL-NH_2) was synthesized using standard FMOC (9-fluorenylmethyloxycarbonyl) solid-state chemistry on a Millipore 9050 synthesizer (Millipore, Billerica, MA). In crude peptide preparations, a predominant peak was observed when analyzed by high-performance liquid chromatography with acetonitrile/water gradients. During high-performance liquid chromatography purification, the main peak was collected and the identity of the product confirmed by matrix-assisted laser desorption ionization mass spectrometry. The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE), 1-palmitoyl$_{d31}$-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG-d31), 1-palmitoyl$_{d31}$-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE-d31), cholesterol, and *Escherichia coli* total lipid extract were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Ergosterol was from Sigma (St. Louis, MO). All other reagents were analytical grade or better.

Sample preparation for solid-state NMR

For solid-state NMR, samples with different lipid compositions were prepared (molar ratios in parentheses): POPC/POPG-d31 (75:25) and POPE/POPG-d31 (75:25). Further samples were prepared in which the amount of phospholipid was kept constant as sterols were added in amounts relative to the initial phospholipid composition (i.e., 10, 20, 30, and 40%), giving final sterol molar concentrations of 9, 17, 23, and 29%, respectively. One further sample was prepared wherein 20 mg *E. coli* total lipid extract was mixed with 2 mg POPE-d31. For the binary and tertiary lipid mixtures, a total of ~5 mg of lipids per sample were dissolved and mixed in chloroform and dried under rotor-evaporation at room temperature. To remove all organic solvent, the lipid films were exposed to vacuum overnight. The films were then rehydrated with 5 ml of 0.1-M Tris and 0.1 M KCl buffer at pH 7.5 at room temperature. Samples were subjected to five rapid freeze-thaw cycles for further sample homogenization and then centrifuged at 21,000 $\times$ *g* for 20 min at room temperature. The pellets, containing lipid vesicles, were transferred to Bruker 4-mm MAS rotors (Bruker, Karlsruhe, Germany) for NMR measurements. The samples were then resuspended in 5 ml buffer and pleurocidin was added at 2 mol %. The samples were briefly sonicated in a bath sonicator to improve exposure of all lipids to the peptide and then subjected to five more freeze-thaw cycles before being transferred to MAS rotors, as above.

Tryptophan fluorescence spectroscopy

Emission spectra of the intrinsic fluorescence of Trp-2 were acquired using a Fluorolog 3-22 spectrometer (HORIBA Jobin-Yvon, Longjumeau, France). Vesicle suspensions were prepared as for solid-state NMR experiments above in the absence of peptide, except that the freeze-thaw cycles were omitted, yielding large multilamellar vesicles. Vesicles containing POPC/POPG (75:25) and POPE/POPG (75:25) were prepared at a concentration of 5 mg/ml. From these suspensions, 150 μl was added to 0.85 ml PBS buffer, and then peptide in solution (2 mg/ml in PBS) was added to produce a final peptide concentration of ~0.01 mg/ml. A peptide/lipid molar ratio of 1:40 was maintained. Tryptophan emission spectra of the lipid/peptide suspension were acquired by scanning from 310 to 450 nm using an excitation wavelength of 295 nm and a spectral bandwidth of 5 nm for both excitation and emission. A spectrum of the aqueous peptide was acquired at a peptide concentration of 0.1 mg/ml in the same buffer. For fluorescence quenching experiments, 30% acrylamide solution was added stepwise to a final concentration of 0.18 M, and at each step, equilibration of the sample was ensured. All spectra are an average of three scans. The temperature was maintained at either 310 or 298 K by connecting the cuvette holder to an external water bath.

Circular dichroism

Spectra were acquired on a Jasco J-810 spectrometer (Jasco, Tokyo, Japan) with samples maintained at 310 K. Spectra were recorded from 250 to 190 nm using a spectral bandwidth of 1 nm and a scan rate of 100 nm/min. Samples were prepared as for the fluorescence experiments above, but with the lipid suspension undiluted. From the lipid suspension, 240 μl was added to a 1-mm cuvette and then 12 μl peptide solution (2 mg/ml) was added and thoroughly mixed. Spectra were treated using Jasco spectra analysis software, where a spectrum of the peptide-free suspension was subtracted and Means Movement smoothing with a convolution width of 5 points was applied. Secondary-structure analysis was performed using CDPro (30).

Dye-release assay

Large unilamellar vesicles (LUV) loaded with calcein were prepared by mechanical extrusion. Three lipid mixtures: POPE/POPG (75:25), POPE/POPG (75:25) supplemented with 40% of cholesterol, and POPE/POPG (75:25) supplemented with 40% of ergosterol, were dissolved separately in chloroform/methanol. The solutions were dried and then hydrated in PBS buffer (50 mM, pH 7.4) with 50 mM of calcein ions (Calcein disodium salt, Fluka, Switzerland) before undergoing several freeze-thaw cycles and then extrusion (11 times) through a 200-nm-pore membrane (Avestin, Ottawa, Canada). The calcein-entrapped vesicles were separated from the dye solution by gel filtration on a Sephadex G-50 column (2.5 $\times$ 3.5 mm) (Sigma) loaded with PBS buffer (50 mM, pH 7.4) supplemented with 75 mM NaCl to compensate for the change in osmolarity induced by the presence of calcein molecules and Na^+ counterions. The concentrations of the LUV suspensions eluting from the column were determined by comparing 100% dye release from suspensions before and after the gel filtration step.

Calcein efflux measurements were performed on a Fluorolog 3-22 spectrometer (Spex Jobin-Yvon). In a typical experiment, an aliquot of the LUV solution was added to 1.5 ml PBS buffer (50 mM and 75 mM NaCl, pH 7.4) in a quartz cuvette and equilibrated for some minutes at 310 K inside the spectrometer. We added 7 μl of pleurocidin solution (2 mg/ml) into the cuvette with the sample excited at $\lambda_{exc} = 480$ nm, and the intensity of fluorescence (I) was recorded at $\lambda_{fluo} = 515$ nm for ~10 min. A spectral bandwidth of 1 nm was used for both excitation and emission. The percentage of calcein released from the vesicles ($I_{\%}$) was calculated according to the formula $I_{\%} = 100 \times (I - I_0) / (I_{Max} - I_0)$, where I_0 represents the intensity of fluorescence before adding the peptide to the solution and I_{Max} is the maximum intensity observed after dissolving the vesicle with 10 μl of 10% Triton X-100. Care was taken to maintain constant I_{Max} to allow quantitative comparison between the multiple recordings.

Solid-state NMR

^{2}H quadrupole experiments (31) for samples containing POPG-d31 as the labeled lipid were performed at 46.10 MHz on a Bruker Avance 300 NMR spectrometer using a 4-mm MAS probe with spectral width 200 kHz and with recycle delay, echo delay, acquisition time, and 90° pulse lengths of 0.3 s, 42 μs, 2 ms, and 5 μs, respectively. The temperature was maintained at 310 K to keep the bilayers in their liquid-crystalline phase. During processing, the first 40 points were removed to start Fourier-transformation at the beginning of the echo. Spectra were zero-filled to 8192 points and 50-Hz exponential line-broadening was applied. Smoothed and/or averaged deuterium order-parameter profiles were obtained from symmetrized and dePaked ^{2}H-NMR powder spectra of POPG-d31 using published procedures (32–34).

RESULTS

Tryptophan fluorescence spectroscopy

Intrinsic fluorescence of the Trp-2 residue in pleurocidin can be used as a sensitive reporter of the environment experienced by the peptide when interacting with vesicles of varying lipid compositions. We used two binary lipid mixtures as a starting point, comprising POPG as the anionic component and either POPC or POPE as the zwitterionic component, and then increased the concentration of cholesterol in the prepared model membranes. Ergosterol was found to contribute a relatively strong background signal and was therefore unsuitable for inclusion in this aspect of the study. Three parameters from the fluorescence experiments are used here to characterize the peptide-lipid interactions and these include the emission maxima, emission intensity, and accessibility to the aqueous quencher, acrylamide, where values with shorter wavelengths, greater intensity, and a reduced accessibility, respectively, represent a more hydrophobic environment, as is found within the hydrophobic core of the membrane. At 310 K, corresponding to physiological temperature for a bacterium within a human host, it can be seen that the addition of increasing amounts of cholesterol to POPC/POPG membranes leads to a reduction in intensity of the fluorescence emission spectra, which accompanies a red shift in the maxima (Fig. 1 *A*). The corresponding Stern-Vollmer plots (Fig. 1 *B*) show a small but noticeable trend of increasing accessibility to acrylamide. These results indicate that, on average, the peptide experiences a less hydrophobic environment for each stepwise addition of cholesterol. Similar results are obtained when POPE is used as the zwitterionic component (Fig. 1, *C* and *D*); however, the red shifts and reductions in peak intensity (Fig. 1 *C*) are much more reduced, whereas the slope on the Stern-Vollmer plots (Fig. 1 *D*) is greater than for the corresponding membranes containing POPC (Fig. 1, *A* and *B*). The emission maxima obtained in the spectra for samples containing POPE are all at relatively long wavelengths, between 364 and 367 nm, indicating that at 310 K the peptide is quite accessible to the external aqueous environment although still associated with the hydrophobic membrane, as, for comparison, the emission maximum for peptide in aqueous solution is at 374 nm (Stern-Vollmer plot for pleurocidin in solution is available as supplementary information). This is confirmed by the Stern-Vollmer plots, which indicate a higher level of accessibility of the peptide to the aqueous quencher (Fig. 1, *C* and *D*), although again, the slope of the Stern-Vollmer plot for pleurocidin in solution is over 1.5 times greater than those for the peptide in the presence of lipid. Nevertheless, to obtain a clearer picture of the effect of adding cholesterol, the experiments were repeated at the lower temperature of 298 K. At this temperature, the membranes remain in the liquid-crystalline phase, but the lipids become more ordered; indeed, a comparison of averaged lipid-chain order parameters from this and our previous work (28) indicates that in POPE/POPG (3:1) membranes, the order of the anionic lipid chains increases by ~7% when the temperature decreases from 310 to 298 K. At this lower temperature, the emission maxima are all blue-shifted to lower wavelengths, indicating that the peptide experiences a more hydrophobic environment at 298 K than at 310 K (Fig. 2, *A* and *C*). For POPC/POPG liposomes, the reduction in emission intensity with increasing cholesterol concentration is much more modest at the lower temperature, whereas the red shifts observed at 310 K are not apparent (Fig. 2 *A*). The corresponding Stern-Vollmer plots (Fig. 2 *B*) show a slight increase in accessibility to acrylamide at higher cholesterol concentrations only and confirm that under these conditions, the addition of cholesterol does not have a large effect on pleurocidin insertion into the membrane. In contrast, the effect on pleurocidin insertion of stepwise addition of cholesterol to POPE/POPG membranes is much clearer under these conditions. When the cholesterol content is increased, large reductions in emission maxima with concomitant red shifts are observed (Fig. 2 *C*) which are reflected in clear increases in the slope of the corresponding Stern-Vollmer plots (Fig. 2 *D*). Taken together, these data show that when the concentration of cholesterol is increased in mixed anionic membranes, the peptide experiences, on average, a less hydrophobic environment and is thus being increasingly excluded from the hydrophobic core of the membrane. In addition, the fluorescence studies performed at both temperatures indicate that the addition of cholesterol to mixed anionic membranes has a greater effect on pleurocidin insertion when the zwitterionic lipid is POPE rather than POPC.

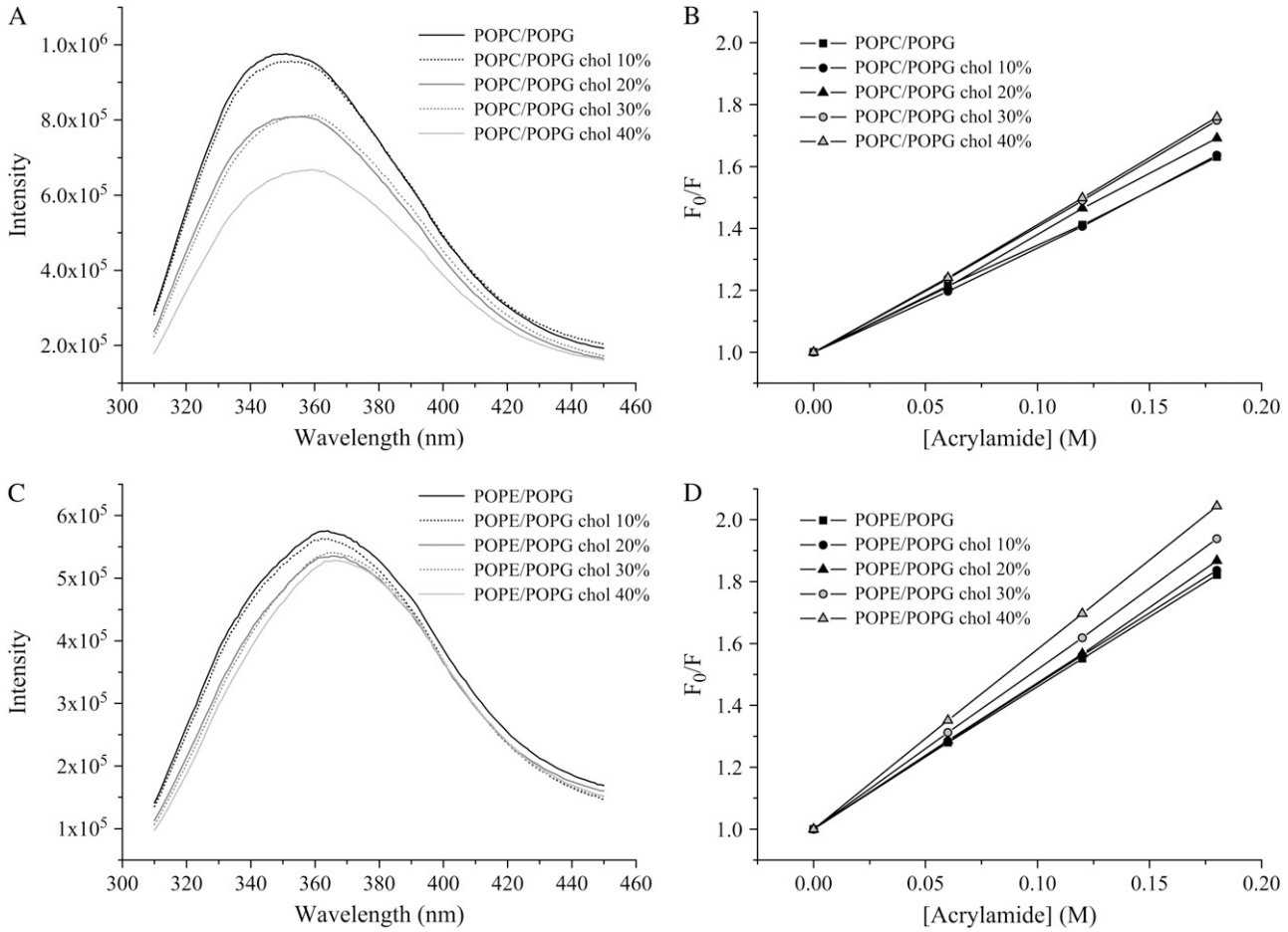

FIGURE 1 Intrinsic tryptophan fluorescence spectra of pleurocidin Trp-2 in the presence of liposomes composed of either POPC/POPG (*A*) or POPE/POPG (*C*) and increasing concentrations of cholesterol at neutral pH, 310 K, and peptide/lipid ratio 1:40. The Stern-Vollmer plots of the effect of adding an aqueous acrylamide quencher reveals the depth of pleurocidin penetration into the same liposomes comprising either POPC (*B*) or POPE (*D*) as the zwitterionic lipid.

Circular dichroism

In a similar fashion, circular dichroism (CD) measurements of the peptide in the presence of lipid vesicles of differing composition provide information on the average secondary structure adopted by the peptide. Pleurocidin is known to adopt an α-helical structure in the presence of anionic liposomes, whereas in aqueous solution the peptide is unstructured (16). Therefore, if pleurocidin is excluded from the membrane by the presence of either cholesterol or ergosterol, then the expected α-helical content will be reduced. Circular dichroism spectra of pleurocidin in POPC/POPG membranes and the same membranes with elevated levels of either ergosterol or cholesterol indicate that the peptide maintains a largely α-helical structure (Fig. 3). Small differences are observable between the spectra, particularly between 200 and 210 nm, but analysis using the CDPro software package (30) was unable to distinguish any consistent trends in alteration of secondary structure. Therefore, despite being more exposed to the aqueous environment in membranes containing elevated sterol levels, pleurocidin retains its secondary structure, and thus, on average, the peptide remains membrane-bound. The low sensitivity of these measurements prevented similar measurements being obtained for POPE/POPG membranes, which were observed to aggregate when peptide was added at the necessarily high concentration.

^{2}H solid-state NMR

Deuterium NMR has been shown to be an effective means of monitoring the changes in lipid chain order when either cholesterol (35,36) or ergosterol (37,38) is added to membranes containing the monounsaturated lipid POPC. Here we apply the technique to membranes containing a mixture of monounsaturated lipids with varying headgroups and with anionic POPG-d31 as the labeled reporter of lipid chain order. The anionic lipid component is selected to carry the deuterium label, as we have observed previously that pleurocidin interacts more strongly with this component of mixed membranes (28). The binary lipid mixtures contain both zwitterionic and anionic lipids and the tertiary mixtures

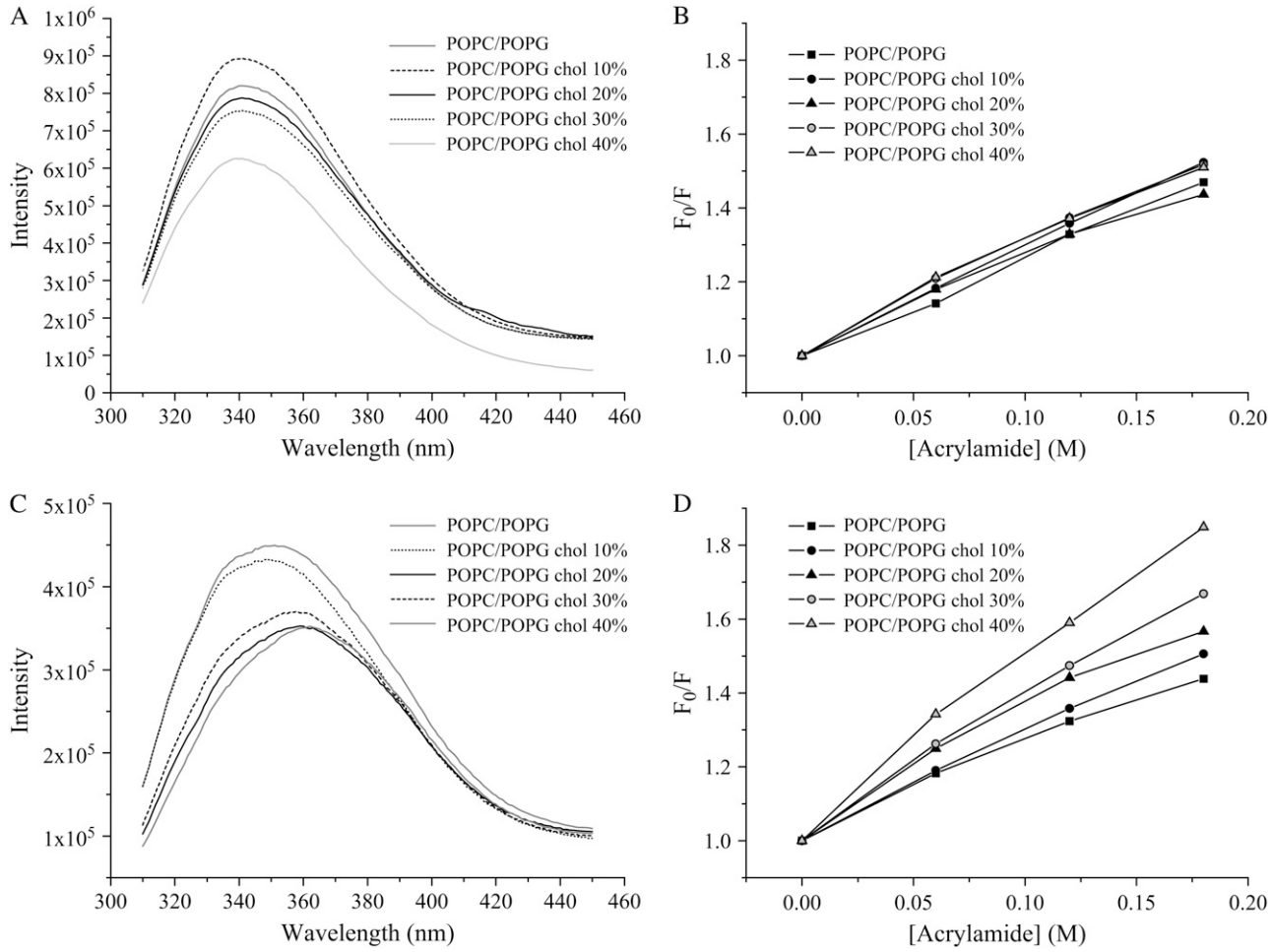

FIGURE 2 Intrinsic tryptophan fluorescence spectra of pleurocidin Trp-2, as in Fig. 1, but at 298 K, where the model membranes can be expected to be more ordered. The red shift and reduction in fluorescence intensity observed in the presence of increasing concentrations of cholesterol in the liposomes is more noticeable for liposomes containing POPE as the zwitterionic lipid (*C*) compared with POPC (*A*). The Stern-Vollmer plots of the effect of adding an aqueous acrylamide quencher reveals the depth of pleurocidin penetration into the same liposomes comprising either POPC (*B*) or POPE (*D*) as the zwitterionic lipid.

contain, in addition, either cholesterol or ergosterol. Wideline ^{2}H echo spectra were obtained of the lipid mixes with an additional 0, 10, 20, 30, or 40 mol % of sterol at 310 K, and averaged order parameters were calculated from the dePaked spectra. The average order parameters obtained for POPG-d31 in the binary and tertiary lipid mixtures show the response of the lipid chain order to increasing concentrations of sterol for each of the four systems studied (Fig. 4). The addition of either cholesterol or ergosterol to mixed POPC/POPG membranes causes a concentration-dependent increase in lipid chain order that is somewhat greater with cholesterol than with ergosterol, a finding that is in agreement with previous studies of neutral POPC-containing membranes (37,38). Previous studies of POPC/ergosterol membranes have shown an apparent saturation of the lipid chain order at a sterol concentration of 25 mol % (38,39). Adding an extra 40 mol % sterol to the phospholipid mixture corresponds to a final sterol concentration of 29% of the total lipid, and for this POPC/POPG membrane, there is no apparent saturation of the lipid chain order of the anionic lipid in the conditions used here. Furthermore, the same study reported the existence of two coexisting liquid-crystalline phases at temperatures up to 304 K (38). In this study, performed with mixed POPC/POPG membranes and at an elevated temperature of 310 K, we saw no evidence of such a phenomenon. Interestingly, however, we observed clear differences in lipid chain ordering and the response to increased sterol concentration between membranes containing either POPC or POPE as the zwitterionic lipid component (Fig. 4). First, the average lipid chain order of POPG-d31 in membranes containing POPE is ~29% greater than that in membranes containing POPC as the zwitterionic lipid. Second, although the addition of either cholesterol or ergosterol does cause an increase in lipid chain order, a clear saturation of the effect is observed with an additional 20–30 mol % ergosterol, but not with additional cholesterol. Thus, elevated levels of cholesterol in such mixed membranes will have a greater ordering effect than ergosterol, whereas membranes

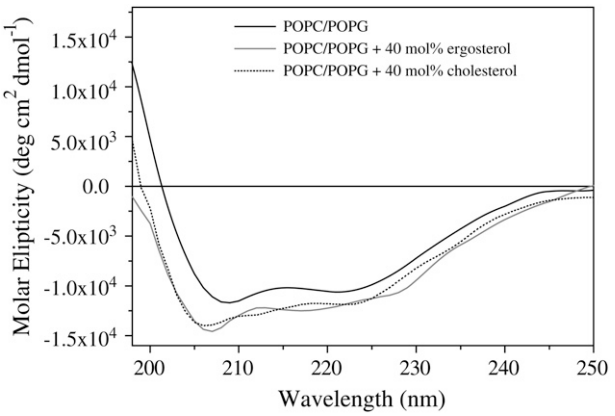

FIGURE 3 Circular dichroism spectra of pleurocidin in the presence of liposomes of varying lipid composition at neutral pH, 310 K, and peptide/lipid ratio 1:40. Spectra of peptide-liposomes containing POPC as the zwitterionic lipid reveal that pleurocidin retains an α-helical secondary structure even in the presence of sterol at an additional 40 mol %.

that are largely composed of PE lipids, in preference to PC lipids, will also have more ordered hydrophobic cores.

The large discrepancy between the average lipid chain order observed for POPC/POPG and POPE/POPG membranes calls into question which model is more appropriate for representing natural membranes. To our knowledge, order parameters are not known for natural membranes and a detailed study of this is beyond the scope of this work. However, we obtained an estimate of the lipid chain order expected for the bacterium *E. coli* by adding POPE-d31 as a labeled reporter to *E. coli* total lipid extract. Total *E. coli* lipid extract contains 57.5% PE by weight (40), and therefore, the effect of adding 2 mg POPE-d31 to 20 mg total lipid should be minimized. In our previous study of the interaction of pleurocidin with POPE/POPG membranes, we showed that spectra of membranes carrying either POPE-d31 or POPG-d31 as reporter were very similar (28). The spectrum of vesicles made from the *E. coli* lipid mix is itself compared with that of POPE/POPG-d31 (Fig. 5) and can be seen to be not too dissimilar, indicating that POPE/POPG and not POPC/POPG model membranes are a much more reliable mimic of the natural bacterial membrane in terms of the lipid chain acyl order at least.

The membrane-destabilizing effect of pleurocidin can also be measured by ^{2}H NMR methods in the presence of elevated levels of sterol. The wideline ^{2}H echo spectra (Fig. 6) and the corresponding order-parameter profiles calculated relative to peptide-free membranes (Fig. 7) reveal how effective pleurocidin remains at destabilizing the anionic lipid acyl chains in the absence or presence of either cholesterol or ergosterol. Pleurocidin at 2 mol % has a modest chain-disordering effect on POPG-d31 in mixed POPC/POPG vesicles (Figs. 6 *A* and 7 *A*). However, since the lipid acyl chains in these membranes are already rather disordered and the relevance of this model membrane is uncertain, a comparison of the membranes with elevated sterol concentration is more revealing. ^{2}H NMR spectra of membranes containing an additional 40 mol % of either cholesterol (Fig. 6 *C*) or ergosterol (Fig. 6 *E*) show that although these sterols have been shown, using optical methods, to inhibit the penetration of pleurocidin into the hydrophobic core, the peptide is nonetheless capable of reducing the lipid acyl chain order of the anionic lipids. Furthermore, a quantitative comparison of this chain destabilization (Fig. 7 *A*) reveals the reduction

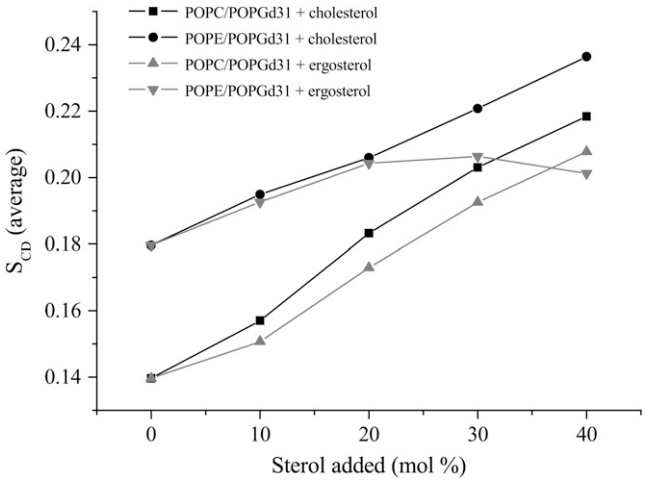

FIGURE 4 Average order parameters of binary and tertiary lipid membranes obtained from dePaked ^{2}H echo spectra of POPG-d31 containing liposomes at pH 7.5 and 310 K. Original spectra, obtained on a Bruker Avance 300 spectrometer, and smoothed order-parameter profiles are available in the Supplementary Material.

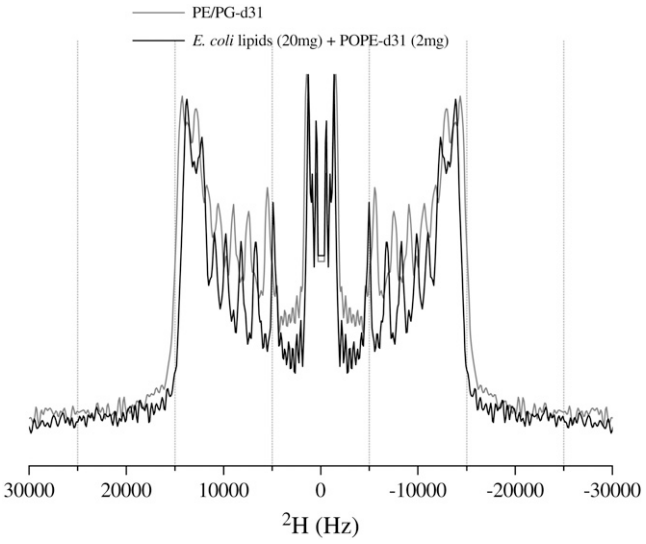

FIGURE 5 Comparison of the ^{2}H spectrum obtained for POPE/POPG-d31 (3:1) and 2 mg of POPE-d31 added to 20 mg of total *E. coli* lipid extract. The similarity of the spectra indicate that model membranes composed of POPE/POPG are a good mimic for the natural *E. coli* membranes targeted by antimicrobial peptides.

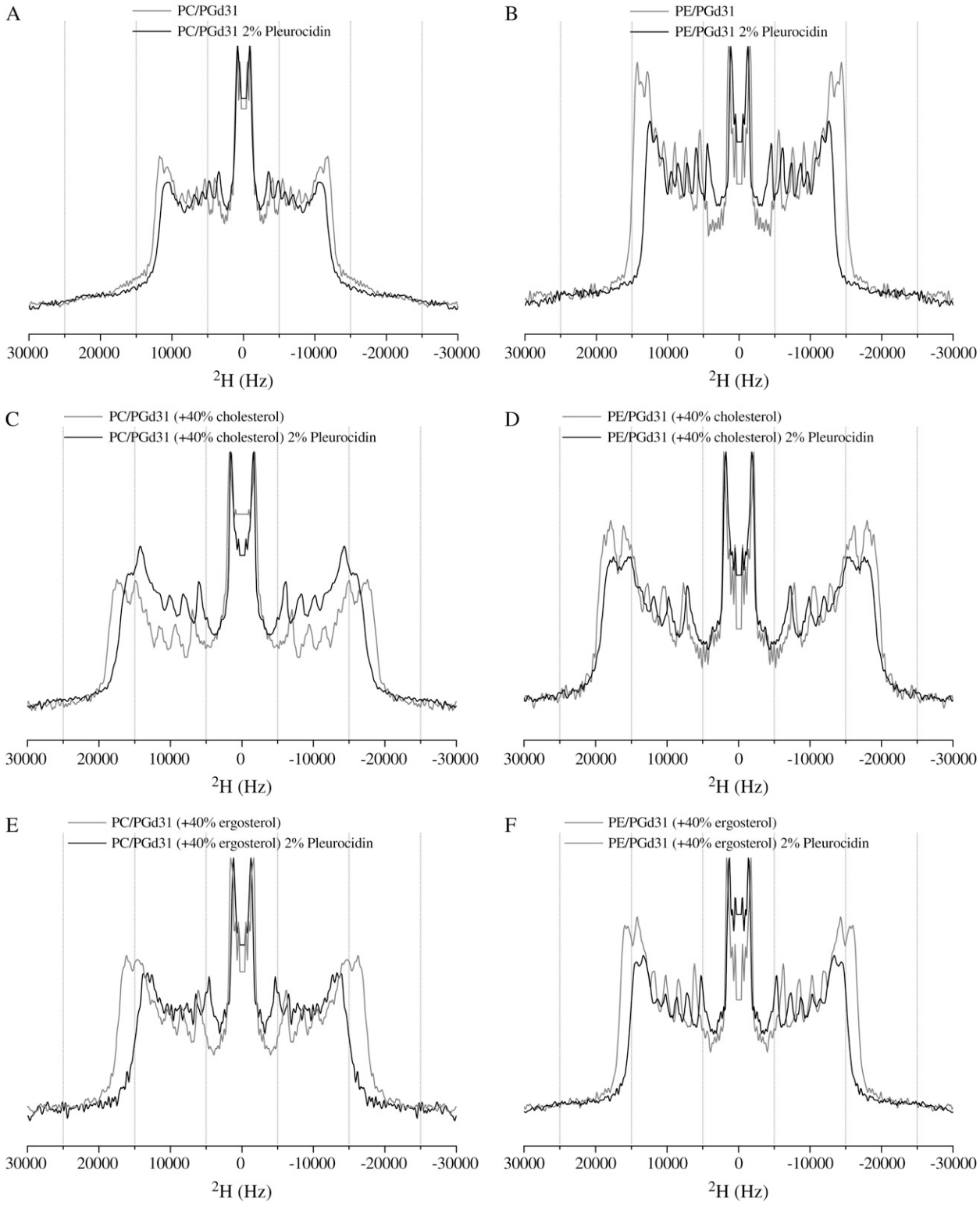

FIGURE 6 Effect of adding pleurocidin to liposomes containing either POPC (*A*, *C*, and *E*) or POPE (*B*, *D*, and *F*) as the zwitterionic-lipid component is observed by acquiring ^{2}H spectra of the deuterium-labeled anionic POPG-d31. Liposomes are either sterol free (*A* and *B*) or contain an additional 40 mol % cholesterol (*C* and *D*) or ergosterol (*E* and *F*). Spectra were recorded on a Bruker Avance 300 spectrometer at 310 K. Spectra of liposomes are shown in the absence (*shaded lines*) and presence (*solid lines*) of 2 mol % pleurocidin.

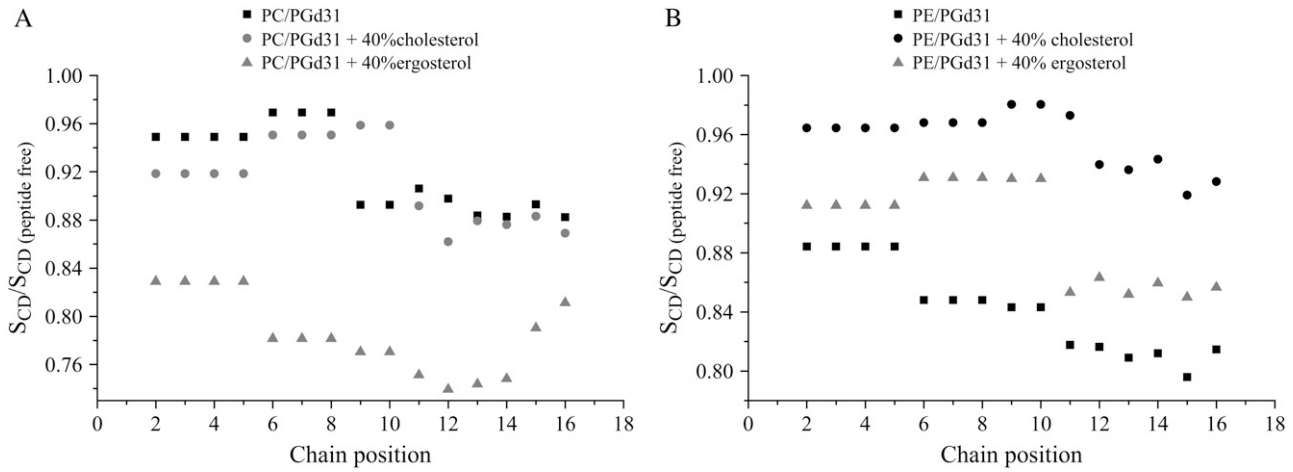

FIGURE 7 Smoothed order parameters shown for spectra in Fig. 5 with liposomes incubated with 2 mol % pleurocidin are calculated relative to profiles for peptide-free liposomes.

of lipid chain acyl order to be much greater in the presence of ergosterol than of cholesterol. When POPE replaces POPC as the zwitterionic lipid component a clear lipid-destabilizing effect can be seen in the presence of 2 mol % pleurocidin (Figs. 6 *B* and 7 *B*), indicating that despite the exposure of the peptide to the aqueous environment at 310 K, as determined by fluorescence measurements, pleurocidin remains associated with the membrane and capable of a strong interaction with the anionic lipid component. Again, a quantitative comparison of the chain-disrupting effects of pleurocidin in the presence of cholesterol (Figs. 6 *D* and 7 *B*) or ergosterol (Figs. 6 *F* and 7 *A*) reveals that the activity of the peptide is much reduced in the presence of cholesterol when compared with ergosterol, and indeed, in this case the activity of pleurocidin on the anionic lipid component is almost completely attenuated. Notably, the chain-ordering effect of elevated levels of cholesterol is greater in both cases than that of ergosterol, whereas the greatest increases in sterol-induced chain ordering correspond to the greatest reductions in peptide-induced acyl-chain disruption.

Dye-release assay

Confirmation of the effects of incorporating elevated levels of either ergosterol or cholesterol in the membranes was obtained by studying pore formation in liposomes by means of a dye-release assay. Liposomes comprised of POPE/POPG and an additional 40 mol % of either cholesterol or ergosterol were challenged by pleurocidin. A previous study showed that pleurocidin causes release of ~30% of the dye from negatively charged liposomes at the peptide/lipid ratios used in this study (16). Here, we observe a similar release of the calcein dye from POPE/POPG vesicles (Fig. 8). It is interesting that the presence of elevated levels of ergosterol reduces the amount of dye released when the liposomes are challenged by pleurocidin. However, when ergosterol is replaced by cholesterol, the release of dye is almost completely attenuated (Fig. 8).

DISCUSSION

The exact nature of how cationic amphipathic peptides kill their microbial targets is a matter of some controversy, with a debate ongoing as to the relative contributions of intracellular targeting and membrane disruption to the overall killing strategy for each different antimicrobial peptide (1). Antimicrobial peptides are therefore diverse not only in structure but also, potentially, in their mechanism of action. Nevertheless, the interaction between the bacterial cell membrane and antimicrobial peptides is thought to be an important determinant of the peptide activity. Although information re-

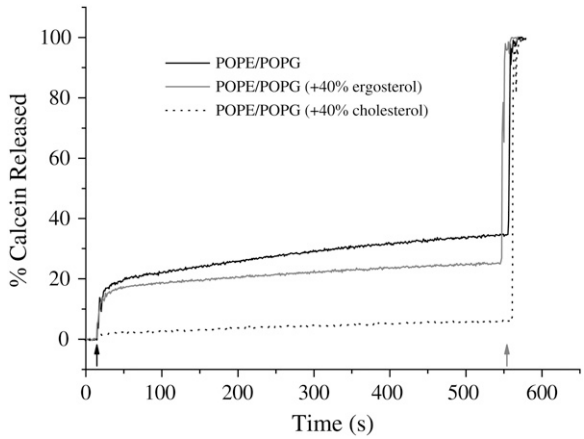

FIGURE 8 Comparison of pore formation in anionic liposomes, assessed by monitoring the release of fluorescent calcein from large unilamellar vesicles when challenged with pleurocidin. The experiment was performed at 310 K and pH 7.4 and the times of addition of pleurocidin and Triton X-100 are indicated by solid and shaded arrows, respectively.

garding the membrane interaction of some classes of peptide, such as defensins, is scarce, considerably more attention has been focused on the membrane interaction of linear cationic peptides such as magainin and pleurocidin. Despite a large number of studies, there is, however, not yet a consensus on how peptides from this class interact with cell membranes. Solid-state NMR studies of aligned samples have shown that at peptide/lipid ratios where channel conductance is observed, a large number of amphipathic helical peptides including magainin (41), pleurocidin (28), piscidins (42), LL-37 (43), granulysin (44), MSI-843 (45)/ and LAH4 (21,46,47) have either been shown or proposed to maintain a surface orientation in their active conformation. Models for the action of antimicrobial peptides on model membranes include the Carpet mechanism (48) and pore-forming models (49). The observation of a surface alignment of the peptides conflicts with existing models that propose the formation of either barrel-stave, or toroidal, pores, where a peptide orientation parallel to the membrane normal is required (49,50), although in the case of toroidal pores, pore formation may be only transient. The NMR data, however, agrees very well with a recent in silico study that predicts the formation of a disordered toroidal pore, at a comparable peptide/lipid ratio, with one peptide in the pore lumen and all peptides maintaining an orientation roughly parallel to the membrane surface (22). This model is useful for considering both a pore-forming and an intracellular target bactericidal strategy for linear cationic antibiotic peptides, since it shows that at such peptide/lipid ratios, the peptide is capable of causing sufficient membrane disruption that sizeable pores do form and the peptide can migrate from one leaflet of the membrane to another with the membrane remaining otherwise intact (22). Recent studies of the mechanism of action of the amphipathic cell penetrating peptide Tp-10 using fluorescence methods provide further experimental support for this model (51). Interestingly, the molecular dynamics simulations also revealed that the disorder in the lipid acyl chains of those lipids in contact with the peptides increased, whereas the remainder of the bilayer-forming lipids were unaffected (22), a finding that agrees excellently with our (28,47,52) and others' (53,54) experimental observations in model membrane systems studied by solid-state ^{2}H NMR. We showed that pleurocidin (28) and designed cationic helical peptides (47,52) cause strong reductions in lipid acyl chain order in the anionic but not the zwitterionic lipid component in mixed membranes, indicating that the peptide has a strong effect on the lipids associated with it but not on other lipids in the membrane. Whether or not the local reduction of bilayer order is a result of or a cause of pore formation and, hence, cell viability, it is a useful probe of the effects of altering the membrane composition and we have shown that the method is sufficiently sensitive to detect small changes in chain order, for example, as a result of altering peptide structure (55). Hence, here we have assessed the role of the zwitterionic lipid and the sterol component in modulating the membrane-disrupting effect of pleurocidin, which we use as a representative of the linear cationic amphipathic class of antimicrobial peptides. Specifically, using fluorescence and circular dichroism techniques, we have studied the binding of pleurocidin to a variety of mixed membranes and have then probed lipid acyl chain order in the anionic lipids, the effect on this of insertion of pleurocidin, and how this is related to pore formation and the bactericidal strategy of the peptide.

The intrinsic tryptophan fluorescence measurements presented here show that the presence of sterols in the membranes does alter the location of pleurocidin in the membrane, with increasing amounts of cholesterol increasing the exposure of the peptide to the external aqueous environment. The CD measurements indicate, however, that despite this change in environment, the peptide retains its secondary structure, whereas solid-state NMR measurements show that it is capable of destabilizing the anionic lipid acyl chains but to a more limited extent. Cholesterol has been known for some time to inhibit the lytic activity of the amphipathic α-helical peptide magainin 2 (56) and early ^{2}H solid-state NMR measurements indicated that cholesterol also affected the membrane interaction of magainin 2 (57). Here, though, we can present a detailed molecular understanding of how sterols can reduce the local membrane-destabilizing effect of the antimicrobial peptide pleurocidin, which is also closely related to the pore forming capability of the peptide. Higher concentrations of sterol increase the order of the acyl chains in the model membranes and by quantifying both the increase in chain order due to the sterol and the local chain disordering effect of the peptide we can see that those membranes that become more ordered, in particular those containing cholesterol in preference to ergosterol or POPE in preference to POPC, are the most resistant to the chain-disordering action of pleurocidin. At a molecular level, the effect of inhibiting the local disruption of chain order, which is linked to the formation of disordered toroidal pores (22), can reduce or even prevent pore formation and the concomitant release of cell contents or attenuation of the membrane potential. Furthermore, since it has been proposed that linear cationic antimicrobial peptides translocate from one side of the membrane to another through such pores (22,51), the inhibition of their formation by elevated levels of cholesterol would also inhibit the entry of antimicrobial peptides into a cell and help to protect from any intracellular killing mechanism. Hence, the presence of sterols, and in particular cholesterol, may protect against many of the proposed (1) killing strategies of such antimicrobial peptides. Cholesterol and ergosterol are structurally similar but differ in that ergosterol has two additional double bonds (at positions C_7-C_8 and C_{22}-C_{23}) and a methyl group at C_{24} of the side chain (38,39). By comparing the membrane ordering effects of cholesterol and ergosterol with an intermediate form, it was shown that the structure of both the fused rings and the more flexible tail contribute to

determining lipid acyl chain order (38). There may be a number of reasons for these structural differences and why one sterol is chosen over any other in any given organism. In yeast, for example, the evolution of the structure of ergosterol has led to enhanced membrane disorder without the organism being reliant on the synthesis of unsaturated fatty acids (58), but it seems likely that adopting ergosterol over cholesterol can cost an organism in terms of resistance to the action of antimicrobials. The clear differences in the capabilities of cholesterol and ergosterol to order POPC/POPG, and more noticeably POPE/POPG membranes, and the interactions of these membranes with pleurocidin, provide a molecular view of how ergosterol, which is the major sterol of lower eukaryotes such as certain protozoa, yeast, and other fungi (58) is often incapable of offering sufficient protection from linear cationic antimicrobial peptides.

A variety of bacteria have been shown to be capable of developing some level of resistance to antimicrobial peptides (59,60), employing a variety of techniques, including adaptations to membrane lipids and membrane fluidity (61,62). In light of this, the relative compositions of biological membranes and their contributions to protection against the action of antimicrobial peptides, which continue to be developed as therapeutics (63), or toxicity of related vector or cell-penetrating peptides should be an area of increased interest.

This study shows how alterations in lipid composition and membrane order can affect the action of pleurocidin on its target membrane. As discussed above, pleurocidin has been shown to behave similarly to a number of other linear cationic amphipathic antimicrobial peptides in terms of its topology and effect on the membrane and, as such, is likely to be representative of this class of peptide. Future studies will determine how far this molecular view can be extended to other classes of antimicrobial peptides, which may be structurally and functionally distinct from those studied here, and how the interaction of an antimicrobial peptide with a target membrane fits in with the other known properties of antimicrobial peptides, which are increasingly identified as having a key modulatory role in the innate immune response (64,65).

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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