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The effect of gramicidin A on phospholipid bilayers

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Abstract. The helical polypeptide, gramicidin A has been widely studied as a model for the interactions of hydrophobic proteins with lipid bilayer membranes. Many reports are now available of the physical effects of mixing gramicidin A with phospholipid membranes, however, the interpretation of these data remains unclear. The purpose of this communication is to examine the controversial claim that high concentrations of gramicidin A′ cause disorder within the L_α phase of phosphatidylcholine-water dispersions. Solid-state nuclear magnetic resonance (NMR), density gradient and X-ray diffraction techniques are used to confirm the existence of such an effect and mechanisms are discussed which account for the known effects of gramicidin A on lipid bilayers.

Key words: Lipid-protein interactions, gramicidin A′, integral membrane polypeptide

Introduction

When attempting to understand the interactions which occur between the lipids and proteins within biological membranes, a major obstacle is the structural complexity of the membrane proteins. In order to circumvent this difficulty, small hydrophobic polypeptides, such as gramicidin A, provide a more manageable system for studying these interactions, and yield information which is useful when interpreting the results of experiments employing larger protein molecules.

Gramicidin A′ is a naturally occurring mixture of linear gramicidins, rich in, and closely related to gramicidin A (Rice and Oldfield 1979; Veatch et al. 1974). It is now generally accepted that gramicidin A in phospholipid bilayers exists as a Π_{LD} helical dimer which substantially partitions into the hydrophobic region of the bilayer. As this is not the primary interest of the present communication we refer the reader to Wallace et al. (1981) Hawkes et al. (1984), Sychev and Ivanov (1984), Arseniev et al. (1985), and Weinstein et al. (1985), for a discussion of the different conformational models proposed for gramicidin A, and Kim and Clementi (1985), Etchebest et al. (1985), Kim et al. (1985), and Prasad et al. (1986), for a discussion of their ion conducting properties.

In the present communication we examine the claim that high concentration of gramicidin A′ cause disorder within the L_α phase of phosphatidylcholine-water dispersions. We report an X-ray diffraction study of 1: 6 gramicidin A′ – dimyristoylphosphatidylcholine (DMPC) dispersion and show that it is in the L_α phase at 30 °C. We also report a study by density gradient centrifugation which demonstrates a homogeneous dispersal of gramicidin A′ within the phospholipid. Finally we report a study by solid state NMR of aligned stacks of lipid-gramicidin A′ multilayers which demonstrates the inherent nature of the disorder induced by the polypeptide.

The present data shows that the disorder is an intrinsic property of the mixed bilayer rather than a change in the morphology of the lipid aggregate as suggested by Cortijo and Chapman (1981), Pink et al. (1981), Cortijo et al. (1982) and Killian et al. (1985 a and b).

Experimental procedures

Materials

Gramicidin A′ was purchased from Koch-Light Chemicals, Colnbrook, Herts., UK as gramicidin (pure). DMPC was purchased from Calbiochem, Los Angeles, California. Dipalmitoylphosphatidylcholine (DPPC) with both carbonyl carbons substituted by carbon-13 was purchased from Sedary Research Labs Inc., Ontario, Canada. All materials ran as single spots by thin layer chromatography (TLC) on SiO_2. The DPPC and DMPC were run in $ChCl_3$, CH_3OH and

H_2O in the ratio 65:25:4. The gramicidin A′ was run in dioxane-water, 99:1. All TLC spots were developed in iodine vapour. The gramicidin A′ was additionally visualised in tryptophan reagent (N,N-dimethyl amino benzaldehyde) spray. An amino acid analysis was carried out on the gramicidin A′ which showed it to contain approximately 75% gramicidin A and 25% of other linear gramicidins, B (7%), C (16%) and D (3%). Dispersions of gramicidin A′ with the different lipids studied here were prepared with equal weights of water to total solids using powders of the appropriate mixtures of polypeptide and lipid lyophilysed from benzene-methanol solutions (95:5).

X-ray diffraction

Low angle X-ray diffraction patterns were recorded from aqueous dispersions of gramicidin A′-DMPC. These mixtures were dispersed with a range of concentrations of water. Both pure lipids and mixtures with a mole ratio of approximately one polypeptide to six phospholipids were studied. The dispersions were initially homogenized by centrifugation back and forth through a 1–2 mm restriction in a sealed glass tube at temperatures 10°–15 °C above the main transition temperature. These tubes were then opened and their contents transferred to a series of thin walled (0.01 mm) glass capillaries (Karl Hilgenberg, Glaswarenfabrik, D-3509 Malsfeld, Fed. Rep. Germany). The capillaries were sealed in a gas flame and mounted in an X-ray camera in a stream of controlled temperature air. The diffraction patterns were recorded with a pin-hole camera on Fuji standard X-ray film at a sample to film distance of 40 mm. A Hilger Watts X-ray generator provided a 40 μm diameter beam of Cu K_α radiation using an acceleration voltage of 30 kV.

Particular care was taken to ensure that the water content of the lipid powder was known prior to its dispersal in added water. The lipid was stored at reduced pressure using a diffusion pump and a liquid N_2 cold trap for approximately one week. On exposure to atmospheric water vapour it rapidly absorbed an amount of water equivalent to half a water molecule per lipid molecule. This occurred over a period of a few minutes. Over a period of days it absorbed an additional two to three water molecules per phospholipid depending on humidity. Based on these studies it was deduced that after prolonged storage at reduced pressure and a brief exposure to atmospheric moisture, the starting material for hydrated dispersions was the monohydrate. Allowing for this water of hydration the monohydrate molecular weight was taken as 696 for DMPC. The water of hydration was included as a contribution to the measured quantity of added water. By a similar process the gramicidin A′ was found to contain two water molecules per polypeptide. This resulted in a dihydrate molecular weight of 1918.

Calculation of molecular area

The primary information derived from the X-ray diffraction data is the molecular area (S) occupied per polypeptide-lipid unit in the lamellar plane. S was calculated using the expression

$$(S \cdot d_l)/2 = \text{Volume of water per polypeptide-lipid unit} + \text{Volume of lipid per polypeptide-lipid unit} + \text{Volume of polypeptide per polypeptide-lipid unit},$$

where S is the area per polypeptide-lipid unit, and $d_l/2$ is half the experimentally observed long spacing.

The volume of water per lipid-polypeptide unit

$$= \frac{MW_{\text{water}} \cdot \text{Weight of water}}{N_A \cdot \varrho_w \cdot \text{Weight of lipid}} \times \frac{MW_{\text{lipid}} + R \cdot MW_{\text{peptide}}}{MW_{\text{water}}},$$

where N_A is Avogadro's number, ϱ_w is the density of the water, taken here as 1.00 kg/l and R the mole ratio of polypeptide to lipid (a number less than one in the present context).

The volume of lipid per lipid-polypeptide unit

$$= \frac{MW_{\text{lipid}}}{N_A \cdot \varrho_l},$$

where ϱ_l is the density of the lipid, taken here as 1.000 kg/l for DMPC.

The volume of the polypeptide per lipid-polypeptide unit

$$= \frac{R \cdot MW_{\text{lipid}}}{N_A \cdot \varrho_p},$$

where ϱ_p is the density of the polypeptide taken here as 1.6 kg/l (present study).

In order to determine the fully hydrated area it is necessary to perform a series of estimates of molecular area at increasing water contents (Janiak et al. 1979). The limiting area is reached when the addition of further water fails to produce an increase in the long spacing.

Density gradients

Density gradient centrifugation measurements were performed using Urografin-water mixtures (Schering AG, Berlin) in 12 ml centrifuge tubes at 30 °C. The samples were run in a field of 20,000 g av. for 35 min.

The polypeptide-lipid mixtures migrate to the same level whether applied to the gradient as a dry powder or as a prehydrated dispersion. Substituting Metrizamide (Aldrich, Milwaukee, WI) solutions for Urografin did not alter the position to which the dispersions migrated. Picnometric measurement of the gramicidin A′ density in benzene at 30 °C yielded a value of 1.19 kg/l compared to 1.16 kg/l by density gradient measurement.

Nuclear magnetic resonance

Solutions of gramicidin A′-DMPC in the ratio of 1:6 dissolved in $CHCl_3$ were deposited onto a series of glass coverslips. The coverslips were approximately 2 cm × 0.7 cm and approximately 2 mg of gramicidin A′-DMPC was added to each slide. The samples were pumped overnight at 20 °C to eliminate the solvent $CHCl_3$. Next day 2 to 3 ml of water were added to each slide and 30–40 slides stacked to form an aligned multilamellar array of gramicidin A′-DMPC sandwiched between the glass slides. An equal quantity of water was syringed around the edge of the slides once the stack had been formed and the array of slides sealed inside a 10 mm diameter NMR tube using a gas flame and extreme care not to heat the sample. Similar stacks were prepared of DMPC without gramicidin A′. These stacks were studied by proton, carbon and phosphorus NMR. At angles near the "magic angle" proton resonances derived from the water, choline methyl and phospholipid methylene groups were resolved and could be used to determine the hydration level of the aligned patches of the dispersion. The pure lipid dispersions were almost totally aligned by the glass slides. However, in the presence of gramicidin A′ only a part of the sample was aligned. The nonaligned powder pattern derived from the remainder of the sample was clearly visible beneath the peaks due to the aligned material. The range of frequencies swept out by the carbon-13 and phosphorus-31 resonances in the patches of aligned lipid was the same as in the powder pattern spectra indicating that the intrinsic properties of both the aligned and non-aligned material were the same.

Spectra were obtained using a Bruker CPX-300 NMR Spectrometer operating at 300 MHz, for protons, 75.46 MHz for carbon-13 and 121.46 MHz for phosphorus-31. The proton and phosphorus spectra were obtained using a conventional cross polarization sequence (Pines et al. 1973). The instrumental details are given in the figure captions.

Differential scanning calorimetry

In order that a direct comparison could be made between the present dispersions and those of other workers, scanning calorimetry measurements were obtained on dispersions of DMPC mixed with varying amounts of gramicidin A′. A Perkin-Elmer DSC-2 scanning calorimeter was used at a scan rate of 2.5 °C/min over a range of temperatures from − 20° to 70 °C. The dispersions were sealed in Perkin-Elmer aluminium volatile-sample pans. The results obtained were in excellent agreement with those of Chapman et al. (1977) and will not be discussed further.

Results

X-ray diffraction

Figure 1 shows the variation in the measured lamellar phase long spacing obtained from DMPC in water and gramicidin A′-DMPC dispersions at a polypeptide to lipid ratio of approximately 1:6. These data are shown as a function of the concentration of water within the dispersions, expressed as the weight of water per weight of total solids plus water. The molecular areas derived from these data are 0.91 nm^2 for the gramicidin A′-DMPC unit and 0.64 nm^2 for DMPC. Studies were also performed on DMPC dispersions containing higher concentrations of gramicidin A′.

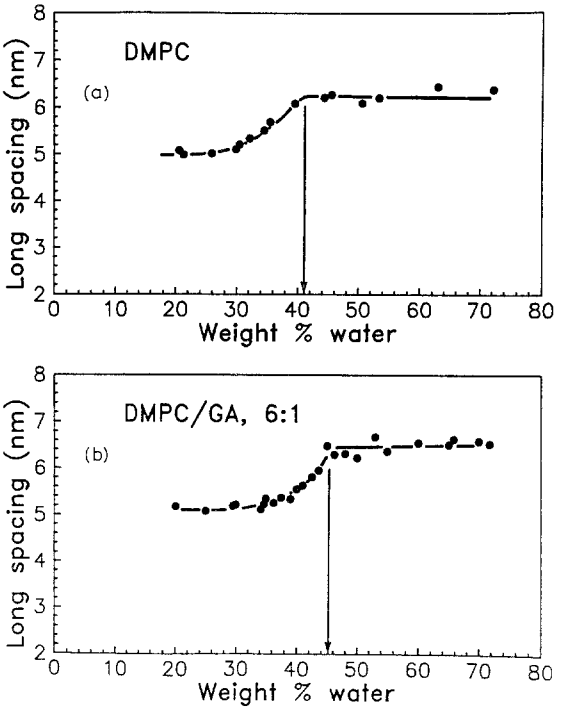

Fig. 1. a X-ray long spacing as a function of the weight percent water concentration for DMPC dispersions at 38 °C. The arrow denotes the concentration used to determine the limiting area. **b** X-ray long spacing as a function of the weight percent water concentration for a DMPC-gramicidin A′ dispersion at 38 °C. The concentration of gramicidin A′ was in the ratio 1 polypeptide per 6.07 DMPC molecules. Again the *arrow* denotes the concentration used to determine the limiting area

Table 1. X-ray diffraction long spacing derived from gramicidin A′-DMPC dispersions which unless specified were in excess water (greater than 50% by weight water) at 36 °C

Gramicidin A′-DMPC	Reflections [nm]					
Pure DMPC (30% by weight water)	5.60	–	2.77	–	1.84	–
1:5.0	5.36	–	2.68	–	–	–
1:3.5	5.24	–	2.65	–	–	–
1:3.0	3.63	2.13	–	–	–	–
1:2.5	3.4	1.93	–	–	–	–
1:2.0	3.4	–	–	1.36	–	–
1:1.5	3.4	–	–	1.35	–	–
1:1.0	3.4	–	–	1.35	–	–
Dry gramicidin A′ powder	–	–	–	1.33	–	1.23
Reflection ratios:	1/1	$(1/3)^{1/2}$	1/2	–	1/3	–

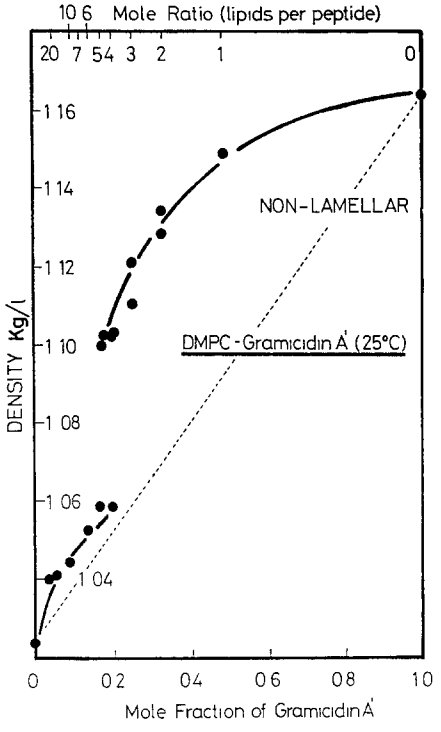

Fig. 2. Densities derived from the density gradient centrifugation bands of gramicidin A′-DMPC dispersions at 25 °C. The dotted line represents the expected density for ideal mixing of pure lipid and gramicidin A′ under the same conditions. The designations, lamellar and non-lamellar are drawn from the discussion within the text and have not been determined from the present density data

The results of this work are summarized in Table 1 and indicate a non-lamellar phase structure at 1:3 and a loss of long range order by 1:1 mole ratio of gramicidin A′ per lipid. At these concentrations the density gradient and X-ray data show that massive phase separation has occurred rendering the ratios an approximation at best. The formation of the non-lamellar phase is associated with a reduction in the first order spacing from approximately 5.2 nm to 3.6 nm, suggesting an exclusion of water. Although the non-lamellar phase yielded only two diffraction orders under our experimental conditions the first and second orders were in the ratio 1: $(3)^{-1/2}$ indicating the possibility of an hexagonal structure.

Gramicidin A′ powder yielded many diffraction rings. In particular intense rings occurred in the region of 1.35 nm and 1.23 nm. These spacings correspond to the (100) and (110) planes of the $P_{2_12_12_1}$ cell reported for gramicidin A by Koeppe et al. (1978). At a gramicidin A′ to DMPC ratio of 1:1 diffraction rings corresponding to crystalline gramicidin A′ were visible.

Density gradients

Examples of the results obtained from the density gradients are shown as plots of density versus gramicidin A′ concentration in Fig. 2. All bands were found to contain both lipid and gramicidin A′ by TLC. The dotted line shown in Fig. 2 represents the density expected for ideal mixing. The density obtained for the pure lipid agreed to within 1% with the published value of Nagle and Wilkinson (1978). Since these authors used flotation in $D_2O - H_2O$ mixtures to estimate the density it appears that the gradient solution penetrates the aqueous channels within the dispersions. Above the transition temperature for a concentration of one polypeptide per six phospholipids, the dispersions ran as a single band with a density which was consistent with that expected from an average of the component densities. This indicates that at the macroscopic level at least, the lipid and gramicidin A′ are homogeneously dispersed. From the NMR data, discussed in the next section, the absence of multiple splittings is evidence for homogeneous dispersal at the microscopic level. However, at mole concentrations of greater than 1:5, the apparent density of the dispersion increased sharply, suggesting a change in phase. Killian et al. (1985b), have reported that the hexagonal II phase induced in dioleoylphosphatidylcholine-water

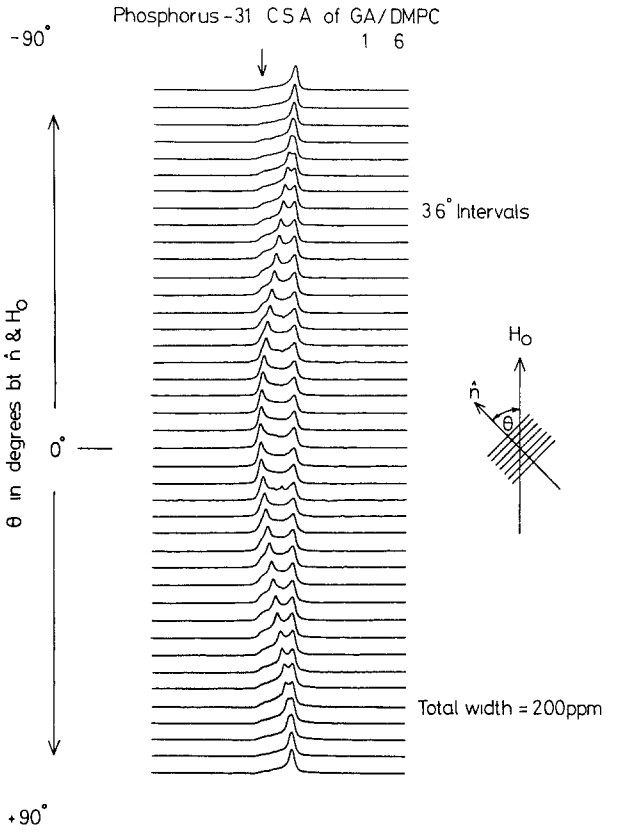

Fig. 3. Phosphorus-31 NMR spectra from a partially aligned sample of gramicidin A′-DMPC in the mole ratio of 1:6. The spectra were recorded at 30 °C using a 4 μs 90° pulse duration, a 4 s recycle delay and a decoupling field strength of 40 kHz The angular increments were 3.6°. The total anisotropy was − 30 ppm

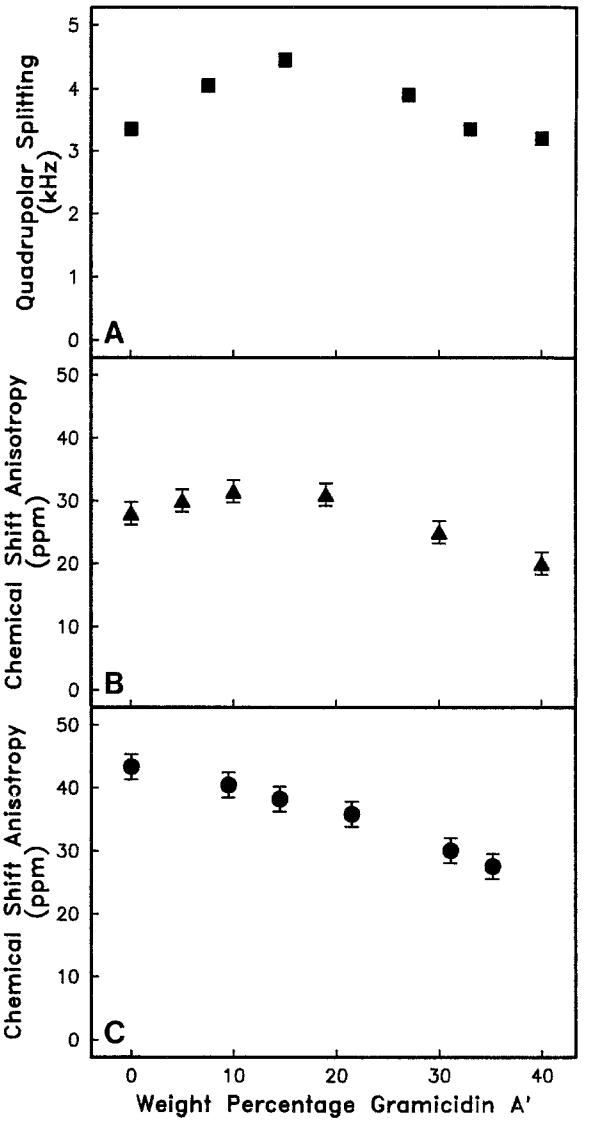

Fig. 4. The variation of phosphorus-31 and carbon-13 CSA of the phosphate headgroup and sn-1 carbonyl, and the variation of the deuterium-2 quadrupolar splitting of the terminal methyl of the hydrocarbon chains of DMPC as a function of the mole ratio of gramicidin A′ per DMPC molecule. The phosphorus-31 CSA were measured as part of the present study, the carbon-13 CSA, were taken from Cornell and Keniry (1983), and the deuterium-2 data from Rice and Oldfield (1979)

dispersions is very rich in gramicidin A, with a mole ratio of polypeptide to lipid of 1:1. In the present study, the density just above the critical concentration of 1:5, corresponds to that expected for a 1:1 mixture of the lipid and gramicidin A′. At higher concentrations the apparent density of the dispersion increases still further, due probably to the inclusion of aggregates of gramicidin A′ in the dispersion. Evidence for such aggregates is seen in the X-ray patterns of the 1: 2 and 1:1 dispersions, in which peaks associated with crystalline gramicidin A′ are clearly present. At lower temperatures near 15 °C, aggregation is present at concentrations down to 1:10 polypeptides per lipid molecule and results in a substantial spread of the band formed on the density gradient.

Nuclear magnetic resonance

The phosphorus-31 NMR spectrum obtained from a non-aligned DMPC dispersion was compared with the spectrum obained from similar dispersions containing various mole ratios of gramicidin A′-DMPC. The effect of the gramicidin A′ is to broaden the phosphorus-31 resonance and to reduce the chemical shift anisotropy (CSA). The variation of CSA as a function of gramicidin A′ concentration is shown in Fig. 4 c. In order to ensure that this reduction in CSA is not a result of morphological changes in the dispersion, produced by the addition of gramicidin A′, measurements were made on a dispersion which had been partially aligned between glass cover slips. Figure 3 shows the superposition of the powder spectrum derived from the non-aligned material, and the orientation dependent signal derived from the aligned material. The range of frequencies covered by the orientation depen-

dent and powder signal apear to be the same at approximately −30 ppm. This demonstrates that the disorder induced by the gramicidin A′ is a result of its direct interaction with the phospholipid and not a consequence of an alteration in the average curvature of the liposomes.

Discussion

The interaction of gramicidin A with phospholipids

An unexpected result first observed by Rice and Oldfield (1979) is that gramicidin A′ appears to either order or disorder the surrounding lipid, depending on its concentration in a lipid bilayer. According to this interpretation, the addition of gramicidin A′ initially produces an increase in order of the hydrophobic region of the phospholipid molecule. At higher concentrations, above one polypeptide per fifteen lipid molecules, this effect is reversed and the entire dispersion becomes progressively disordered. The phosphorus-31 CSA shown in Fig. 4 c are consistent with the polar region of the bilayer simply disordering as the gramicidin A′ concentration is increased. Published accounts of the reduction in the phosphorus-31 CSA when substantial amounts of gramicidin A′ are dispersed with the lipid have been reported by Rajan et al. (1981), and Killian et al. (1985 a and b). The more complex behaviour of the carbonyl group, carbon-13 CSA has been reported by Cornell and Keniry (1983). The concentration dependence of the carbonyl CSA is remarkably similar to the dependence of the deuterium quadrupolar splittings reported by Rice and Oldfield (1979). A similar pattern of order followed by disorder has been observed using infrared spectroscopy by Lee et al. (1984). Tanaka and Freed (1985) have published a series of Electron Spin Resonance (ESR) studies of aligned lipid-gramicidin A′ dispersions which show that at low concentrations, the gramicidin A′ induces disorder in the hydrocarbon chains of the L_α phase lipids. However, owing to the water content of these samples being only 3 mole H_2O per mole of phospholipid it is difficult to compare their dynamics with those of the present study.

Since 1979 a number of suggestions have been made to account for the effect of gramicidin A on phospholipid dispersions. The three main models are paraphrased as follows:

The rough surface of gramicidin A disorders the boundary lipids which then present a smooth rigid surface to the remaining lipids which they order (Rice and Oldfield 1979).

Due to the rapid molecular interchange between the boundary and remaining lipids, only a single, averaged NMR spectrum is obtained. Although similar two site models are now widely used to explain the effects of lipid-protein interactions on an NMR spectrum of lipid molecules, Pink et al. (1981), question the concept that a disordered boundary layer of lipid should increase the order of the remainder of the lipid dispersion.

Gramicidin A disturbs the morphology of the lipid dispersions producing highly curved surfaces, which in the presence of rapid translational diffusion average the NMR splittings and only appears to produce disorder (Cortijo and Chapman 1981; Pink et al. 1981; Cortijo et al. 1982; Killian et al. 1985 a and b).

A direct contradiction of this interpretation may be seen in the reduced phosphorus-31 CSA that we have obtained from the aligned gramicidin A′–phospholipid dispersions in which tumbling is not possible. Even without this direct evidence, the model was unlikely since the radius of curvature necessary to produce a reduction in the CSA is of the order of a few tens of nanometers. Further demonstration that curvature is not the origin of the apparent disordering effects of gramicidin A′ is seen by comparing the results of Rice and Oldfield (1979) with those of Cornell and Keniry (1983) (Fig. 4 a and b). These two studies show that a similar profile of order parameters is obtained by both deuterium NMR and carbon-13 NMR as the concentration of gramicidin A′ in DMPC is increased. This is despite an order of magnitude difference in the CSA of the carbon-13 carbonyl group and the deuterium quadrupolar splittings from the labelled methylenes at the 2, 3, 4, 6 or 8 methylene positions down the hydrocarbon chain.

The results of Lee et al. (1984) further argue against the curvature model. Using infra-red spectroscopy these authors show that on increasing the gramicidin A′ concentration in the fluid phase of DMPC multilayers, the ratio of the *trans* to *gauche* isomers down the hydrocarbon chain peaks at intermediate polypeptide to lipid ratios. At higher concentrations of gramicidin A′ the ratio decreases and at 1:5 is below that of pure lipid. As the timescale defined by an infra-red spectrum is shorter than any possible diffusive process, the changes in the *trans-gauche* ratio are consistent with the addition of high concentrations of gramicidin A′ causing disorder in the hydrocarbon chains of the phospholipid.

If lipid is adjacent to one gramicidin A molecule it is ordered. If lipid is sandwiched by two or more gramicidin A molecules it is disordered (Pink et al. 1981).

As a alternative to the Rice and Oldfield model, Pink et al. (1981) have suggested that the presence of gramicidin A′ creates a range of specific environments, each of which possesses a characteristic NMR splitting. Again as the lipid is undergoing rapid exchange between all sites in the system, only a single averaged spectrum is observed. The origin of the various sites

was derived from a suggestion by Chapman et al. (1977). These authors proposed that the lipids sandwiched between two or more gramicidin A′ molecules are the source of the restricted component seen in the ESR spectrum of spin labelled lipids in the presence of high concentrations of gramicidin A′. However, the concentration range over which this effect is seen only occurs above a mole ratio of 1:5 polypeptides per phospholipid. Since this concentration is very close to the phase boundary between the lamellar and non-lamellar phases seen in the present study, we suggest that the broad ESR spectral component in fact arises from a disruption of the L_α phase structure. This interpretation begs the question of the origin of similar effects seen for membrane bound proteins dispersed in lipid bilayers. Restricting ourselves to the concentration range from 1:5 to pure lipid, gramicidin A′ has virtually no effect on the ESR spectra obtained from spin labelled phospholipids.

In a following article we address the physical mechanisms underlying the order in these gramicidin A-DMPC dispersions and propose a model which accounts for the experimental data presented here.

Conclusion

In this report we have shown:

(*a*) That gramicidin A′-DMPC dispersions remain lamellar at 30 °C up to a polypeptide to lipid ratio of $1:4 \pm 0.5$.
(*b*) That at higher concentrations of poplypeptide a non-lamellar phase is formed. Although possibly an hexagonal II structure, this new phase is dissimilar to the hexagonal II structure formed with the longer chainlength lipids.
(*c*) That at a concentration of 1:6 the gramicidin A′ induces an intrinsic disorder in the lipid bilayer. Morphological changes in the structure of the dispersion are not the origin of this effect.

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