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Importance of the tryptophans of gramicidin for its lipid structure modulating activity in lysophosphatidylcholine and phosphatidylethanolamine model membranes
A comparative study employing gramicidin analogs and a synthetic α-helical hydrophobic polypeptide

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The importance of the tryptophan residues of gramicidin for the lipid structure modulating activity of this pentadecapeptide was investigated by studying the interaction of gramicidin analogs A, B, C (which have a tryptophan, phenylalanine and tyrosine in position 11, respectively) and tryptophan-*N*-formylated gramicidin (in which the four tryptophan residues have been formylated) with several phospholipid systems. In addition an α-helical model pentadecapeptide (P15) was studied to further test the specificity of the gramicidin–lipid interaction. DSC experiments showed that all the gramicidin analogs produced a significant decrease in the gel to liquid-crystalline transition enthalpy of dipalmitoylphosphatidylcholine. The P15 peptide was much less effective in this respect. In dielaidoylphosphatidylethanolamine the gel → liquid-crystalline transition enthalpy was much less affected by the incorporation of these molecules. In this lipid system tryptophan-*N*-formylated gramicidin was found to be the most ineffective. ^{31}P-NMR and small angle X-ray diffraction experiments showed that the ability of the peptides to induce bilayer structures in palmitoyllysophosphatidylcholine and H_{II} phase promotion in dielaidoylphosphatidylethanolamine systems follows the order: gramicidin A′ (natural mixture) ≈ gramicidin A > gramicidin B ≈ gramicidin C > tryptophan-*N*-formylated gramicidin > P15. These results support the hypothesis that the shape of gramicidin and its aggregational behaviour, in which the tryptophan residues play an essential role, are major determinants in the unique lipid structure modulating activity of gramicidin.

Introduction

Gramicidins are linear hydrophobic pentadecapeptides, prpoduced by *Bacillus brevis* strain ATCC 8185 [1]. They contain an alternating poly-L- and -D-amino acid sequence which permit the polypeptide to adopt an organization in β-type of helices. The C- and N-termini are blocked by, respectively, an ethanolamine and a formyl group. The structure of gramicidin A, which amounts to 80% in the natural mixture (gramicidin A′) is

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Abbreviations: lyso PC, lysophosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DEPE, dielaidoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; Pipes, 1,4-piperazinediethanesulfonic acid; DSC, differential scanning calorimetry; T_m, temperature at the maximum of the thermotropic peak; ^{31}P-NMR, ^{31}P-nuclear magnetic resonance.

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HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$

In the less abundant gramicidin species the Trp-11 is replaced by a phenylalanine (gramicidin B, 5%) or a tyrosine residue (gramicidin C, 15%) [2].

Gramicidin has been widely used as a model for the hydrophobic part of intrinsic membrane proteins [3,4]. It is capable of forming aqueous channels which are believed to be membrane-spanning helical dimers [5,6]. Furthermore the peptide can strongly modulate lipid structure (for review see Ref. 7).

Gramicidin A′ has a pronounced ability to promote H_{II} phase formation in phosphatidylethanolamine systems [8] which is manifested by a lowering of the bilayer $\rightarrow H_{II}$ transition temperature of the lipid. In dielaidoylphosphatidylethanolamine systems no gramicidin–lipid interactions seem to occur in the gel state, suggesting extensive segregation between the peptide and the lipid and the formation of gramicidin aggregates which are supposed to be responsible for inducing the H_{II} phase [9]. The polypeptide can also induce the formation of the H_{II} phase in model membranes of typical bilayer preferring lipids such as phosphatidylcholines when the chain length exceeds 16 carbon atoms [10], and the negatively charged dioleoylphosphatidylserine and dioleoylphosphatidylglycerol [18]. It has been claimed that a specific hydrated conformation of gramicidin in combination with peptide aggregation is essential for gramicidin-induced H_{II} phase formation in phosphatidylcholine systems [11].

Lysophosphatidylcholine, which prefers a micellar organization upon aqueous dispersion, forms lamellar structures with gramicidin A′ [12] when a mixed peptide-lysophosphatidylcholine film is hydrated. Analysis of these data in terms of the shape-structure concept of polymorphism together with a theoretical conformational analysis [13], suggested a localization of the C-terminal part of the molecule in the interior of the lipid bilayer [14]. Furthermore, the lack of any significant perturbation of gramicidin on the acyl chain order of these systems [14] has been taken as indication of the presence of gramicidin aggregates. Heat incorporation of gramicidin into lysophosphatidylcholine also results in formation of bilayers [15] in which the gramicidin molecules are aggregates [16] but now they are present as N-terminal linked dimers [17] thus with the C-terminus at the bilayer/water interface.

Recently it has been shown that the presence of ionizable groups at the C- and N-terminal part of gramicidin A′ do not interfere with the specific interactions of the peptide with lipids [18], but that the tryptophan residues of the gramicidin molecule play an important role in the interaction with dioleoylphosphatidylcholine. Formylation of them results in a complete loss of H_{II} phase formation [19]. Even a single replacement of tryptophan-9 or -11 by phenylalanine results in a large reduction in extent of H_{II} phase formation for this lipid [20].

In order to further understand the molecular mechanism of the lipid structure modulating activity of gramicidin, we will first study in this paper the role that the tryptophan residues of gramicidin play in bilayer induction in lysophosphatidylcholine and H_{II} phase promotion in dielaidoylphosphatidylethanolamine systems by means of investigating the interaction of purified gramicidin species A, B, C and tryptophan-*N*-formylated gramicidin with lysophosphatidylcholine and dielaidoylphosphatidylethanolamine model membranes, using differential scanning calorimetry, ^{31}P-nuclear magnetic resonance and small X-ray diffraction techniques.

Since organization in β-helices with an external hydrophobic surface is confined to polypeptides with alternating D- and L-amino acids, and since in biological membranes the hydrophobic membrane spanning part of integral proteins appears to be predominantly organized in α-helices [21,22], we thought it of interest to comparatively study the effect of a synthetic hydrophobic pentadecapeptide consisting of L-amino acids on the structural organization of different phospholipid systems. For this purpose we used a hydrophobic model of the antibiotic alamethicin [23], the fifteen amino acid polypeptide Boc-(L-Ala-Aib-L-Ala-Aib-L-Ala)$_3$-OMe (where Boc = *t*-butyloxycarbonyl, Ala = alanine, Aib = aminoisobutyric acid and OMe = methoxy), called P15, which was shown to be largely present as α-types helices in lipophilic solutions [24] due to the high amino-

isobutyric acid content of the peptide [25]. This polypeptide and several analogues with different number of repeat units have been found to form voltage-dependent ion-conducting channels in black lipid membranes [26].

Materials and Methods

Gramicidin A′ was obtained from Sigma (St. Louis, MO, U.S.A.). Tryptophan-*N*-formylated gramicidin, in which the indole protons of all the tryptophan residues are replaced by a formyl group, was prepared by incubation of gramicidin A′ in a HCl saturated formic acid solution as described in Ref. 19. Gramicidin species A, B and C were purified from the natural mixture by preparative reversed phase high performance liquid chromatography (HPLC) following the procedure described before [20]. The purity of these gramicidin analogs was checked by thin-layer chromatography (HPTLC) and their identity confirmed by ^{1}H-NMR as described in Ref. 20. The amount of gramicidin analogs was determined either directly by weighing, or indirectly by determination of the absorbance at 280 nm in methanolic solution. The molar absorption coefficient for the peptides was found to be: 20900 $cm^{-1} \cdot M^{-1}$ for gramicidin A (mol.wt. 1885), 15860 $cm^{-1} \cdot M^{-1}$ for gramicidin B (mol.wt. 1845), 19290 $cm^{-1} \cdot M^{-1}$ for gramicidin C (mol.wt. 1861) and 11820 $cm^{-1} \cdot M^{-1}$ for tryptophan-*N*-formylated gramicidin (mol.wt. 1986); these values are in close agreement with those previously reported [20].

The fifteen amino acid synthetic polypeptide (P15) was obtained from Dr. Boheim (Ruhr-Universität, F.R.G.) and it was synthesized by Dr. Jung (Institute of Biophysical Chemistry, University of Tübingen, F.R.G.). The calculated molecular weight agreed within 10% with the molecular weight determined by amino acid analysis using glycine as an internal standard.

1-Palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine, lysoPC), 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (dielaidoylphosphatidylethanolamine, DEPE), 1,2-dioleyl-*sn*-glycero-3-phosphocholine (dioleylphosphatidylcholine, DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine, DPPC) were synthesized according to the procedures described in Refs. 27 and 28. The lipids were purified by HPLC as described in Ref. 28 and judged chromatographically pure by the use of HPTLC.

All other chemicals were of analytical grade.

Differential scanning calorimetry (DSC)

Samples of 5 μmol of DEPE or DPPC and the appropriate amount of gramicidin analogs or P15 in chloroform/methanol (1 : 1, v/v) were dried under a stream of N_2 and stored overnight under vacuum to remove the last traces of solvent. The samples were then dispersed in 1 ml of 100 mM NaCl, 25 mM Pipes, 0.1 mM EDTA buffer (pH 7.4 at 45 °C). Subsequently the samples were spun for 30 min at 4 °C at 17000 rpm. The pellet was then carefully transferred to stainless steel capsules, and measured in a perkin-Elmer DSC-4 calorimeter equipped with a Perkin-Elmer Thermal Analysis Data Station, using an empty capsule as reference. The instrument was calibrated using indium as standard. The samples were scanned with a heating rate of 5 Cdeg per min. The range of temperatures studied was from 20 °C to 75 °C (DEPE samples) and from 15 °C to 55 °C (DPPC samples). Successive scans yielded identical thermograms, the second scan was usually used for transition enthalpy calculations. After the measurements, the capsules were opened and the samples were dissolved in chloroform/methanol (1 : 1, v/v). After subsequent perchloric acid hydrolysis, the amount of phospholipid originally present in the sample was determined by the method of Fiske and SubbaRow [30].

^{31}P-Nuclear magnetic resonance (^{31}P-NMR)

10 μmol of lysoPC or 40–50 μmol of DEPE or DOPC and the appropriate amount of gramicidin analog or P15 were mixed in a final volume of 300 μl of chloroform/methanol (1 : 1, v/v) (or chloroform in the case of DEPE samples) in a small tube (5 cm length, 8 mm outer diameter) and evaporated under vacuum to dryness in a rotavapor, followed by overnight storage under vacuum to remove the last traces of solvent. 50 μl of destilled water in the case of lysoPC samples or an amount of buffer (100 mM NaCl, 25 mM Pipes, 0.1 mM EDTA (pH 7.4)) corresponding to the weight of the dry sam-

ple in the case of DEPE and DOPC samples, was added to the obtained lipid film, The samples were kept for 60 min at 37° C for lysoPC and DOPC or 45° C for DEPE samples to hydrate the phospholipid. The samples were centrifuged at 25° C during 60 min at 3000 rpm to settle the hydrated lipid at the bottom of the tube. The tube was then put inside a conventional 10 mm NMR tube, and proton noise decoupled 121.5 MHz ^{31}P-NMR spectra were recorded on a Bruker MSL-300 spectrometer, as described before [31]. A spectral width of 40 KHz, a 14 μs 90° pulse, a memory of 4K data points and an interpulse delay of 1 s were used. 2000 free induction decays were accumulated in case of lysoPC samples and 4000 in case of DEPE and DOPC. Prior to Fourier transformation an exponential multiplication was applied resulting in a 100 Hz line broadening. The relative amount of bilayer signal in the ^{31}P-NMR spectra of lysoPC systems and the percentage of bilayer and H_{II} phase component in the ^{31}P-NMR spectra of DEPE systems were calculated as described previously [12,20]. 0 ppm corresponds to the chemical shift of the ^{31}P-NMR resonance position of pure lysoPC micelles.

Small angle X-ray diffraction

X-ray diffraction experiments were performed on samples used for NMR on a Kratky camera with a 10 × 0.2 mm Cu-K_α beam (40 kV, 20 mA) equipped with a position-sensitive detector (Leti) interfaced to a microcomputer as described before [9]. X-ray diffraction profiles at different temperatures were obtained from 5–10 min exposure times after 15 min of temperature equilibration.

Incorporation of P15 into phospholipid systems

The incorporation of P15 into the phospholipid structures was studied by preparing different types of P15/phospholipid mixtures as described above and calculating by means of amino acid analysis (carried out on a LKB 4151 Alpha plus, Amino acid analyser) the fraction of the total amount of polypeptide which was associated with the phospholipid.

It was found that P15 in a dried film in the absence of lipid, remained almost quantitatively associated with the glass wall of the tube upon hydration, in agreement with the low aqueous solubility expected for this polypeptide. In contrast, when P15 was present in a mixed film which DPPC or lysoPC all of the peptide and lipid came into solution upon hydration. In case of DPPC upon subsequent centrifugation at 4° C for 60 min at 17000 rpm most of the peptide (75%) and lipid (96%) pelleted. For the micellar lysoPC virtually all peptide (93%) and lipid (97%) remained in the supernatant. These experiments thus demonstrate an association of P15 with the different model membrane systems.

Results

Interaction of gramicidin analogs and lysoPC systems

The comparative structural effect of incorporation of different amounts of tryptophan-*N*-formylated gramicidin and gramicidins A, B and C on lysoPC systems was studied by means of ^{31}P-NMR.

Pure lysoPC forms micelles in aqueous solution, which due to fast tumbling and lateral diffusion of the lipids, give rise to a narrow isotropic ^{31}P-NMR signal [12] (see also Fig. 6C). When the phospholipid is mixed with all the gramicidin analogs a second spectral component becomes visible which is characterized by a high-field peak and a low-field shoulder with a residual chemical shift anisotropy ($\Delta\sigma$) of about 19 ppm as was reported before for gramicidin A' [12] (see also Fig. 6B).

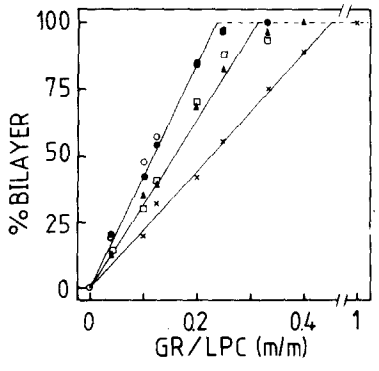

Fig. 1. Relative amount of bilayer type of ^{31}P-NMR signal in aqueous solutions of gramicidin/lysoPC at various molar ratios. Gramicidin A' (○), gramicidin A (●), gramicidin B (▲), gramicidin C (□), tryptophan-*N*-formylated gramicidin (×). The data were obtained from spectra recorded at 25° C.

Such asymmetrical spectra are typical for phospholipids organized in extended lamellar structures in which the chemical shift anisotropy is only partially averaged by a rapid axial rotation of the phosphate moiety. The small value of $\Delta\sigma$ found in these systems is in good agreement with those previously reported [12,13] for lysoPC in a variety of different bilayers and indicate an increased motional freedom of the lipid head group due to increased rotational possibilities around the glycerol C_2-C_3 bond [32,33].

From the spectra of the various samples the relative amount of 'bilayer signal' was obtained (Fig. 1). It can be seen that although all the gramicidin analogs used were able to promote bilayer formation in lysoPC systems, clear differences can be observed between them. The molecular efficiency for bilayer formation follows the order gramicidin A′ ≈ gramicidin A > gramicidin B ≈ gramicidin C > tryptophan-*N*-formylated gramicidin.

In all cases there is a linear increase of the bilayer component upon increasing the gramicidin analog content suggesting the formation of a stoichiometric complex. Using a linear regression method it could be calculated that in case of gramicidin A′ and gramicidin A on average each peptide molecule causes 4.1 ± 0.09 lysoPC molecules to adopt a structure giving rise to a bilayer type of ^{31}P-NMR signal (in close agreement with the value of 3.9 ± 0.1 reported before for gramicidin A′ by Killian et al. [12]). This value was smaller for gramicidin B and C for which a stoichiometry was found for bilayer formation of 3.1 ± 0.08 lysoPC molecules per molecule of gramicidin B or C. Tryptophan-*N*-formylated gramicidin is the least effective in this bilayer promoting ability, causing only 2.2 ± 0.04 lysoPC molecules per peptide molecule to adopt a bilayer structure.

Interaction between gramicidin analogs and DPPC

In order to get insight in possible differences in mode of interaction of the various gramicidin analogs with the acyl chains of phosphatidylcholines, DSC experiments were carried out using DPPC as a test lipid. In this PC species gramicidin cannot induce the H_{II} phase because of its limited chain length.

Fig. 2A shows the enthalpy of the main gel to liquid crystalline transition of DPPC as a function of the gramicidin (analogs) content. The enthalpy for the transition of pure DPPC systems was estimated to be 8 ± 0.5 kcal/mol and the T_m 41.5 ± 0.3°C in agreement with literature [34]. All the gramicidin analogs behave in a similar way, the incorporation of low amounts of gramicidin produces an initial more of less proportional decrease of the transition enthalpy. When the concentration of gramicidin reaches a molar ratio of peptide to lipid of 15 : 1, incorporation of higher amounts of gramicidin only results in a slightly further decrease of the transition enthalpy producing a deflection in the curve. Such behaviour was previously observed for gramicidin A′ [3,9] whereby the observed deflection in the enthalpy curve was interpreted in terms of the onset of a peptide aggregation proces. Again similar to the natural mixture of gramicidins, all analogs broaden the range of the thermotropic transition but do not affect the positions of the peak maximum (T_m being 41.5 ± 0.4°C) and abolish the pretransition in such a way that it cannot be detected anymore at a molar ratio (DPPC/gramicidin) below to 100–50 : 1 (data not shown).

Interaction between gramicidin analogs and DEPE

We next studied the effect of these gramicidin analogs on the thermotropic phase transitions of DEPE. Fig. 2B shows the enthalpy of the gel to liquid-crystalline and that of the bilayer to H_{II} phase transition of DEPE as a function of the gramicidin (analogs) content. For pure DEPE the enthalpies for both transitions were estimated to be 7.3 ± 0.5 kcal/mol and 0.5 ± 0.07 kcal/mol and the T_m values 39.7 ± 0.4°C and 63.9 ± 1°C, respectively, in agreement with previous data [35]. Incorporation of gramicidin species A, B and C shows only a minor effect on the enthalpy of the gel to liquid-crystalline transition, even the presence of the largest amount of polypeptide (DEPE/gramicidin molar ratio of 5 : 1) only produces a small decrease of the transition enthalpy. The chain melting transition profile is not visibly affected (the T_m being 40 ± 0.4°C), except that, similar to what was previously reported for gramicidin A′ [9], in the samples with the highest amounts of gramicidin A, B or C a slightly differ-

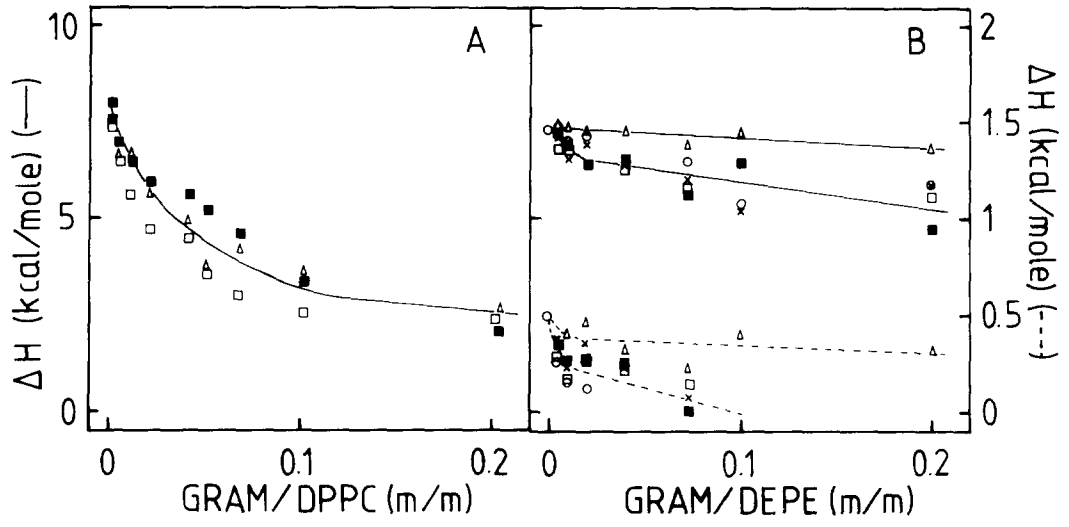

Fig. 2. Effect of incorporation of gramicidin analogs on the enthalpy of the gel to liquid-crystalline transition of DPPC (A), and on the enthalpy of the gel to liquid-crystalline (————) and bilayer to H_{II} phase transition (- - - - - -) of DEPE (B). Gramicidin A' (○), gramicidin A (□), gramicidin B (■), gramicidin C (×), tryptophan-*N*-formylated gramicidin (△).

ent line shape was observed, in that the onset of the melting process, defined as the temperature at which the heating curve begins to deviate from the baseline, is shifted to lower temperatures (data not shown).

The effect of gramicidin A, B and C on the bilayer to H_{II} phase transition is more pronounced. Incorporation of increasing amounts of the polypeptide results in a decrease of the transition enthalpy in such a way that at a molar ratio (DEPE/gramicidin) of 10:1 this transition cannot be observed anymore. The same behaviour was previously reported for gramicidin A' in [9], and it was interpreted that upon gramicidin incorporation part of the DEPE molecules, most likely those interacting with the peptide, give rise to a broad bilayer to H_{II} phase transition which is shifted to lower temperatures and which due to its width and low energy content cannot be detected in the thermograms; the remainder of the DEPE molecules still shows an unperturbed transition with a transition temperature not significantly changed with respect to the pure phospholipid ($T_m = 64.3 \pm 1°C$). Upon increasing the gramicidin content the fraction of these molecules decrease and so decreases the enthalpy of the detectable transition, such that eventually only a very broad bilayer to H_{II} phase transition is present which already starts even below the gel to liquid-crystalline phase transition.

Whereas in DPPC systems the thermotropic behaviour of all gramicidin analogs is rather similar, in DEPE tryptophan-*N*-formylated gramicidin has clearly a different effect than gramicidin A, B and C. Even at a molar ratio of 5:1 (DEPE/tryptophan-*N*-formylated gramicidin) the presence of this compound has no effect on the profile (data not shown) or on the enthalpy of the gel to liquid-crystalline transition. Also the bilayer to hexagonal H_{II} phase transition is not drastically altered. At the highest amount (5:1 molar ratio) tested the transition temperature is not significantly shifted and the enthalpy of the process is 0.32 kcal/mol.

The effect of tryptophan-*N*-formylated gramicidin and gramicidin A, B and C on the bilayer to H_{II} phase transition of DEPE was further investigated by means of ^{31}P-NMR.

DEPE when organized in bilayer structures gives rise to an asymmetrical ^{31}P-NMR line shape with a high-field peak and a low-field shoulder and a $\Delta\sigma$ of 40 ppm [36]. When the phospholipid is organized in the H_{II} phase, due to additional rapid lateral diffusion around the tubes of which this phase is composed, a spectrum results with a reversed asymmetry i.e. a low-field peak and high-field shoulder, with a 2-fold reduction in absolute value of $\Delta\sigma$. When gramicidin A' is incorporated in DEPE systems [9] the characteristic spectrum corresponding to the hexagonal struc-

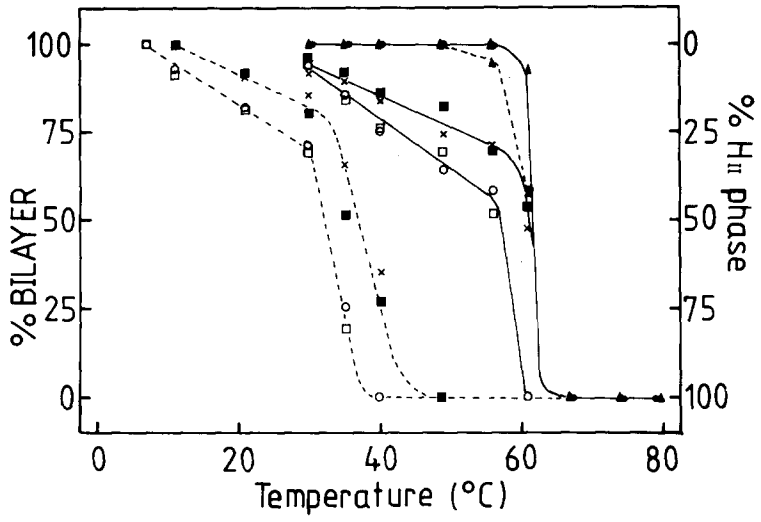

Fig. 3. Percentages of bilayer and H_{II} phase component in the ^{31}P-NMR spectra of pure DEPE (●) and DEPE/gramicidin analogs mixtures. Gramicidin A′ (○), gramicidin A (□), gramicidin B (■), gramicidin C (×), tryptophan-*N*-formylated gramicidin (▲). At a DEPE/peptide molar ratio of 50 : 1 (————) and 10 : 1 (- - - - -).

ture appears at temperatures lower than that of the pure phospholipid.

Fig. 3 shows the percentages of bilayer and H_{II} phase component in the ^{31}P-NMR spectra of pure DEPE and those corresponding to DEPE/gramicidin (analogs) mixtures in two different molar ratios as a function of temperature. Pure DEPE shows a sharp transition from a bilayer organization to an hexagonal H_{II} phase at about 63 °C. The presence of gramicidin A at a 50 : 1 DEPE/gramicidin molar ratio, has the same effect as that of gramicidin A′. In these cases the transition begins at lower temperatures and is extended over a large range of temperature, fifty percent of the transition still taking place between 55 °C and 60 °C. This supports the suggestion made above and in Ref. 9. At a higher concentration (molar ratio of DEPE/gramicidin of 10 : 1) H_{II} phase formation already starts at about 15 °C for gramicidin A′ and A. Gramicidin B and C also promote H_{II} phase formation but are slightly but significantly less effective at both concentrations. At a 10 : 1 molar ratio of peptide to lipid complete H_{II} formation was obtained at about 38 °C for gramicidin A and at 45 °C for gramicidins B and C. Tryptophan-*N*-formylated gramicidin does not promote H_{II} phase formation at a molar ratio of 50 : 1 (peptide/lipid) and has only a minor effect at the higher ratio of 10 : 1.

To further characterize the interaction between gramicidin analogs and DEPE, small angle X-ray diffraction was used. Fig. 4 shows the diffraction pattern profiles at 40 °C corresponding to pure DEPE and those of DEPE/gramicidin analogs systems at a 10 : 1 molar ratio. Pure DEPE (Fig. 4A) shows only one reflection at about 55 Å (Table I), which is the first-order reflection of the multilamellar DEPE in the liquid-crystalline state. That in DEPE in this phase no higher reflections are found, was previously reported [9,37]. The ability of gramicidin A′, A, B and C to promote H_{II} phase formation in DEPE systems is clearly seen in Fig. 4B–E by the presence of two reflections with repeat distances which relate as 1 to $1/\sqrt{3}$, charactersitic of phospholipid organized in hexagonal structures (Table I). In the case of gramicidin C (Fig. 4E) a minor peak was found

TABLE I

X-RAY DIFFRACTION DATA OF PURE DEPE AND DEPE/GRAMICIDIN (ANALOGS) SYSTEMS AT A PHOSPHOLIPID/PEPTIDE MOLAR RATIO OF 10 : 1, AT 40 °C

Sample	Reflections (Å)		
Pure DEPE		54.6	
DEPE/gramicidin A′	62.5		36.4
DEPE/gramicidin A	61.2		35.4
DEPE/gramicidin B	64.2		36.4
DEPE/gramicidin C	64.2	54.6	37.3
DEPE/tryptophan-*N*-formylated gramicidin		55.1	

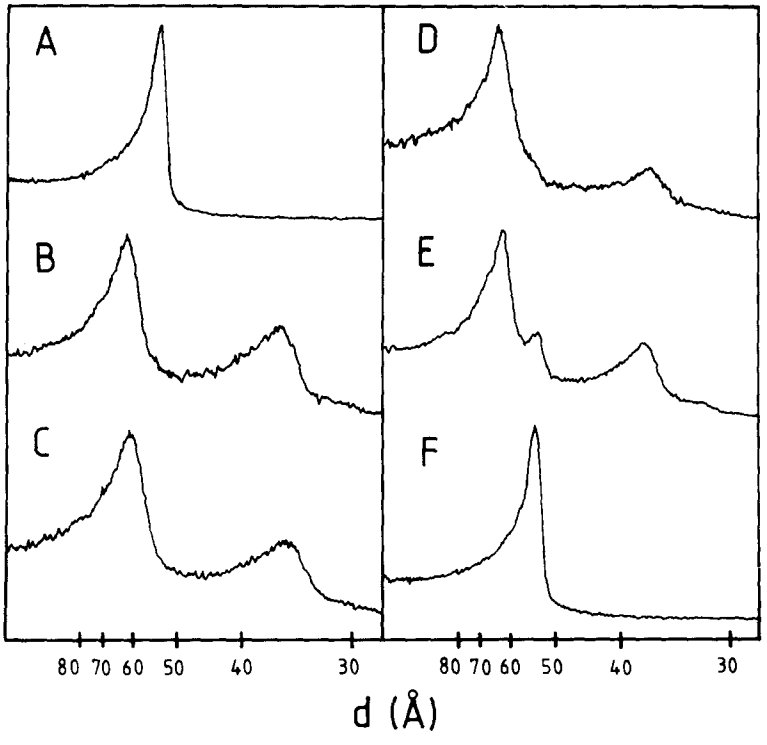

Fig. 4. Small angle X-ray diffraction patterns at 40 °C of DEPE (A) and of DEPE/gramicidin analogs at a 10:1 molar ratio. Gramicidin A′ (B), gramicidin A (C), gramicidin B (D), gramicidin C (E), tryptophan-*N*-formylated gramicidin (F).

between the two H_{II} phase reflections with a *d*-value similar to the first-order reflection found for the pure multilamellar liquid crystalline DEPE (for gramicidin B in this region also a small shoulder can be detected). This reflection most likely arises from a small amount of DEPE molecules that at this temperature remain organized in the liquid-crystalline lamellar state. Furthermore it can be seen in Fig. 4F that the diffraction profile of the DEPE/tryptophan-*N*-formylated gramicidin sample is quite similar to the one shown for pure DEPE (Fig. 4A), supporting the interpretation of the DSC and ^{31}P-NMR results that the tryptophan-*N*-formylated gramicidin does not promote H_{II} phase formation in this phospholipid system.

Interaction between P15 and phospholipid systems

Using differential scanning calorimetry we studied the effect of P15 on the thermotropic transitions of phosphatidylcholine and phosphatidylethanolamine systems. Fig. 5A shows the enthalpies for the pretransition and the main gel to liquid-crystalline transition of different DPPC/P15 mixtures. The presence of increasing amounts of P15 gradually decreases the enthalpy of the pretransition as well as the temperature at which it takes place. The pretransition vanished at a 15:1 DPPC/P15 molar ratio. The polypeptide has much less effect on the main transition than gramicidin A. Increasing concentrations of P15 do not change the main transition temperature and only slightly decreases its enthalpy, till 6.4 kcal/mol for the sample with the highest amount of P15 (molar ratio 5:1).

Fig. 5B shows the enthalpy for the gel to liquid crystalline and for the bilayer to H_{II} transitions of different DEPE/P15 mixtures. It can be seen that the presence of P15 slightly lowers the ΔH of the gel to liquid-crystalline transition to 6.5 kcal/mol. The bilayer to H_{II} phase transition is more affected but in contrast to gramicidin A, it does not disappear at high peptide concentrations. In these samples the decrease in the bilayer to H_{II} phase transition enthalpy is accompanied by a decrease in the T_m of this transition from approx. 64 °C for pure DEPE to 61.5 °C for the 5:1 molar ratio sample (data not shown).

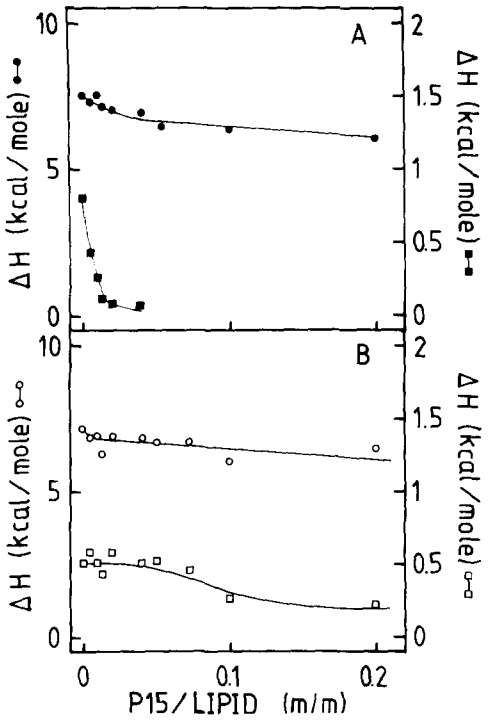

Fig. 5. Effect of P15 incorporation on the enthalpy of the gel to liquid-crystalline main transition (●) and pretransition (■) of DPPC (A) and on the enthalpy of the gel to liquid-crystalline (○) and bilayer to H_{II} (□) phase transitions of DEPE (B).

When the DEPE/P15 system was studied by means of ^{31}P-NMR as a function of temperature (at a DEPE/15 molar ratio of 10:1) the main observation was that in contrast to gramicidin, this polypeptide does not promote the formation of a H_{II} phase. This was deduced from the lack of H_{II} signal present in the ^{31}P-NMR spectrum at temperatures lower than the bilayer to H_{II} transition temperature of the pure DEPE. However we found the presence of an isotropic spectral component that begins to appear at about 55 °C which is lower than the H_{II} phase transition temperature of pure DEPE and which is also present above the transition temperature. We interpreted this isotropic signal as arising from phospholipids which due to the presence of P15 are prevented to organise in extended hexagonal or lamellar structures, and instead of this organize themselves in structures giving rise to an isotropic peak. Small angle X-ray diffraction experiments did not gave us additional information concerning the exact structural organization of the phospholipid giving rise to the isotropic signal.

When P15 was incorporated in DOPC at a molar ratio of 10:1 the only effect observed in the ^{31}P-NMR spectra was an increase in line width of the asymmetrical 'bilayer' type of line shape, which measured as the width at half height was ≈ 4 ppm and ≈ 10 ppm for the DOPC and DOPC/P15 sample, respectively. This demonstrates that P15 cannot like gramicidin promote H_{II} phase formation in the DOPC system.

We further investigated the effect of P15 on the

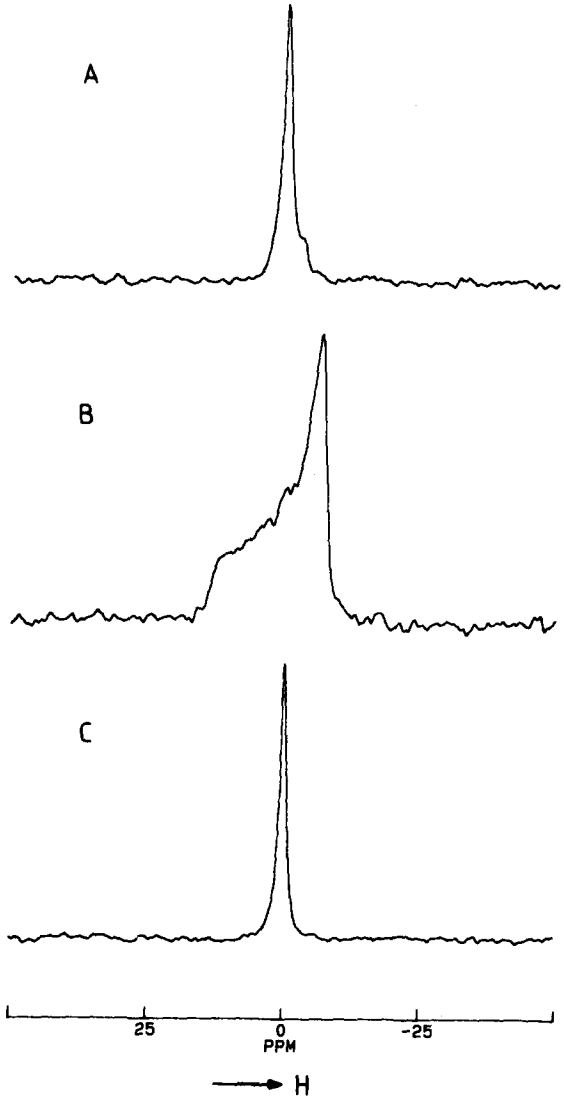

Fig. 6. ^{31}P-NMR spectra of lysoPC/P15 at 4:1 molar ratio (A), lysoPC/gramicidin A′ at 4:1 molar ratio (B) and pure lysoPC (C). The spectra were recorded at 25 °C.

polymorphism of lysoPC. Fig. 6 compares the ^{31}P-NMR spectra corresponding to pure lysoPC micelles and those of lysoPC/gramicidin A' and lysoPC/P15 mixtures at a 4 : 1 lysoPC/polypeptide molar ratio. As we mentioned in the preceeding sections, the presence of gramicidin in lysoPC systems (Fig. 6B) induces the formation of extended bilayer structures with the characteristic ^{31}P-NMR spectrum. When P15 is incorporated in lysoPC systems (Fig. 6A) the majority of the ^{31}P-NMR spectrum corresponds to an isotropic signal closely similar to the one found for pure lysoPC (Fig. 6C), hence indicating that the phospholipid is not organized in extended bilayers. The broadening of the base of the peak indicates the presene of some lysoPC undergoing restricted motion. The lysoPC/P15 sample showed visual differences when compared with the pure lysoPC one, whereas the latter forms a clear solution in water, the lysoPC/P15 was slightly turbid but much less than the corresponding lysoPC/gramicidin sample. Freeze-fracture electron microscopy of a lysoPC/P15 (4 : 1 molar ratio) system (carried out as described in Ref. 12) showed besides the very occasionally presence of some undefined large structures no lipid fracture planes, consistent with a predominantly micellar organization of the peptide-lysoPC complex and the nearly complete recovery of the lipid and peptide in the supernatant after centrifugation (see experimental section).

Discussion

In this study, the importance of the tryptophan residues of gramicidin for the peptide lipid structure modulating activity of the peptide has been investigated using gramicidin analogs in which Trp-11 (gramicidin A) was replaced by either phenylalanine (gramicidin B) or tyrosine (gramicidin C) or in which all four tryptophans are formylated on the indol ring nitrogen (tryptophan-*N*-formylated gramicidin). Furthermore P15 a tryptophan-free α-helical model peptide with an identical amount of hydrophobic amino acid residues and similar to gramicidin with blocked N- and C-termini was studied to further test the specificity of the gramicidin-induced changes in lipid organization. In all lipid systems explored with these analogs (this study and Ref. 20), i.e. DPPC, DOPC, DEPE and lysoPC, the peptides, incorporated via hydration of mixed peptide-lipid films, appear to be associated with the model membrane, most likely in the hydrophobic part, as can be inferred from either binding experiments or by their effects exerted on the properties of these lipids and in line with the type of behaviour that can be expected for uncharged very apolar molecules.

We first discuss the effect of the peptides on acyl chain packing as deduced from our calorimetric experiments. From the influence of the peptides on the gel → liquid-crystalline phase transition properties of DPPC it can be suggested that all gramicidin analogs in a similar manner fluidize the acyl chains in the gel state of the lipid. The P15 peptide is much less effective in this respect. Extrapolation from permeability experiments with black lipid membranes suggests that the P15 peptide can span the lipid bilayer [38]. The difference in effect of P15 and the gramicidin analogs on DPPC acyl chain packing might be caused by the more shoother outer surface of the P15 peptide causing less acyl chain packing perturbation most likely in combination with the high tendency of P15 to aggregate in the plane of the membrane thereby reducing the effective peptide-lipid contact area. In favor of this latter interpretation is the notion that even at very low concentrations this peptide forms voltage-dependent channels which are thought to consist of peptide oligomers [38]. That the peptide nevertheless shows interaction with the lipid is indicated by its effect on the pretransition, which in general is more sensitive than the main transition to the presence of other compounds in the bilayer. Aggregation of the gramicidin analogs occurs to a similar extent for the different species as can be deduced by the less efficient reduction of ΔH of the gel → liquid-crystalline phase transition at higher (lipid/peptide < 15 : 1) concentrations. Thus, the modifications brought about in the peptides tryptophans do not seem to grossly influence the interaction of gramicidin A with the acyl chains of DPPC in the gel state. However it should be realized that this behaviour might depend on the nature of the acyl chains as in the liquid-crystalline state of DOPC centrifugation experiments indicated similar aggregational behaviour of gramicidin A and C but a

different behaviour for gramicidin B and tryptophan-*N*-formylated gramicidin [20].

The gel → liquid crystalline phase transition of DEPE is much less affected by incorporation of gramicidin A′ [9]. This was interpreted to result from the stronger intermolecular headgroup interactions (H-bonding, electrostatic) of phosphatidylethanolamines causing peptide aggregation and thus less peptide–lipid interaction. The gramicidins A′, A, B and C and the P15 peptide behave similarly but the tryptophan-*N*-formylated gramicidin is even more ineffective in preventing acyl chains crystallization in this system, suggesting an additional stronger tendency to self associate of this analog.

We would like now to consider the effect of the peptides on the macroscopic organization of the lipids. The bilayer stabilising effect of gramicidin A′ in lysoPC and the H_{II} phase promotion in PC and PE systems have been rationalized on the basis of the shape-structure concept of polymorphism [39], by assuming that gramicidin has a cone shape due to the presence of the four bulky tryptophans all located at the C-terminal part of the peptide. In these latter lipid systems an additional involvement of peptide aggregation in inducing the H_{II} phase was shown [9,11].

The results of the present investigation demonstrate that the ability of the peptide to induce bilayer structure in lysoPC and H_{II} phase formation in DEPE systems follows the order: gramicidin A′ ≈ gramicidin A > gramicidin B ≈ gramicidin C > tryptophan-*N*-formylated gramicidin > P15 peptide. For H_{II} phase induction in DOPC systems an almost similar order of gramicidin A′ ≈ gramicidin A > gramicidin C > gramicidin B > tryptophan-*N*-formylated gramicidin ≈ P15 peptide was found (Ref. 20 and this study). The similarity in order of efficiency to induce these phase changes in such different lipid systems suggests that some fundamental properties of gramicidin A must underly these lipid-structure modulating effects. We consider the shape or the peptide and its ability to aggregate into specific structures to be the most prominent candidates. Within the shape-structure concept the differences between gramicidin A, B and C are consistent with the changes in size of the bulky C-terminus of the peptide. On substituting at position 11 the tryptophan residue for a tyrosine or phenylalanine the size of the residue at this position will decrease. The difference in aggregational behaviour of gramicidin B might further contribute to the reduced ability of the peptide to induce the H_{II} phase in DOPC [20]. The results of the tryptophan-*N*-formylated gramicidin cannot be interpreted within the shape-structure concept since the size of the tryptophans and thus of the C-terminus is increased by formylation. In this case we suggest that the very strong tendency of this analog to aggregate in the model membrane in multimolecular structures different from gramicidin (this study and Ref. 20), possibly in combination with another conformational behaviour of the peptide monomer might be responsible for its drastically reduced lipid-structure modulating activity. It should be explicitely stated that the actual conformation of gramicidin A and its analogs in the different lipid systems investigated is unknown and that possible differences in conformation between the analogs might contribute to differences in their interactions with the lipids.

The P15 peptide is the least efficient in influencing the structure of the lipids in the different systems explored. The shape of this peptide can be considered to be more cylindrical than gramicidin A which is consistent with this behaviour. Moreover, its apparent much stronger tendency to aggregate in the model membranes (at least in the diacyl lipid systems studied) will certainly contribute to its limited effect on the macroscopic structure of DEPE and DOPC systems. Since the P15 peptide resembles the antibiotic alamethicin, it is of interest to compare the results obtained with the P15 peptide with those previously reported for the antibiotic [40]. DMPC/alamethicin systems and NMR techniques indicated no effect on lipid polymorphism and a highly aggregated state of the peptide which is in fair agreement with the results shown above.

In conclusion, the present results further demonstrate the importance of the tryptophan residues for the ability of gramicidin to affect membrane lipid organization and support the view [20] that the shape of the gramicidin molecule and its aggregational behaviour both involving the peptides tryptophans are major determinants in this lipid-structure modulating activity.

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