Biochimica et Biophysica Acta 857 (1986) 13–27
Elsevier

BBA 73068

Gramicidin-induced hexagonal H_{II} phase formation in negatively charged phospholipids and the effect of N- and C-terminal modification of gramicidin on its interaction with zwitterionic phospholipids

J. Antoinette Killian [a], Carmen W. van den Berg [a], Huibert Tournois [a], Stef Keur [a], Arend J. Slotboom [a], Gustaaf J.M. van Scharrenburg [a] and Ben de Kruijff [b]

[a] *Department of Biochemistry and* [b] *Institute of Molecular Biology, State University of Utrecht, Padualaan 8, Transitorium III, NL-3584 CH Utrecht (The Netherlands)*

(Received November 26th, 1985)

Key words: Gramicidin; Anionic phospholipid; Phase transition; Gramicidin analog; ^{31}P-NMR; Small-angle X-ray diffraction

The effect of gramicidin on macroscopic structure of the negatively charged membrane phospholipids cardiolipin, dioleoylphosphatidylglycerol and dioleoylphosphatidylserine in aqueous dispersions was investigated and compared with the effect of gramicidin on dioleoylphosphatidylcholine. It was shown by small-angle X-ray diffraction, ^{31}P nuclear magnetic resonance and freeze-fracture electron microscopy that in all these lipid systems gramicidin is able to induce the formation of a hexagonal H_{II} phase. ^{31}P-NMR measurements indicated that the extent of H_{II} phase formation in the various lipids ranged from about 40% to 60% upon gramicidin incorporation in a molar ratio of peptide to lipid of 1:10. Next, the following charged analogues of gramicidin were prepared: desformylgramicidin, *N*-succinylgramicidin and *O*-succinylgramicidin. The synthesis was verified with ^{13}C-NMR and the effect of these analogues on lipid structure was investigated. It was shown that, as with gramicidin itself, the analogues induce H_{II} phase formation in dioleoylphosphatidylcholine, lower and broaden the bilayer-to-H_{II} phase transition in dielaidoylphosphatidylethanolamine and form lamellar structures upon codispersion with palmitoyllysophosphatidylcholine. Differential scanning calorimetry measurements indicated that, again like gramicidin, in phosphatidylethanolamine the energy content of the gel-to-liquid-crystalline phase transition is not affected by incorporation of the analogues, whereas in phosphatidylcholine a reduction of the transition enthalpy is found. These observations were explained in terms of a similar tendency to self-associate for gramicidin and its charged analogues. The results are discussed in the light of the various factors which have been suggested to be of importance for the modulation of lipid structure by gramicidin.

Introduction

Gramicidin is a linear hydrophobic peptide, consisting of 15 alternating L and D amino acids. The structure of gramicidin was originally elucidated by Sarges and Witkop [1]. A well-known property of gramicidin is its ability to form

Abbreviations: lyso-PC, palmitoyllysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; DEPE, dielaidoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; DOPS, dioleoylphosphatidylserine; DMSO, dimethyl sulphoxide.

cation-selective transmembrane channels in model and biological membranes (for recent review, see Anderson [2]). In its channel configuration the peptide is believed to be present as an N to N terminal helical dimer [3,4]. As a membrane-spanning hydrophobic peptide, gramicidin has been widely used as a model for the hydrophobic part of intrinsic membrane proteins in studies of lipid-protein interactions [5,6]. It was in these kinds of study that another interesting property of gramicidin manifested itself. The peptide appeared to be extremely effective as modulator of lipid phase behavior. When mixed with lysophosphatidylcholine (lyso-PC), which in excess water organizes in micelles, a lamellar complex is formed [7–9] of one gramicidin molecule with four lyso-PC molecules [8]. Upon incorporation in phosphatidylethanolamines (PEs) the peptide strongly promotes H_{II} phase formation, in that it lowers the onset temperature of the bilayer to hexagonal H_{II} phase transition [10,11]. However, the most intriguing observation was that gramicidin is able to induce H_{II} phase formation in the typical bilayer-forming phosphatidylcholines (PCs) when the acyl chain length exceeds 16 C atoms [12].

Since integral membrane proteins such as chlorophyllase [13], glycophorin [14] and the membrane-spanning 23-amino-acid-long hydrophobic segment of this protein [15] do not induce H_{II} phases in model membrane systems, but in contrast act as bilayer stabilizers, the gramicidin molecule must have specific features allowing it to interact in a unique manner with the surrounding lipids. These most likely include its hydrophobicity and lipid-headgroup-dehydrating capacity [16], its irregular outer contour (cone-shaped in the $\beta^{6\text{-}3}$ helical conformation) and its tendency to self-associate in a particular, as yet unknown, hydrated conformation [16].

This paper addresses two aspects of the lipid structure modulating activity of the peptide. Firstly, until now, H_{II} phase formation only was observed in PE and PC systems. We thought it of interest to see whether this effect also occurs for other bilayer-forming lipids. Therefore we have studied in this paper by means of ^{31}P nuclear magnetic resonance (^{31}P-NMR), small angle X-ray diffraction and freeze-fracture electron microscopy the effect of gramicidin on the phase behavior of different negatively charged membrane phospholipids. This choice of lipids is of particular interest, since it can be epected that due to electrostatic interheadgroup repulsive forces a location of the lipid molecules around the narrow aqueous channels in the H_{II} phase will be energetically unfavorable.

Secondly, we recently demonstrated that chemical modification of the four tryptophan residues in gramicidin by *N*-formylation of the indole moieties results in a complete loss of H_{II} phase formation in dioleoylphosphatidylcholine (DOPC) model membranes [17]. To see whether this effect is specific for modification of the tryptophan residues and in order to gain more insight into the importance of the chemical structure of gramicidin for its lipid-structure-modulating activity, we prepared desformylgramicidin, *N*-succinylgramicidin and *O*-succinylgramicidin and we compared the interaction of gramicidin and these ionizable groups bearing N- and C-terminal modified analogues with PC, PE and lyso-PC. The lipid phase behavior was studied with small angle X-ray diffraction and ^{31}P-NMR. Differential scanning calorimetry (DSC) was used to study the effect of the gramicidin analogues on the thermodynamic properties of PE and PC systems.

The results are discussed in the light of the various models proposed for the lipid-structure-modulating activity of the peptide.

Materials and Methods

Chemicals

Gramicidin from *Bacillus brevis*, which is a mixture of gramicidins A, B and C in a molar ratio of approx. 80 : 5 : 15, was obtained from Sigma (St. Louis, U.S.A.). Gramicidins B and C differ from gramicidin A in that the tryptophan at position 11 is replaced by, respectively, phenylalanine and tyrosine. The mixture was used without further purification. Preliminary experiments demonstrated a quantitatively similar effect on lipid phase behavior for the natural mixture of gramicidins as compared to gramicidin A.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC) were synthesized accord-

ing to the procedure of Van Deenen and De Haas [18]. 1-Palmitoyl-*sn*-glycero-3-phosphocholine (lyso-PC) was obtained via hydrolysis of DPPC by the action of pancreatic phospholipase A_2. 1,2-Dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS) were obtained from the corresponding phosphatidylcholines by the phospholipase-D-catalyzed base-exchange reaction [19] and converted to their sodium salts as described by Smaal et al. [20] for cardiolipin. All these lipids were purified by preparative high-performance liquid chromatography [21]. Cardiolipin was obtained from Avanti Polar Lipids (Birmingham, AL). All lipids were judged pure from high-performance thin-layer chromatography on silica-gel, using chloroform/ methanol/ water/ ammonia (68 : 28 : 2 : 2, by volume) as eluent.

^{13}C-enriched succinic acid (90% at both methylene positions) was purchased from Stohler Isotope Chemicals (U.S.A.). Dimethyl sulfoxide (DMSO-d_6), 99.8% deuterated, and 4-dimethylaminopyridine were obtained from Merck (Darmstadt). *N*,*N*-Dicyclohexylcarbodiimide and hydroxybenzotriazol were obtained from Aldrich (Beerse, Belgium).

All other reagents were of analytical grade. Methanol, dichloromethane and dimethylformamide were freshly distilled before use.

Quantification of gramicidin and its analogues

The amount of peptide was determined either directly by weighing, or indirectly by determination of the absorbance at 280 nm of the peptide in a methanolic solution. The absorbance was linear with the gramicidin concentration in the range of 10–30 μM (Shimadzu Spectrophotometer UV-110-02). For the natural gramicidin mixture a molar extinction coefficient of 19100 $M^{-1} \cdot cm^{-1}$ was measured, based on an average molecular weight of 1876. Similar molar extinction coefficients were measured for the N- and C-terminal-modified gramicidin analogues.

High-performance thin-layer chromatography of gramicidin (analogues)

Thin-layer chromatography of gramicidin and derivatives was carried out on silica HPTLC plates (Merck, Darmstadt) with chloroform/methanol/water (65 : 25 : 4, by volume) as eluent. Spots were visualized by spraying the plates with 10% sulfuric acid in H_2O, followed by charring at 180°C. For the unmodified gramicidin mixture under these conditions, one main spot was observed, of gramicidin A (R_F 0.74), and two minor spots originating from gramicidins B and C (R_F 0.75 and 0.71). The presence of these different species could also be detected in the gramicidin analogues. In addition, and in agreement with observations of other authors [1,22], other faint spots occasionally occurred, which were ascribed to the presence of different conformations of the peptide [22].

For detection of primary amino groups the plates were sprayed with a solution of 1 g ninhydrin in 100 ml butanol, saturated with water.

Desformylgramicidin

Desformylgramicidin was prepared via a modification of the procedure of Sarges and Witkop [1].

To a solution of 500 mg of gramicidin in 40 ml of methanol, 10 ml of a 5 M HCl solution was freshly prepared by passing HCl gas through methanol until the appropriate weight was attained. The mixture was allowed to stand for 2–3 h at room temperature, after which the reaction was almost complete as judged from HPTLC (gramicidin: R_F 0.74, ninhydrin negative, desformylgramicidin: R_F 0.58, ninhydrin positive). Subsequently, methanol was added to a final volume of 500 ml, whereafter the peptide was isolated via a Bligh and Dyer extraction [23], followed by washing of the chloroform phase with twice 500 ml of water. The solvent was evaporated under reduced pressure in a rotatory evaporator, whereby as much of isopropanol was added as necessary to remove all traces of water. The residue was dissolved in 5 ml methanol and applied to a Dowex 50W-X2 resin, 200–400 mesh (Merck, Darmstadt) column (200 × 10 mm), in the H^+ form equilibrated with methanol and equipped with a cooling jacket. Unreacted gramicidin was eluted with 300 ml methanol (detection at 280 nm). Desformylgramicidin was eluted from the column with 250 ml methanol/25% ammonia in H_2O (400 : 80, v/v). Since during this latter step

heat develops, the column was temporarily cooled with running tap water. HPTLC showed one spot of desformylgramicidin. After removal of the solvent, as indicated above, and storage under high vacuum, the desformylgramicidin was obtained as a transparent layer in a yield of 350–400 mg.

N-Succinylgramicidin

A method for the synthesis of *N*-succinylgramicidin has been described before in detail by Bamberg et al. [24]. The procedure involved the formation of *N,O*-bisuccinyldesformylgramicidin via a reaction of desformylgramicidin with succinic anhydride in pyridin, followed by hydrolysis of the ester bond.

We now describe the following, more direct method, in which we synthesize *N*-succinylgramicidin from desformylgramicidin and succinic acid, with *N,N*-dicyclohexylcarbodiimide as activator and hydroxybenzotriazol as a trapping agent.

To 150 mg desformylgramicidin was added a 3-fold molar excess of, successively, *N*-methylmorpholine, succinic acid, hydroxybenzotriazol and *N,N*-dicyclohexylcarbodiimide, each dissolved in approximately 1 ml of dimethylformamide/dichloromethane (1 : 2, v/v) to a final volume of 4.5 ml. The mixture was allowed to stand overnight at 4°C, after which the reaction was almost complete as judged from HPTLC (*N*-succinylgramicidin: R_F 0.78, ninhydrin negative). Subsequently, the mixture was acidified by addition of 4 ml 5 M HCl in methanol and applied to a column (75 × 11 mm) filled with Dowex 50W-X2 in the H^+ form. Elution with 60 ml methanol at room temperature yielded *N*-succinylgramicidin with some contaminants. Under these conditions unreacted desformylgramicidin was retained on the column. After removal of the solvents, the crude *N*-succinylgramicidin was dissolved in 3 ml of methanol and purified on a Sephadex LH20 (Pharmacia, Sweden) column (2500 × 15 mm) in 50 mg portions. The column was running in methanol at a flow rate of 16 ml/h. *N*-Succinylgramicidin (determined by HPTLC) came off the column after an elution volume of 190 ml. After removing of the solvent and redissolving the residue in 1 ml of methanol, the *N*-succinylgramicidin was rechromatographed on the same column and now eluted as a single peak. After evaporation of the solvent and further drying under high vacuum a final average amount of approx. 110 mg of the product was obtained. HPTLC showed one spot of *N*-succinylgramicidin.

^{13}C-enriched *N*-succinylgramicidin was prepared similarly using ^{13}C-enriched succinic acid.

O-Succinylgramicidin

The synthesis of *O*-succinylgramicidin, based on a reaction of gramicidin with the corresponding anhydride in pyridine [24], has been described before [24,25]. However, in view of the high yield we obtained in the synthesis of *N*-succinylgramicidin, we used an analogous method, which involves the 4-dimethylaminopyridine-catalyzed reaction of gramicidin with succinic acid, using *N,N*-dicyclohexylcarbodiimide as an activator.

25 mg gramicidin was dissolved in 125 µl dimethylformamide/triethylamine (4 : 1, v/v). Successively, a 20-fold molar excess of succinic acid was added and 3-fold molar excesses of 4-dimethylaminopyridine and *N,N*-dicyclohexylcarbodiimide, each dissolved in dimethylformamide/triethylamine (4 : 1, v/v). The reaction mixture, with a final volume of 500 µl, was incubated at 37°C for 6 h. After completion of the reaction, as judged from HPTLC (*O*-succinylgramicidin: R_F 0.67, ninhydrin negative), the mixture was applied to a Sephadex LH 20 column (2500 × 15 mm) and eluted with methanol at room temperature. After an elution volume of 175 ml the *O*-succinylgramicidin-containing fractions came off the column. These fractions were combined and dried. The residue was dissolved in 1 ml methanol and rechromatographed on the same column. This time a single *O*-succinylgramicidin peak was obtained in a final average yield of 20 mg. HPTLC of this compound showed one spot of *O*-succinylgramicidin.

^{13}C-enriched *O*-succinylgramicidin was prepared similarly, except that an 8-fold excess of (^{13}C-enriched) succinic acid was added and the reaction mixture was incubated for 24 h at 37°C. Under these conditions the reaction was approx. 85% complete as judged from HPTLC.

^{13}C nuclear magnetic resonance (^{13}C-NMR)

10–30 mg peptide, or 1–2 mg in the case of ^{13}C-enriched derivatives, was dissolved in 1 ml of DMSO-d_6 and the solution was transferred to a 10 mm NMR tube. ^{13}C-NMR spectra were recorded using gated ^{1}H-decoupling at 50.3 MHz on a Bruker WP200 spectrometer at 40°C. 10–50K free induction decays were accumulated using 8K data points, a spectral width of 10 kHz, an 18 μs 90° R_F pulse and an interpulse time of 1 s. Prior to Fourier transformation, an exponential multiplication was applied to the accumulated free induction decays, resulting in a 5 Hz line-broadening.

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

Proton-noise-decoupled ^{31}P-NMR spectra were recorded on a Bruker WP200 or WM200 spectrometer at 81.0 MHz, as described before [11]. All spectra were recorded at 25°C, with the exception of DEPE-containing samples, which were recorded over a temperature range of 25–70°C. Relative amounts of bilayer or H_{II} component present in the spectra were obtained with similar results either by computer subtraction of the pure bilayer type of spectra, followed by integration, or by estimation of the bilayer lineshape and measurement of the area of the bilayer component in relation to the total area of the spectrum with the use of a Hewlett-Packard digitizer (type 9864A). The maximal error was estimated to be 10%.

Dispersions of gramicidin with the negatively charged lipids cardiolipin, DOPG and DOPS were prepared as follows. 70 μmol lipid and the appropriate amount of gramicidin were dissolved in chloroform/methanol (1 : 1, v/v). In view of the limited solubility of DOPS in this solvent during evaporation, samples containing this lipid were dissolved in chloroform only. The solvent was removed by rotation under a stream of nitrogen at 40°C and the samples were further dried overnight under high vacuum. The dry lipid/peptide films were dispersed in 5 ml buffer (100 mM NaCl/10 mM Tris-HCl (pH 7.4)) at room temperature, and subsequently centrifuged for 15 min at 4°C at 30 000 × g. The pellets were resuspended in 1 ml of the same buffer, containing 25% $^{2}H_2O$, and transferred to 10 mm NMR tubes.

Homogeneous mixed films of DOPC with gramicidin and its analogues were prepared as follows. The appropriate amounts of lipid (60 μmol) and peptide were dissolved in chloroform/methanol (2 : 1, v/v) in a small pyrex tube (5 × 0.8 cm) and slowly evaporated to dryness by rotation under a stream of N_2 at 40°C. After overnight storage under high vacuum, an amount of buffer (150 mM NaCl/10 mM Tris-HCl/0.2 mM EDTA (pH 7.0)) was added, corresponding to 75% of the sample weight. After incubation for 30 min at 37°C, the sample was centrifuged at 5000 × g in a swing-out rotor at 40°C for several hours. The pellet was washed once carefully with 2 ml of buffer. The tube then was transferred into a 10 mm NMR tube.

In case of DEPE and lyso-PC, dry lipid/peptide films were prepared in 10 mm NMR tubes and were dispersed in 1.2 ml of buffer (150 mM NaCl/10 mM Tris-HCl/0.2 mM EDTA (pH 7.0)) by gently vortexing at 40°C and at room temperature, respectively.

Using these procedures, the pH of all samples prior to measurement did not significantly deviate from the pH of the buffers used.

Small-angle X-ray diffraction

The X-ray diffraction profile was obtained over a period of 5–10 min exposure at 25°C, using a Kratky camera with a 10 × 0.2 mm CuK_α beam (40 kV, 20 mA) equipped with a position-sensitive detector (LETI). For measurement of the interaction of gramicidin with negatively charged lipids, pelleted NMR samples were used. In other cases samples were prepared freshly, but with similar results, as follows. 10 μmol lipid and the appropriate amount of peptide in chloroform/methanol (2 : 1, v/v) were dried by rotation under reduced pressure. Then 1 ml of buffer (150 mM NaCl/10 mM Tris-HCl/0.2 mM EDTA (pH 7.0)) was added to the dry film and the sample was dispersed by gently vortexing at room temperature, followed by centrifugation for 15 min at 30 000 × g at 4°C. The pellet was washed once with an equal volume of buffer and then mounted in a slit (16 × 1.5 × 1.5 mm) in a steel cuvette between two sheets of cellophane.

Differential scanning calorimetry (DSC)

DSC measurements were performed on a Per-

kin Elmer DSC 2 as described before [11]. Dispersions were prepared as described for the X-ray measurements, except that, this time, after centrifugation the pellet was not washed. Samples of DEPE and DPCC were dispersed at 40°C and at 45°C, respectively.

Results

Interaction of gramicidin with negatively charged lipids

Upon dispersion in aqueous solution at neutral pH and in the presence of 100 mM NaCl, anionic membrane phospholipids prefer a bilayer organization [26]. Under these conditions we investigated the effect of gramicidin incorporation on lipid structure of cardiolipin, DOPC and DOPS. In the first experiment, the phase behavior of cardiolipin was monitored upon titration with gramicidin. In the absence of the peptide, ^{31}P-NMR showed a typical bilayer type of spectrum with a low-field shoulder and a high-field peak and a chemical shift anisotropy of about 27 ppm, in agreement with earlier published observations [27,28]. As is shown in Fig. 1, the relative amount of this bilayer component decreases upon incorporation of increasing amounts of gramicidin. This decrease was parallelled by the gradual appearance of a second spectral component with a reversed asymmetry and a reduced chemical shift anisotropy, as is characteristic for an H_{II} phase [29]. In addition, a small and rather broad isotropic component was observed, the intensity of which, in all spectra, was estimated to be less than 10% of the total spectral intensity. At a molar ratio of gramicidin to cardiolipin of 1 : 10 it was thus calculated that more than 50% of the lipids are organized in the H_{II} phase.

This molar ratio of peptide to lipid was used to investigate further the effect of gramicidin on lipid structure in DOPG and DOPS dispersions. The results were compared with the DOPC system, in which the gramicidin-induced H_{II} phase formation is well documented [16]. Whereas these lipids in the absence of the peptide showed a pure bilayer type of spectrum, in agreement with previous observations [27,30,31], it was found that an H_{II} component appeared in all samples upon gramicidin incorporation. In addition, in DOPS and DOPG dispersions, an isotropic component was observed. The first column in Table I shows the percentage of the remaining bilayer component in the various samples. In DOPC dispersions a decrease of the chemical shift anisotropy of approx. 10% was observed upon gramicidin incorporation in a molar ratio of peptide to lipid of 1 : 10, in agreement with previous observations [16]. In DOPG and DOPS, decreases were found of about 15% and 10% respectively, whereas in dispersions of cardiolipin, gramicidin incorporation did not seem to affect the chemical shift anisotropy.

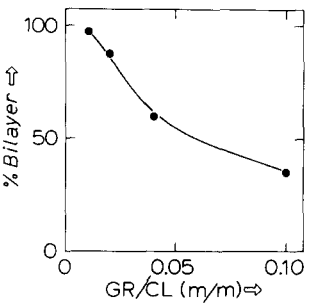


Fig. 1. Percentage of bilayer signal in 81.0 MHz ^{31}P-NMR spectra of aqueous dispersions of cardiolipin as a function of the gramicidin content.

To characterize these samples further, small-angle X-ray diffraction was also used. In general, the diffraction profile of lipids in a multilamellar organization consists of a series of reflections at distances which relate as 1 : 1/2 : 1/3, etc. [32]. In dispersions of pure cardiolipin, DOPS and DOPG, only a broad scattering profile is observed, in agreement with observations of Hauser et al. [33].

TABLE I

^{31}P-NMR ANALYSIS AND X-RAY DIFFRACTION CHARACTERISTICS OF DISPERSIONS OF VARIOUS LIPIDS UPON GRAMICIDIN INCORPORATION IN A 1 : 10 MOLAR RATIO OF PEPTIDE TO LIPID

Lipid	^{31}P-NMR	X-ray diffraction		
	% bilayer	reflections (Å)		tube diameter (Å)
DOPC	56	63.8	36.2	72.4
CL [a]	38	62.0	35.0	70.0
DOPG	54	62.3	35.4	70.8
DOPS	52	60.5	35.9	71.8

[a] Cardiolipin.

In these samples no long-range order could be detected, most likely due to the increased interbilayer repeat distance as a consequence of the presence of a net charge. Upon gramicidin incorporation, however, in addition sharp reflections appeared at distances which relate as $1:1/\sqrt{3}$ (second column, Table I). This relationship is characteristic for the long spacings of the (1,0,0) and the (1,1,0) reflections of the hexagonal H_{II} phase, for which theoretically a more complete series of higher-order reflections can be expected at distances which relate as $1:1/\sqrt{3}:1/2:1/\sqrt{7}$ etc. [32]. However, these higher-order reflections often cannot be detected and, in addition, it should be noted that, although small-angle X-ray diffraction is a useful technique to elucidate the macroscopic organization of lipids, it does not allow quantification of the phases present. From the *d* values of the reflections, the tube-to-tube distance in the H_{II} phase can be derived. Table I, third column, shows that the tube diameters are similar in the various samples.

The ability of gramicidin to induce H_{II} phase formation in cardiolipin, DOPG and DOPS was further demonstrated by freeze-fracture electron microscopy. Extensive areas of H_{II}-type structures were observed. Fig. 2 shows the micrograph of a dispersion of a DOPG/gramicidin (10:1, m/m) mixture. The characteristic striated pattern of the H_{II} phase [34] is clearly visible. A similar morphology was found in the other anionic phospholipid dispersions upon gramicidin incorporation. It is of interest to note that in systems in which bilayer-to-hexagonal-H_{II} phase transitions occur, lipidic particles are in general found as intermediates (for review, see Ref. 34), which often can be visualized as rows of regularly sized pits or particles with a diameter roughly of the order of magnitude of the tube diameter in the H_{II} phase. In gramicidin-induced H_{II} phases to date no such particles have been observed, and they could not be detected in the lipid systems presently investigated, either.

Interaction of gramicidin analogues with various lipids

Characterization of the gramicidin analogues. Structural representations of the various compounds are given in Table II. First, the synthesis

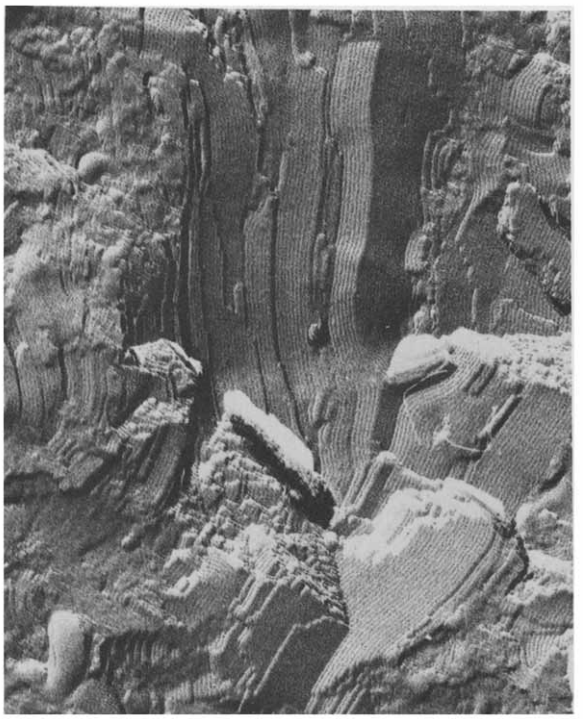


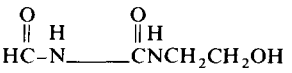
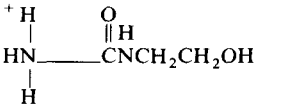
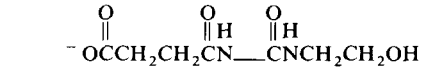
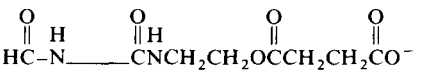
Fig. 2. Freeze-fracture electron micrograph of an aqueous dispersion of DOPG and gramicidin in a 1/10 molar ratio. The sample was quenched from room temperature. Experiments were performed using the jet-freeze method as described previously [38]. Magnification, 88000×.

of these gramicidin derivatives was verified and their purity was checked with the use of ^{13}C-NMR. Fig. 3A shows the spectrum of the natural gramicidin mixture. Assignments and chemical shift positions are given in Table III. The resonances were assigned according to Fossel et al. [35] and, in the aromatic region, also according to Gratwohl and Wüthrich [36].

Deformylation of gramicidin results in a number of spectral changes (Fig. 3B) of which the most obvious difference is the disappearance of the resonance of the N-terminal formyl carbon atom at 161.5 ppm. Further changes are restricted to the Val-1 carbon atoms. In the carbonyl region the Val-1 resonance shifts downfield to 174.8 ppm upon deformylation. The Val-1 $C_\alpha H$ resonance at 56.6 ppm shows a 1–2 ppm downfield shift. Part of the Val $C_\beta H$ resonances at 30.4–30.8 ppm shift approx. 1 ppm downfield and a new resonance occurs, which is due to an upfield shift of the Val-1 $C_\gamma H_3$ resonances.

TABLE II

STRUCTURES OF THE GRAMICIDIN ANALOGUES

Compound	Abbreviation	Structural representation
Gramicidin	GR	
Desformylgramicidin	Desf-GR	
N-Succinylgramicidin	N-Suc-GR	
O-Succinylgramicidin	O-Suc-GR	

Upon subsequent coupling of succinate at the N-terminal part (Fig. 3C), the resonances originating from Val-1 adopt similar chemical shift positions as found for the other valine residues. Both succinate carbonyl resonances are in the region of 171.0 to 172.5 ppm, with the other peptide carbonyl resonances. The methylene resonances of succinate, which are only partially resolved, appear at 29 ppm. Their position was verified by the ^{13}C-NMR spectrum of the ^{13}C-enriched *N*-succinyl compound (data not shown), which exhibited a similar partially resolved pattern with peaks at 29.0 and 29.3 ppm.

Fig. 3D shows the ^{13}C-NMR spectrum of *O*-succinylgramicidin. At the N-terminal part the resonances are similar to those of the unmodified compound, including the presence of the *N*-formyl resonance. Changes in the spectrum are now restricted to the additional appearance of the succinate resonances (the carbonyl resonances in the range of 171.0 to 172.5 ppm and the methylene carbon resonances at 28.9 and 29.1 ppm) and to the ethanolamine part (a 1 ppm downfield shift of the methylene resonances at 60.0 ppm and a shift of the resonance at 41.8 ppm to the region in which the DMSO resonances occur at about 40 ppm). The positions of the methylene carbon atoms could be verified after ^{13}C enrichment (data not shown).

In all spectra, the direction and magnitude of the changes in chemical shift position were in accordance with those expected on the basis of elementary additivity rules for substituent effects [37]. Since no other changes could be detected, these data confirm the synthesis and demonstrate the purity of the various gramicidin analogues.

Effect of the gramicidin analogues on lipid structure

The effect of gramicidin and its analogues on the lipid phase behavior of DOPC was investigated with small-angle X-ray diffraction and ^{31}P-NMR.

Fig. 4 shows the diffraction profiles of pure DOPC and of the various lipid/peptide mixtures in a molar ratio of 10 : 1. The distances of the reflections in a pure DOPC dispersion (Fig. 4A) have a $1 : 1/2$ relationship, which is characteristic for the long-range order as found in multilamellar structures. In contrast, incorporation of gramicidin (Fig. 4B) or any of its analogues (Fig. 4C–E) results in the appearance of the additional H_{II}-phase-specific $(1/\sqrt{3})$ reflection, which clearly shows that all three analogues are able to induce H_{II} phase formation in DOPC, similar to gramicidin. The *d*-values of these reflections as well as the tube diameter in the H_{II} phase calculated therefrom are given in Table IV. It may be noted that, in agreement with previous data [16,38], in the DOPC/gramicidin mixture the first-order (1,0,0) reflections of the lamellar and the H_{II} phases are similar. The (1,1,0) reflection in the H_{II} phase has a rather high intensity, as has been observed before in gramicidin/PC systems [38]. The reason for this is at yet unknown. When

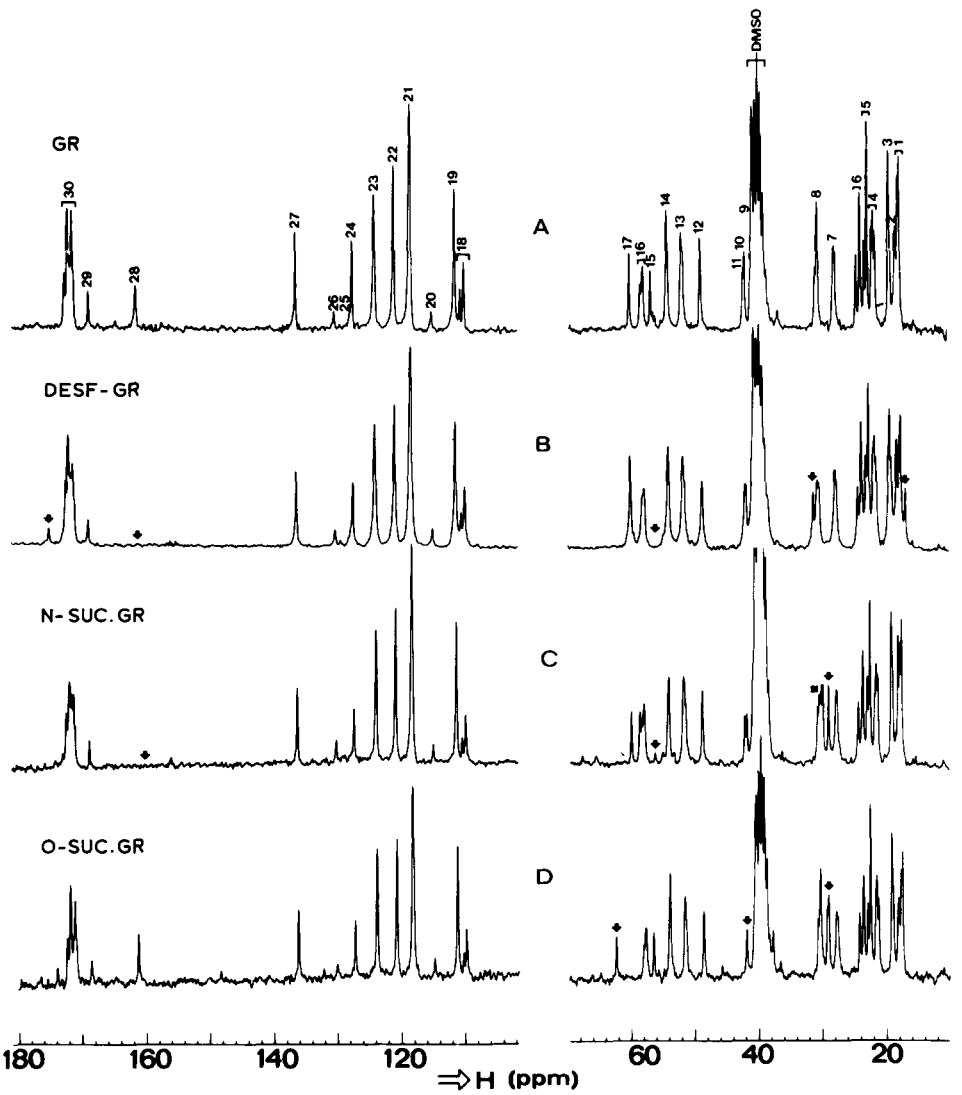
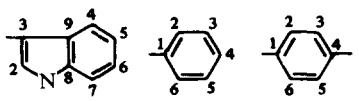


Fig. 3. 50.3 MHz natural abundance ^{13}C-NMR spectra of gramicidin (A), desformylgramicidin (B), *N*-succinylgramicidin (C) and *O*-succinylgramicidin (D) in DMSO-d_6. 0 ppm corresponds to the chemical shift position of TMS. Assignments of the resonances are given in Table III.

desformylgramicidin is incorporated (Fig. 4C) the intensities are altered and an additional reflection is observed at approximately half the distance of the first-order reflection. From Table IV one may notice that the distance of the first order reflection is slightly increased as compared to the repeat distance in the DOPC/gramicidin sample and, furthermore, that the values of the long spacings of this reflection and the (1,1,0) reflection of the H_{II} phase do not exactly relate as $1:1/\sqrt{3}$. Together with the forthcoming ^{31}P-NMR data we interpret the diffraction pattern as the first- and second-order reflection of a bilayer with a lamellar repeat distance of 65.1 Å and the (1,1,0) reflection of an H_{II} component of which the (1,0,0) reflection cannot be discerned from the rather broad diffraction peak at 65 Å. The diffraction profile of the *N*-succinylgramicidin/DOPC sample (Fig. 4D) was similarly interpreted as showing the first-order reflection of a bilayer with a slightly increased interlamellar repeat distance, and the (1,0,0) and (1,1,0) reflections of an H_{II} phase. In

TABLE III

CHEMICAL SHIFT POSITIONS OF THE C ATOMS OF GRAMICIDIN IN DMSO-d_6 AS SHOWN IN FIG. 3



Trp Phe Tyr

	C atom	Chemical shift position (ppm)
1	Val $C_\gamma H_3$	17.7–17.9
2	Ala $C_\beta H_3$	18.2–18.4
3	Val $C_{\gamma 1} H_3$	19.1–19.3
4	Leu $C_{\delta 2} H_3$	21.4–21.7–22.0
5	Leu $C_{\delta 1} H_3$	22.6–23.0
6	Leu $C_\gamma H$	23.7–24.4
7	Trp β-CH	27.7–27.9
8	Val β-CH	30.4–30.8
9	Leu β-$\underline{C}H_2$	40.9
10	$NH\underline{C}H_2$	41.8
11	Gly α-CH	42.0
12	Ala α-CH	48.8
13	Leu α-CH	51.5–51.7
14	Trp α-CH	54.0
15	Val-1α-CH	56.6
16	Val-6,7,8α-CH	57.8–58.2
17	$\underline{C}H_2OH$	60.0
18	Trp C3	109.8–109.9–110.5
19	Trp C7	111.4
20	Tyr C3/C5	115.0
21	Trp C4,C6	118.4
22, 23	Trp C2,C5	120.9–124.0
24	Trp C9	127.4
25	Phe C2/C6	127.8
26	Phe C3/C5, Tyr C2/C6	130.2–130.6
27	Trp C8	136.4
28	Formyl C=O	161.5
29	Gly C=O	168.0
30	Peptide backbone C=O	171–172.5

the sample with *O*-succinylgramicidin (Fig. 4E), two broad reflections are observed with distances having a $1:1/\sqrt{3}$ relationship. Since in this sample the tube diameter in the H_{II} phase is slightly increased (Table IV), the first-order reflection might now originate from both an H_{II} phase and a bilayer with a slightly increased lamellar repeat distance.

The ability of the various derivatives to induce H_{II} phase formation in DOPC model membranes was confirmed and quantified with the use of ^{31}P-NMR. Whereas for a pure DOPC dispersion a typical bilayer type of spectrum is observed (Fig. 5A), incorporation of gramicidin (Fig. 5B) or one of its analogues (Fig. 5C–E) results in the appearance of a second spectral component with a low-field peak at about −7 ppm, which is characteristic for lipids organized in the H_{II} phase [29]. The chemical shift anisotropy of a pure DOPC dispersion is approx. 44 ppm and decreases upon incorporation of gramicidin with about 10%, in agreement with previous observations [16]. Incorporation of the gramicidin analogues results in a similar decrease of the chemical shift anisotropy. The extent of H_{II} phase formation induced by the various peptides is given in the second column of Table IV.

We next used ^{31}P-NMR to compare further the H_{II} phase promoting effect of gramicidin and these derivatives in DEPE in a molar ratio of peptide to lipid of 1:50. Fig. 6 shows the percentage of H_{II} phase in the various samples as a function of temperature. Pure DEPE undergoes a

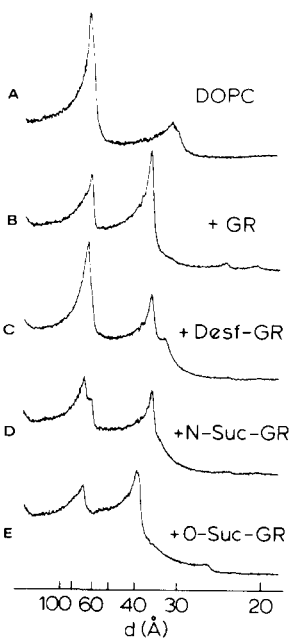


Fig. 4. Small-angle X-ray diffraction patterns of aqueous dispersions of DOPC and of mixtures of DOPC with gramicidin (A), desformylgramicidin (B), *N*-succinylgramicidin (C) and *O*-succinylgramicidin (D) in a molar ration of peptide to lipid of 1:10.

TABLE IV

X-RAY DIFFRACTION CHARACTERISTICS AND ^{31}P-NMR ANALYSIS OF AQUEOUS DISPERSIONS OF DOPC AND MIXTURES OF DOPC AND GRAMICIDIN (ANALOGUES) IN A MOLAR RATIO, PEPTIDE TO LIPID, OF 1 : 10

The tube diameter was obtained by multiplying the distance of the (1,1,0) reflection in the H_{II} phase by a factor of 2.

	X-ray diffraction				^{31}P-NMR
	reflections (Å)			tube diameter (Å)	% H_{II} phase
DOPC	64.4		31.7	-	0
DOPC/GR	63.8	36.2		72.4	44
DOPC/Desf. GR	65.1	35.7	32.8	71.4	33
DOPC/N-Suc. GR	68.1/82.2	35.6		71.2	40
DOPC/O-Suc-GR	69.0	39.3		78.6	31

bilayer to hexagonal H_{II} phase transition at $\pm 60°C$. The H_{II}-phase-promoting effect of gramicidin, observed as a shift of the transition to lower temperatures, agrees well with previous data [10,11]. All three derivatives appear to behave in approximately the same way as gramicidin.

Finally, the lipid-phase-modulating activity of these derivatives in lyso-PC dispersions was investigated. Pure lyso-PC gives rise to a sharp isotropic ^{31}P-NMR signal due to the fast tumbling of the lipid micelles. Incorporation of gramicidin in a 1 : 4 molar ratio leads to a quantitative bilayer organization for all lipids, as evidenced by the ^{31}P-NMR lineshape (Fig. 7A), in agreement with previous data [8,9]. A characteristic feature of this spectrum is the decrease of the chemical shift anisotropy by a factor of about 2, as compared to the values reported for diacylphospholipids. A decreased chemical shift anisotropy was observed earlier in ^{31}P-NMR spectra of lyso-PC in bilayers with cholesterol [9,39], with palmitic acid [40] or with DPPC [9,41] and was proposed to originate mainly from increased motional freedom of the phospholipid headgroup due to a larger flexibility of the glycerol backbone in the absence of the *sn*-2 acyl chain [39,41]. All three derivatives show an effect similar to that of gramicidin (Fig. 7B–D) and form lamellar structures upon codispersion with lyso-PC. The small isotropic components present in the lyso-PC/*N*-succinylgramicidin and the lyso-PC/*O*-succinylgramicidin samples can most

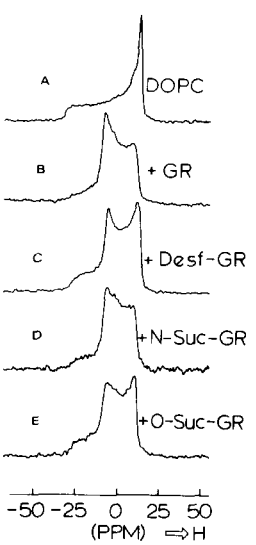


Fig. 5. Proton-noise-decoupled 81.0 MHz ^{31}P-NMR spectra of aqueous dispersions of DOPC (A) and of mixtures of DOPC with gramicidin (B), desformylgramicidin (C), *N*-succinylgramicidin (D) and *O*-succinylgramicidin (E) in a molar ratio of peptide to lipid of 1 : 10.

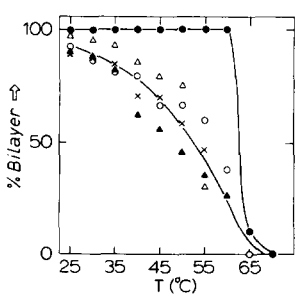


Fig. 6. Temperature dependency of the bilayer-to-H_{II} phase transition of dispersions of DEPE (●———●) and of DEPE and gramicidin (○ ——— ○), desformylgramicidin (× ——— ×), *N*-succinylgramicidin (△———△) and *O*-succinylgramicidin (▲———▲) in molar ratios of peptide to lipid of 1 : 50.

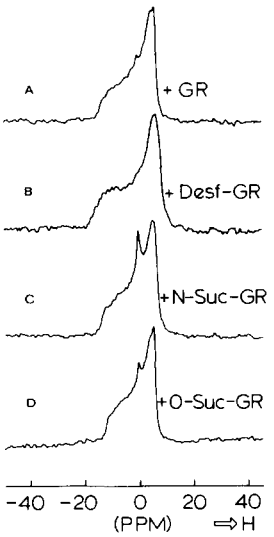


Fig. 7. 81.0 MHz ^{31}P-NMR spectra of aqueous dispersions of lyso-PC with gramicidin (A), desformylgramicidin (B), *N*-succinylgramicidin (C) and *O*-succinylgramicidin (D) in a molar ratio of peptide to lipid of 1:4.

likely be attributed to the presence of some residual lyso-PC micelles. The similarity of the values of the chemical shift anisotropy in these spectra suggest that, when present in bilayers with lyso-PC, gramicidin and its derivatives permit similar motions of the phospholipid headgroup.

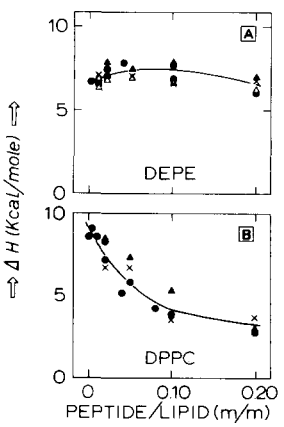


Fig. 8. Enthalpy of the gel-to-liquid-crystalline phase transition of aqueous dispersions of DEPE (A) and DPPC (B) upon incorporation of gramicidin (●———●), desformylgramicidin (×———×), *N*-succinylgramicidin (△———△) and *O*-succinylgramicidin (▲———▲) as a function of the peptide content.

Effect of the gramicidin analogues on the thermodynamic properties of DEPE and DPPC

To gain insight into the bilayer solubility of the various gramicidin analogues, we monitored their effect on the thermodynamic properties of DPPC and DEPE dispersions. Fig. 8A shows the effect of peptide incorporation on the energy content of the gel-to-liquid-crystalline phase transition of DEPE. As can be seen from this figure, all analogues behave in a manner similar to gramicidin [11] and do not show any significant effect on the transition enthalpy. In addition, no effects were observed on the peak width of the transition enthalpy or on the transition temperature.

In contrast, upon incorporation in DPPC, the analogues, like gramicidin [6,11], cause a decrease of the enthalpy of the gel-to-liquid-crystalline phase transition (Fig. 8B), until a limiting value is observed at high concentrations of the peptide. The analogues broaden the range of the transition, but do not affect the position of the peak maximum, as was also found for gramicidin [6,11].

Discussion

Thus far, the ability of gramicidin to induce H_{II} phase formation in PC systems can be considered to be the most intriguing aspect of the lipid-structure-modulating activity of this peptide. The present study not only shows tht gramicidin can induce H_{II} phase formation in other bilayer-preferring lipid systems as well, but it also demonstrates that the driving force underlying this effect must be extremely strong, for the data clearly show that even the presence of a net charge, either at the lipid headgroup or at the peptide itself, does not interfere with H_{II} phase formation.

Let us first discuss the results obtained with negatively charged lipids. In general, interheadgroup repulsive forces make an organization int he hexagonal H_{II} phase for this class of lipids highly unfavorable. However, many examples are known of dispersions of negatively charged lipids in single or in mixed lipid systems, in which bilayer-to-hexagonal-H_{II} transition can be triggered by neutralization of the charge, for instance by protonation at low pH [42], by increasing the salt concentration [43], or by the use of divalent cations [44–46]. To understand the ability

of gramicidin to induce H_{II} phase formation in negatively charged lipids without any charge compensation, two possibilities arise.

First, it has been suggested that in the H_{II} phase, gramicidin is alligned parallel to the lipid acyl chains [10], from which orientation it follows that gramicidin spaces the lipid headgroups, leading to a reduction of the electrostatic repulsive forces in these systems. The probability of this orientation was confirmed in PE systems by the way in which gramicidin affects the order and motion of the lipid polar headgroups [11] and in PC systems by the observation that a fast exchange occurs between water molecules present in the gramicidin channel and those present in the aqueous tubes of the H_{II} phase [16].

A second possibility is that dehydration of the lipid headgroups by gramicidin plays a role. In PE systems it was shown by Seddon et al. [47] that a decreasing water content promotes H_{II} phase formation. However, it was also found that protonation of the PE headgroup facilitates H_{II} phase formation, in spite of the concomitant interheadgroup repulsive forces. Therefore it was suggested that upon protonation dehydration occurs, and that it is the dehydration step which dominates the resultant effect. In our opinion it is very well possible that similar opposite effects occur upon interaction of gramicidin with negatively charged lipids, whereby the dehydrating effect dominates over the increase in interheadgroup repulsion upon H_{II} phase formation. In PC systems it has been shown that at low water content the peptide is indeed preferentially hydrated over the lipid polar headgroup [16]. The related phenomenon that PC lipid films swell less readily upon hydration and are more difficult to disperse when gramicidin is incorporated was also observed in mixtures with cardiolipin, DOPS and DOPG.

Besides the possibility of dehydration as a driving force for the gramicidin-induced H_{II} phase formation, a number of other factors have been suggested to be of importance. Since all these must be related to specific features of the gramicidin molecule, a logical approach to obtain more insight into the mechanism of the gramicidin-induced H_{II} phase formation on a molecular level is by way of chemical modification of the peptide. That this is a promising approach was indicated by the recent observation that formylation of the tryptophan residues completely and reversibly blocks H_{II} phase formation in DOPC systems [17]. However, the present study clearly shows that neither N- or C-terminal modification of gramicidin results in a significant change of the lipid-structure-modulating activity of the peptide. In order to try to interpret these results, we shall discuss the present data in the light of the various factors which have been suggested to play a role in gramicidin-induced H_{II} phase formation.

First, it has been proposed that, due to the presence of four bulky tryptophan residues, all located in its C-terminal part, the gramicidin molecule has a cone shape. In analogy to the shape-structure relationship as postulated for pure lipid dispersions [48], such a cone shape could account for the lipid-structure-modulating activity of gramicidin if the molecule is oriented with the smaller end of the cone towards the lipid/water interface. This orientation is opposite to the reported channel conformation of the peptide [3,4]. If we assume, that the H_{II}-phase-inducing activity of gramicidin in PC and PE and its bilayer stabilization in lyso-PC can be explained by the cone shape of the gramicidin molecule, then it follows that in these lipid systems for all charged derivatives the N-terminal part is oriented towards the lipid/water interface. However, in the case of *O*-succinylgramicidin, we consider it more likely that the molecule orients itself with its negatively charged C-terminal at the lipid/water interface. Such an orientation would imply that the geometrical shape of the gramicidin molecule cannot be responsible for its lipid-structure-modulating activity.

Although the possible role of hydration as the driving force of gramicidin-induced H_{II} phase formation has already been discussed, we should like to remark that, as with gramicidin, for the charged analogues it could be observed that, upon incorporation in DOPC systems, dispersion of the lipids was more difficult and swelling seemed to occur less readily. This observation gave rise to the suggestion that no alteration in the hydration properties had occurred.

Only recently it was suggested that aggregation of gramicidin molecules is important for H_{II} phase formation [11,16]. One indication for such a mech-

anism was obtained in a study on the hydration properties of DOPC/gramicidin mixtures [16] which showed that, at low water content, gramicidin in a hydrated conformation can induce phase separation and formation of gramicidin-rich domains which, upon further hydration, convert into the hexagonal H_{II} phase. Another, more direct, indication for the tendency of gramicidin to aggregate was obtained by DSC measurements on gramicidin/lipid mixtures [11]. In PE systems it was found that incorporation of gramicidin does not affect the energy content of the gel-to-liquid-crystalline phase transition of the lipid, suggesting aggregation of the peptide in the gel phase. Upon melting of the chains it was shown that part of the lipids adopt the H_{II} phase, whereafter the presence of gramicidin could no longer be detected in the bilayer component. In PC systems, DSC measurements showed that incorporation of small amounts of gramicidin results in a decrease of the enthalpy of the gel-to-liquid-crystalline phase transition. At high peptide concentrations, however (gramicidin/lipid > 1 : 20, m/m), no further decrease was observed, which results were interpreted as being indicative of the onset of an aggregation process [6,11]. The similarity in effect of incorporation of gramicidin and its analogues on the gel-to-liquid-crystalline phase transition of PE and PC systems observed in this study suggest that the aggregation behavior of the various gramicidin analogues in these systems is comparable, consistent with the similar H_{II}-phase-promoting effect of these peptides in DOPC.

The present study thus indicates that the geometrical shape of the gramicidin molecule is insufficient to explain all aspects of the lipid-modulating activity of gramicidin. We consider it most likely that, at least for H_{II} phase formation in the bilayer type of lipid systems, other properties of the molecule, such as its dehydrating effect on the lipid polar headgroups [16], its ability to induce a disordering of the lipid acyl chain packing at high peptide concentrations [5,49] and its tendency to self-associate [11,16], are all essential factors. With respect to this latter property, it should be noted that tryptophan-tryptophan stacking interactions could be an additional important or even essential feature. It has been proposed by Spisni et al. [50] that such interactions may be responsible for the formation of regularly ordered gramicidin aggregates in bilayers with lyso-PC. In view of the dramatic effect of modification of the tryptophans of gramicidin on its influence on lipid phase behavior [17], further studies on gramicidin-lipid interactions should be carried out with the use of analogues in which the tryptophan residues are more gently modified. This is likely to be a fruitful approach to obtain a more detailed understanding of the modulating effect of gramicidin on lipid structure.

Acknowledgements

We would like to thank Arie J. Verkleij and Jose Leunissen-Bijvelt for performing the freeze-fracture electronmicroscopy experiments. The NMR measurements on the Bruker WM200 were carried out at the national NMR facility at the University of Nijmegen, supported by the Netherlands Foundation for Chemical Research (SON) with the financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

1 Sarges, R. and Witkop, B. (1985) J. Am. Chem. Soc. 87, 2011–2020
2 Anderson, O.S. (1984) Annu. Rev. Physiol. 46, 531–548
3 Urry, D.W., Goodall, M.C., Glickson, J.D. and Mayers, D.F. (1971) Proc. Natl. Acad. Sci. USA 68, 1907–1911
4 Urry, D.W., Trapane, T.L. and Prasad, W.U. (1983) Science 221, 1064–1067
5 Rice, D. and Oldfield, E. (1979) Biochemistry 18, 3271–3279
6 Chapman, D., Cornell, B.A., Eliasz, A.W. and Perry, A. (1977) J. Mol. Biol. 113, 517–538
7 Pasquali-Ronchetti, I., Spisni, A., Casali, E., Masotti, L. and Urry, D.W. (1983) Biosci. Rep. 3, 127–133
8 Killian, J.A., De Kruijff, B., Van Echteld, C.J.A., Verkleij, A.J., Leunissen-Bijvelt, J. and De Gier, J. (1983) Biochim. Biophys. Acta 738, 141–144
9 Killian, J.A., Borle, F., De Kruijff, B. and Seelig, J. (1986) Biochim. Biophys. Acta 854, 133–142
10 Van Echteld, C.J.A., Van Stigt, R., De Kruijff, B., Leunissen-Bijvelt, J., Verkleij, A.J. and De Gier, J. (1981) Biochim. Biophys. Acta 648, 287–291
11 Killian, J.A. and De Kruijff, B. (1985) Biochemistry 24, 7881–7890
12 Van Echteld, C.J.A., De Kruijff, B., Verkleij, A.J., Leunissen-Bijvelt, J. and De Gier, J. (1982) BBA 592, 126–138
13 Lambers, J.W.J., Verkleij, A.J. and Terpstra, W. (1984) Biochim. Biophys. Acta 786, 1–9

14 Taraschi, T.F., De Kruijff, B., Verkleij, A.J. and Van Echteld, C.J.A. (1982) Biochim. Biophys. Acta 685, 153–161
15 Taraschi, T.F., Van der Steen, A.T.M., De Kruijff, B., Tellier, C. and Verkleij, A.J. (1982) Biochemistry 21, 5756–5764
16 Killian, J.A. and De Kruijff, B. (1985) Biochemistry 24, 7890–7898
17 Killian, J.A., Timmermans, J.W., Keur, S. and De Kruijff, B. (1985) Biochim. Biophys. Acta 820, 154–156
18 Van Deenen, L.L.M. and De Haas, G.H. (1964) Adv. Lipid. Res. 2, 167–234
19 Confurius, P. and Zwaal, R.F.A. (1979) Biochim. Biophys. Acta 488, 36–42
20 Smaal, E.B., Romijn, D., Geurts and Kessel, W.S.M., De Kruijff, B. and De Gier, J. (1985) J. Lipid Res. 26, 634–637
21 Geurts van Kessel, W.S.M., Tieman, M. and Demel, R.A. (1981) Lipids 16, 58–63
22 Veatch, W.R., Fossel, E.T. and Blout, E.R. (1974) Biochemistry 13, 5249–5256
23 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
24 Bamberg, E., Alpes, H., Apell, H.-J., Bradley, R., Harper, B., Quelle, M.-J. and Urry, D.W. (1979) J. Membrane Biol. 50, 257–270
25 Apell, H.-J., Bamberg, E., Alpes, H. and Langer, P. (1977) J. Membrane Biol. 31, 171–188
26 Cullis, P.R., De Kruijff, B., Hope, M.J., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, T.D. and Bally, M.B. (1982) in Membrane Fluidity, Vol. 2, (Aloia, R.C., ed.), pp. 40–79, Academic Press, New York
27 De Kruijff, B. and Cullis, P.R. (1980) Biochim. Biophys. Acta 602, 477–490
28 Nicolay, K., Van der Neut, R., Fok, J.J. and De Kruijff, B. (1985) Biochim. Biophys. Acta 819, 55–65
29 De Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope, M.J., Van Echteld, C.J.A. and Taraschi, T.F. (1985) in The Enzymes of Biological Membranes (Martonosi, A.N., ed.), pp. 131–204, Plenum Press, New York
30 Tilcock, C.P.S. and Cullis, P.R. (1980) Biochim. Biophys. Acta 641, 189–201
31 Farren, S.B. and Cullis, P.R. (1980) Biochem. Biophys. Res. Commun. 97, 182–191
32 Luzzati, V. (1980) in Biological Membranes (Chapman, D., ed.), pp. 71–123, Academic Press, New York
33 Hauser, H. (1984) Biochim. Biophys. Acta 772, 37–50
34 Verkleij, A.J. (1984) Biochim. Biophys. Acta 179, 43–63
35 Fossel, E.T., Veatch, W.R., Ovchinnikov, Y.A. and Blout, E.R. (1974) Biochemistry 13, 5266–5275
36 Gratwohl, C. and Wüthrich, K. (1974) J. Magn. Res. 13, 217
37 Horsley, W., Sternlicht, H. and Cohen, J.S. (1970) J. Am. Chem. Soc. 92, 680
38 Killian, J.A., Verkleij, A.J., Leunissen-Bijvelt, J. and De Kruijff, B. (1985) Biochim. Biophys. Acta 812, 21–26
39 Van Echteld, C.J.A., De Kruijff, B., Mandersloot, J.G. and De Gier, J. (1981) Biochim. Biophys. Acta 649, 211–220
40 Allegrini, P.R., Van Scharrenburg, G., De Haas, G.H. and Seelig, J. (1983) Biochim. Biophys. Acta 731, 448–455
41 Wu, W., Stephenson, F.A., Mason, J.T. and Huang, C. (1984) Lipids 19, 68–71
42 Hope, M.J. and Cullis, P.R. (1980) Biochem. Biophys. Res. Commun. 92, 846–852
43 Seddon, J.M., Kaye, R.D. and Marsh, D. (1983) Biochim. Biophys. Acta 734, 347–352
44 Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta 255, 484–492
45 De Kruijff, B., Verkleij, A.J., Leunissen-Bijvelt, J., Van Echteld, C.J.A., Hille, J. and Rijnbout, H. (1982) Biochim. Biophys. Acta 693, 1–12
46 Farren, S.B. and Cullis, P.R. (1980) Biochem. Biophys. Res. Commun. 97, 182–191
47 Seddon, J.M., Cevc, G., Kaye, M.D. and Marsh, D. (1984) Biochemistry 23, 2634–2644
48 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
49 Lee, D.C., Durrani, A.A. and Chapman, D. (1984) Biochim. Biophys. Acta 769, 49–56
50 Spisni, A., Pasquali-Ronchetti, I., Casali, E., Lindner, L., Cavatorta, P., Masotti, L. and Urry, D.W. (1983) Biochim. Biophys. Acta 732, 58–68