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A mismatch between the length of gramicidin and the lipid acyl chains is a prerequisite for H_{II} phase formation in phosphatidylcholine model membranes

J. Antoinette Killian [1], Kari U. Prasad [2], Dan W. Urry [2] and Ben de Kruijff [3]

[1] *Centre for Biomembranes and Lipid Enzymology and* [3] *Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht (The Netherlands) and* [2] *Laboratory of Molecular Biophysics, School of Medicine, University of Alabama at Birmingham, Birmingham, AL (U.S.A.)*

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Previously it was shown that gramicidin can induce H_{II} phase formation in diacylphosphatidylcholine model membranes only when the lipid acyl chain length exceeds 16 carbon atoms (Van Echteld, C.J.A., De Kruijff, B., Verkleij, A.J., Leunissen-Bijvelt, J. and De Gier, J. (1982) Biochim. Biophys. Acta 692, 126–138). Using ^{31}P-NMR and small angle X-ray diffraction we now demonstrate that upon increasing the length of gramicidin, the peptide loses its ability to induce H_{II} phase formation in di-C18:1$_c$-PC but not in the longer chained di-C22:1$_c$-PC. It is concluded that a mismatch in length between gramicidin and the lipid acyl chains, when the latter would provide excess bilayer thickness, is a prerequisite for H_{II} phase formation in phosphatidylcholine model membranes.

Gramicidin is a peptide antibiotic, produced by *Bacillus brevis* strain ATCC 8185 [1] as a mixture of gramicidins A, B and C [2–4]. The main component, gramicidin A, has the following sequence: HCO-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-$NHCH_2CH_2OH$. In biological as well as in model membranes this very hydrophobic peptide can form cation specific transmembrane channel (for reviews, see Refs. 5 and 6) by hydrogen-bonding of the N-terminal parts of two gramicidin molecules, present in the single-stranded $\beta^{6.3}$ helical configuration [7]. In addition to its channel properties gramicidin also has a dramatic effect on the macroscopic organization of membrane lipids (for reviews, see Refs. 8 and 9). In particular the ability of gramicidin to induce a transition from a bilayer organization of lipids to hexagonal H_{II} phase, as observed in a variety of model membranes [8,10,11] and even in erythrocyte membranes [12] is intriguing, in view of the possible involvement of non-bilayer structures in specific aspects of membrane functioning [13].

Several investigations have been carried out to achieve insight into the molecular mechanism of gramicidin-induced H_{II} phase formation and a detailed mechanistic model was proposed [14], in which one important parameter is the lipid acyl chain length. In earlier studies [10] it was found that gramicidin can induce H_{II} phase formation in phosphatidylcholine (PC) model membranes only when the lipid acyl chains contain more than 16 carbon atoms, in which case the length of the lipid bilayer exceeds the length of the single-stranded, head-to-head dimerized gramicidin transmembrane channel. Therefore it was proposed that for induction of H_{II} phase formation by gramicidin a mismatch in length is required between the peptide and the lipid acyl chain [10]. However, alternatively it is possible that a minimum lipid chain length of 18 carbon atoms is required for H_{II} phase formation in this system. Based on geometric considerations [15] and as experimentally supported by studies in phosphatidylethanolamines [16] it can be argued that longer chained lipids fit better into an H_{II} phase.

In order to obtain further insight into the nature of the lipid acyl chain length dependence of gramicidin-induced H_{II} phase formation, analogs of gramicidin with

Abbreviations: PC, phosphatidylcholine; csa, chemical shift anisotropy; NMR, nuclear magnetic resonance.

Correspondence: J.A. Killian, Centre for Biomembranes and Lipid Enzymology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

an increased chain-length were used and their H_{II} phase inducing activity was measured in di-C18 : 1_c-PC and in the longer chained di-C22 : 1_c-PC. Analogs were used with a D-Leu-L-Ala or D-Leu-L-Ala-D-Leu-L-Ala sequence inserted between L-Ala[5] and D-Val[6] of gramicidin, which analogs are denoted con-D-Leu[5a]-L-Ala[5b]-gramicidin and con-D-Leu[5a]-L-Ala[5b]-D-Leu[5c]-L-Ala[5d]-gramicidin, respectively. These analogs in particular are useful because the insertion does not interfere directly with the N-terminal part of the molecule, which is involved in hydrogen bonding of the dimer, nor with the tryptophan containing C-terminal part which has been shown to be of importance for H_{II} phase induction [9,17] as well as for channel formation [18,19]. Furthermore, by choosing the D-Leu-L-Ala sequence and inserting it between residues five and six, the interaction between side chains, when gramicidin is in the $\beta^{6.3}$ helical configuration, is maximally retained. Both length-analogs have been successfully employed to investigate in more detail the channel characteristics of gramicidin [20]. To get further insight into the role of the tryptophan-containing C-terminal part also a con-D-Leu[16]-L-Trp[17] analog was tested, in which a D-Leu-L-Trp sequence was added to the C-terminal Trp[15] of gramicidin.

Di-C18 : 1_c-PC and di-C22 : 1_c-PC were prepared and purified as described before [21,22]. For comparative purposes a synthetically prepared gramicidin ([13]C-L-Ala[5]-gramicidin A (Ref. 23)) was used as a control, instead of the natural mixture of gramicidins. Gramicidin A length analogs were synthesized [20] as will be described more extensively elsewhere (Prasad, K.U. and Urry, D.W., in preparation). Peptide/lipid dispersions were prepared by first codissolving 50 μmoles of lipid and the appropriate amount of gramicidin (analog) in a small volume of chloroform/methanol (2 : 1, by vol.). Dry, homogeneous films of these samples were obtained in pyrex tubes (length 5 cm, outer diameter 0.8 cm) by evaporation of the solvent under reduced pressure, while slowly rotating the sample. After overnight storage under high vacuum, the samples were hydrated in a volume of water equal to twice the dry sample weight. Samples were equilibrated for 2 h at 40 °C, after which they were centrifuged at 5000 × g at room temperature for 15 min. For the ^{31}P-NMR measurements, carried out on a Bruker MSL 300 spectrometer as described [24], the pyrex tubes were positioned in the magnet such that the pellet was centered in the receiver coil. Small angle X-ray diffraction measurements of the pelleted samples were carried out as described in Ref. 11. All measurements were performed at 25 °C.

Fig. 1 shows the ^{31}P-NMR spectra of dispersions of di-C18 : 1_c-PC in the absence and presence of gramicidin and different length analogs in a 1 : 10 molar ratio of peptide to lipid, which ratio was chosen for comparative purposes [10,11]. For the pure lipid dispersion a

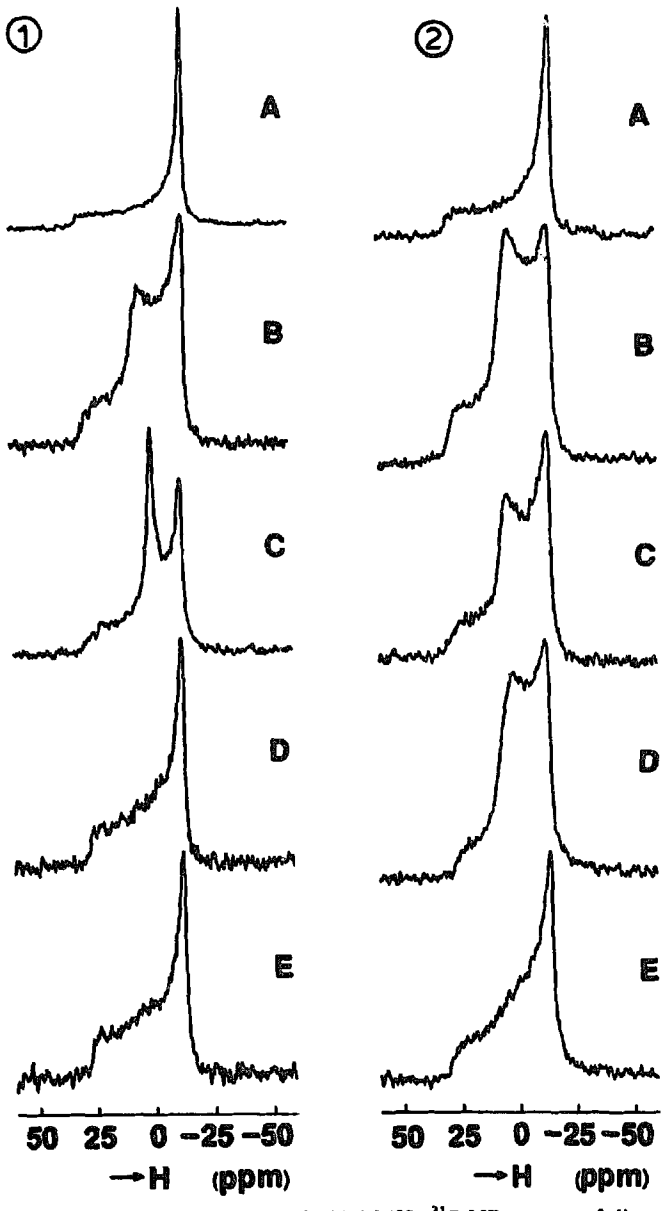

Fig. 1. Proton-noise decoupled 121.4 MHz ^{31}P-NR spectra of dispersions of di-C18 : 1_c-PC (A) and mixtures of di-C18 : 1_c-PC with gramicidin (B), con-D-Leu[5a]-L-Ala[5b]-gramicidin (C), con-D-Leu[5a]-L-Ala[5b]-D-Leu[5c] – L-Ala[5d]-gramicidin (D), and con-D-Leu[16]-L-Trp[17]-gramicidin (E) in a molar ratio of peptide to lipid of 1 : 10.

Fig. 2. Proton-noise decoupled 121.4 MHz ^{31}P-NMR spectra of dispersions of di-C22 : 1_c-PC (A) and mixtures of di-C22 : 2_c-PC with gramicidin (B), con-D-Leu[5a]-L-Ala[5b]-gramicidin (C), con-D-Leu[5a]-L-Ala[5b]-D-Leu[5c]-L-Ala[5d]-gramicidin (D), and con-D-Leu[16]-L-Trp[17]-gramicidin (E) in a molar ratio of peptide to lipid of 1 : 10.

spectrum is obtained with a low-field shoulder and a high-field peak, indicative of a bilayer organization of lipids in the liquid-crystalline phase [25,26]. The chemical shift anisotropy (csa), measured as the distance between the low-field shoulder and high-field peak, is about −45 ppm, in agreement with previous observations [11]. Upon incorporation of the synthetic gramicidin A, like for gramicidin A isolated from the natural mixture of gramicidins [17], a second spectral

component appears (Fig. 1B) with a low-field peak at about 5 ppm, which is characteristic for the presence of lipids in a hexagonal H_{II} phase. This second spectral component is not observed in dispersions of di-C18 : 1_c-PC with any of the gramicidin length analogs. ^{31}P-NMR spectra of mixtures of di-C18 : 1_c-PC with con-D-Leu^{5a}-L-Ala^{5b}-D-Leu^{5c}-L-Ala^{5d}-gramicidin and con-D-Leu^{16}-L-Trp^{17}-gramicidin exhibit a pure bilayer type of spectrum (Figs. 1D and 1E), whereas in the sample with con-D-Leu^{5a}-L-Ala^{5b}-gramicidin in addition an isotropic signal is present (Fig. 1C). In all these peptide-containing samples a decrease of the csa of the bilayer component is observed of about 15%, demonstrating the incorporation of the peptides.

Further structural information on the various samples was obtained with small angle X-ray diffraction. In the di-C18 : 1_c-PC dispersion in the absence of peptide, peak spacings are found in a ratio of 1 : 1/2 (Table IA), characteristic of the long range order as found in multilamellar structures. From the value of the first order (1,0,0) spacing, and assuming a thickness of the water layer including the lipid headgroup, of 38.5 Å [27], a thickness of the lipid layer can be calculated of about 29.4 Å, in close agreement with values obtained by direct methods [28]. Upon incorporation of gramicidin an additional reflection is observed, at a distance which relates as $1/\sqrt{3}$ to the first order (1,0,0) reflection (Table IA), indicating that part of the lipids is now organized in a hexagonal H_{II} phase. The first-order spacings in the H_{II} phase and in the bilayer in the dispersion of gramicidin and di-C18 : 1_c-PC are similar, in agreement with earlier data obtained on gramicidin A from the natural mixture of gramicidins [17]. The H_{II} phase specific (1,1,0) reflection is not observed upon incorporation of any of the gramicidin analogs (Table IA), for which a rather similar diffraction pattern is obtained as for the pure lipid dispersion. The first-order spacings appear not to be affected by the length of the analog used (Table IA). For all samples the structural information provided by the X-ray diffraction data is in full agreement with the interpretation of the ^{31}P-NMR measurements. However, the data do not give any information on the origin of the isotropic ^{31}P-NMR signal as observed in the mixture of the con-D-Leu^{5a}-L-Ala^{5b}-analog with di-C18 : 1_c-PC, which may arise from small particles, from a cubic phase, or from other structures that are possible intermediates in H_{II} phase formation. In this context it is interesting to note that the spectral characteristics of this sample are similar to those observed for a mixture of gramicidin with di-C16 : 1_c-PC [10], in which the length of the peptide [20] and of the lipid [28] are both expected to be about 3 Å shorter. This suggests that the nature of the mismatch between the length of the gramicidin dimer and the bilayer thickness may play a role in the formation of structures that give rise to the appearance of an isotropic ^{31}P-NMR signal and that possibly in both samples the mismatch is large enough to destabilize the bilayer, but not to induce H_{II} phase formation.

For dispersions of gramicidin(analogs) with di-C22 : 1_c-PC a completely different picture is obtained. ^{31}P-NMR measurements on the pure lipid dispersion again show a bilayer type of spectrum, with a csa of about −45 ppm (Fig. 2A), whereas incorporation of gramicidin as well as the con-D-Leu^{5a}-L-Ala^{5b} and con-D-Leu^{5a}-L-Ala^{5b}-D-Leu^{5c}-L-Ala^{5d} analogs now leads to ^{31}P-NMR spectra with a second spectral component, characteristic for the presence of lipids organized in the H_{II} phase (Figs. 2B–2D). Incorporation of the con-D-Leu^{16}-L-Trp^{17} analog results in a ^{31}P-NMR spectrum with a peculiar 'bilayer' type of lineshape (Fig. 2E), with a broad linewidth at half height. Such a lineshape is rather similar to that obtained previously for mixtures of gramicidin and di-C18 : 1_c-PC at low temperatures, in which small angle X-ray diffraction measurements demonstrated the presence of a hexagonal H_{II} phase [29]. In these latter samples the apparent loss of the lineshape characteristic for an H_{II} organization of lipids was attributed to a decrease of the rate of diffusion of the lipids about the gramicidin-rich tubes at lower temperatures. Like in the di-C18 : 1_c-PC dispersions also in the di-C22 : 1c-PC samples a decrease of the csa of the bilayer component was observed upon incorporation of the peptide.

Small angle X-ray diffraction measurements of di-C22 : 1_c-PC dispersions in the absence of peptide show two spacings, which relate as 1 : 1/2 (Fig. 3A and Table IB). In agreement with earlier observations [28,30] an increase is observed of the value of the first order (1,0,0) spacing of about 7 Å, as compared to the value found

TABLE I

Small angle X-ray diffraction characteristics of dispersions of di-C18: 1_c-PC (A) and di-C22: 1_c-PC (B) with gramicidin (analogs) in a 1:10 molar ratio of peptide to lipid

Sample	Reflections (Å)		
	(1,0,0)	(1,1,0)	(2,0,0)
A			
Di-C18 : 1_c-PC	63.9		31.7
+ gramicidin	64.3	36.9	
+ con-D-Leu^{5a}-L-Ala^{5b}-gramicidin	69.3		34.1
+ con-D-Leu^{5a}-L-Ala^{5b}-D-Leu^{5c}-L-Ala^{5d}-gramicidin	66.1		33.6
+ con-D-Leu^{16}-L-Trp^{17}-gramicidin	66.9		33.6
B			
Di-C22 : 1_c-PC	71.4		35.5
+ gramicidin	74.6	44.4	37.2
+ con-D-Leu^{5a}-L-Ala^{5b}-gramicidin	73.6	41.6	37.3
+ con-D-Leu^{5a}-L-Ala^{5b}-D-Leu^{5c}-L-Ala^{5d}-gramicidin	72.7	41.9	36.0
+ con-D-Leu^{16}-L-Trp^{17}-gramicidin	73.2	43.1	36.1

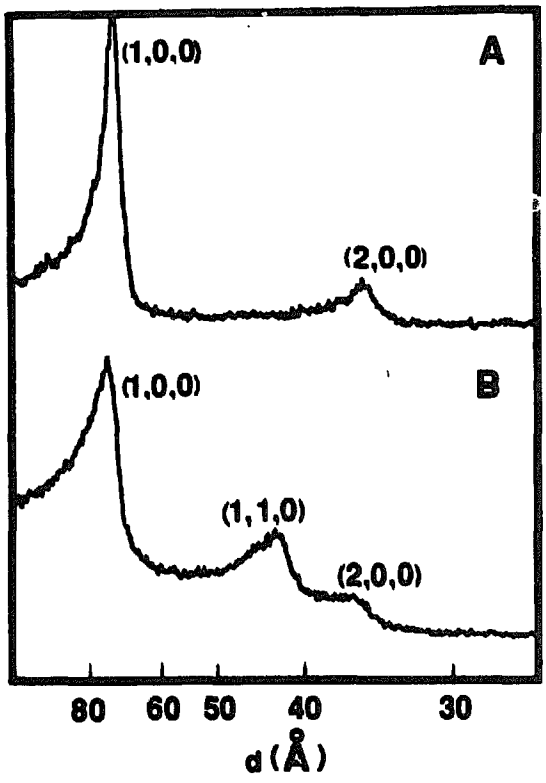

Fig. 3. Small angle X-ray diffraction patterns of dispersions of di-C22 : 1_c-PC (A) and a mixture of con-D-Leu[16]-L-Trp[17]-gramicidin with di-C22 : 1_c-PC in a 1 : 10 molar ratio (B).

for di-C18 : 1_c-PC (Table IA), which is attributed to an increase of the thickness of the lipid layer. In contrast to the situation in di-C18 : 1_c-PC, the H_{II} phase specific (1,1,0) reflection is now observed not only upon incorporation of gramicidin, but also upon incorporation of its length analogs. The ^{31}P-NMR and small angle X-ray diffraction data did not reveal large differences in the extent of H_{II} phase formation in the various samples. As for di-C18 : 1_c-PC also for di-C22 : 1_c-PC the first-order reflections in the bilayer and the hexagonal H_{II} phase appear to be similar and again the values of the first-order spacings are not influenced by the length of the analog used. Since apparently in the mixture of di-C22 : 1_c-PC with con-D-Leu[16]-L-Trp[17]-gramicidin an H_{II} phase is present (Fig. 2E and Table IB), it is likely that also in this sample a loss of the H_{II} phase characteristic ^{31}P-NMR lineshape has occurred, due to a limited rate of diffusion of the phospholipids around the tubes in the H_{II} phase.

The results clearly indicate that a mismatch between the length of the gramicidin dimer (26 Å; Ref. 31) and the lipid bilayer is necessary for H_{II} phase induction in phosphatidylcholine model membranes. Upon increasing the length of a gramicidin molecule to 29 Å or 32 Å upon insertion of a dipeptide or a tetrapeptide respectively [20], H_{II} phase formation is completely blocked in di-C18 : 1_c-PC (lipid bilayer thickness: 27 Å, Ref. 28; about 29.4 Å, this study), but it is restored again upon increasing the lipid chain length in di-C22 : 1_c-PC (lipid bilayer thickness 34 Å, Ref. 28; about 36.9 Å, this study). This rules out the possibility that the requirement of chain lengths more than 16 carbon atoms for gramicidin induced H_{II} phase formation is due only to the ability of the longer chained lipids to fit better into an H_{II} phase.

For the apparent role of the mismatch several possibilities exist. First, the lipids could react to the smaller length of the peptide either by 'dimpling' of the lipids adjacent to gramicidin, or by bending of the headgroups over the channel, both of which are likely to result in a disordering of headgroups and acyl chains and might thereby facilitate H_{II} phase formation. However, a mismatch between a hydrophobic peptide and the thickness of the hydrophobic part of the bilayer in itself is not enough for H_{II} phase induction as illustrated for example by the observation that no H_{II} phase formation occurs in di-C18 : 1_c-PC upon interaction with the hydrophobic, channel-forming, α-helical pentadecapeptide P15 [32], which peptide consists of alanine and aminoisobutyric acid and has a length of only 22.5 Å [33].

Another possibility to explain the role of the mismatch is that the peptide adjusts itself to match the longer lipid length, for instance by dissociation of the hydrogen-bonded N-N dimers, upon which H_{II} phase formation could occur according to the scheme as proposed in Ref. 14. In this model it was suggested that lateral self-association of gramicidin monomers, involving intermolecular tryptophan-tryptophan stacking interactions, plays an important role in gramicidin-induced H_{II} phase formation. The observation that in the H_{II} phase of the con-D-Leu[16]-L-Trp[17] analog with di-C22 : 1_c-PC the lateral diffusion of the lipids around the cylinders of the H_{II} phase appears to be restricted, suggests that indeed such interactions may be involved, for example by formation of specific aggregates, which, due to the presence of one extra tryptophan residue in this sample, are larger or have a stronger intermolecular interaction, thereby hampering rotational diffusion of the lipids about the tubes of the H_{II} phase.

Finally for the role of a mismatch also the possibility should be considered that in the H_{II} phase itself a difference in length between gramicidin and the lipid acyl chains may be required. It is very well possible that organization in the H_{II} phase is more favourable if the lipids are longer than the gramicidin monomers and therefore better able to overcome the constraints [15] of a hexagonal packing of these gramicidin-rich tubes. Several hydrophobic molecules are known that may fill up the volumes between adjacent tubes and promote H_{II} phase formation by relieving the hydrocarbon packing constraints [15,34–36]. However, the results of the present study exclude the possibility that the H_{II} phase inducing activity of gramicidin is simply a result of partitioning of the peptide in the hydrophobic phase,

similar to the proposed mechanism for induction of H_{II} phase formation by *n*-dodecane, as was recently suggested [36].

The results clearly demonstrate that a mismatch in length between gramicidin and lipid is necessary for H_{II} phase formation. This observation is in line with an orientation of gramicidin with its long axis parallel to the acyl chains but it is difficult to reconcile with a localization of the peptide in the hydrophobic interior of the H_{II} phase at the intersection of adjacent tubes.

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