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The influence of acylation on the lipid structure modulating properties of the transmembrane polypeptide gramicidin

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Abstract

In order to get insight into the effect of acylation of a transmembrane polypeptide on the interaction of the polypeptide with the membrane lipids we used ^{31}P-NMR to investigate the influence of acylated gramicidins on the polymorphic phase behavior of hydrated dispersions of 1-palmitoyllysophosphatidylcholine (lyso-PC), 1,2-dioleoylphosphatidylcholine (DOPC) and 1,2-dielaidoylphosphatidylethanolamine (DEPE). Palmitoylgramicidin induces a micelle to extended bilayer organization in lyso-PC with a slightly lower efficiency than the parent gramicidin molecule. In DOPC and DEPE acylgramicidins induce the formation of H_{II} phase at the expense of a bilayer organization with a similar high efficiency as gramicidin. The ability of acylgramicidin to induce lipid mixing between vesicles prepared of DOPC was decreased relative to gramicidin. The results are discussed in the light of the proposed models for gramicidin-induced H_{II} phase formation and emphasize that gramicidin itself has a very strong lipid structure modulating activity.

Key words: Acylgramicidin; Acylprotein; Gramicidin; Lipid polymorphism

1. Introduction

Many transmembrane proteins are found to be palmitoylated [1]. In general the site of palmitoylation is confined to a region of the polypeptide that is already very hydrophobic. Very little is known about the structural and functional consequences of palmitoylation. However, palmitoylation was found to be important for many biological processes [2].

To get more insight in the structural and functional consequences of protein fatty acylation we use the polypeptide antibiotic gramicidin as a model for the transmembrane segment of a membrane spanning protein. The very hydrophobic pentadecapeptide gramicidin is well known for its ability to form channels thereby spanning the bilayer in an N- to N-dimeric helical $\beta^{6.3}$ conformation [3]. Recently an analog of gramicidin was purified from the commercially available mixture of gramicidins with a fatty acid covalently coupled to the C-terminal ethanolamine group. This analog was called gramicidin K [4]. The C-terminal ethanolamine of gramicidin offers an acylation site close to the membrane interface comparable to that of palmitoylated proteins. This site can be used for the synthesis of acylgramicidins [5].

As a transmembrane polypeptide gramicidin experiences strong lipid–protein interactions. For instance, gramicidin has a strong influence on the polymorphic phase behavior of different lipids (for review, see [6]). Suspensions of lyso-PC (a type I-lipid) form micellar structures with a convex surface curvature. It was shown that gramicidin induces the formation of extended lamellar structures in such systems [7]. In mixtures with lipids that have a preference to form structures with a concave surface curvature (type II-lipids), like phosphatidylethanolamines, gramicidin enhances the formation of these structures [8]. Most strikingly gramicidin also strongly influences the polymorphic behavior of bilayer forming lipids, like diacylphosphatidylcholines, if the length of the acyl chains exceeds or equals 16 carbon atoms [9]. For instance, in dioleoylphosphatidylcholine gramicidin induces the inverted hexagonal or H_{II} phase [10]. Consistent with the hy-

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pothesis that structures with a concave surface curvature are intermediates in vesicle fusion, it was shown that gramicidin promotes fusion between large unilamellar vesicles (LUVs) of DOPC [11].

The strong potency of gramicidin to promote type-II lipid structures, makes gramicidin a good model to study the importance of lipid–protein interactions for membrane lipid organization. Such studies are expected to give insight into the postulated importance of type II-lipid structures for membrane organization and functions [12,13].

In this study we report on the consequences of acylation for the lipid structure modulating effect of gramicidin using ^{31}P-NMR. ^{31}P-NMR has been proven [14,15] to be an excellent technique to study the lipid organization in membranes because the line shape of the spectra is directly related to the structural organization of the lipid molecules. We will show that the ability to modulate the lipid organization is not significantly affected upon acylation of gramicidin. This is a surprising result because the lipid structure modulating effect of the peptide is very sensitive to its chemical structure [6]. Moreover, the fatty acid chain must contribute to the hydrocarbon area and therefore theoretically can be expected to influence lipid polymorphism. The implications of these findings for the mechanism of the lipid structure modulating effect of gramicidin are discussed.

2. Materials and methods

Gramicidin A was purified from the commercially available mixture of gramicidins (Sigma, St. Louis, MO) according to Vogt et al. [5]. Acylgramicidins with acyl chains differing in size and unsaturation (lauroyl (12:0), myristoyl (14:0), palmitoyl (16:0), stearoyl (18:0) and oleoyl (18:1$_c$)) were prepared, characterized and handled as described by Vogt et al. [5]. 1-Palmitoyl-*sn*-glycero-3-phosphocholine (lyso-PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) were prepared according to established methods [16,17]. 1-Palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (Pyrene-PC) was obtained from Molecular Probes, Eugene, OR. Lipid concentrations were determined according to Rouser et al. [18]. All other reagents were of analytical grade.

2.1. Preparation of model membrane systems for the studies on lipid polymorphism

A dried film containing 30 μmoles of lipid and the appropriate amounts of acylgramicidin, gramicidin A or as a control situation gramicidin A and equimolar amounts of free fatty acid was dissolved in 400 μl of trifluoroethanol (in the case of lyso-PC) or chloroform/methanol (1:1, v/v) for the other systems. This solution was transferred to NMR sample tubes (inner dimensions 4 × 70 mm). The solvent was carefully removed under reduced pressure. The samples were then stored for at least 15 h under high vacuum. The samples were hydrated with 100 μl of buffer (100 mM NaCl, 0.5 mM NaN_3, 10 mM Tris-HCl, pH 7.0) and stored for at least 3 h at 40°C. The fully hydrated films were centrifuged (3500 × g, 5 min.) to concentrate the samples.

2.2. ^{31}P-NMR measurements

Proton decoupled ^{31}P-NMR spectra were recorded at 121 MHz on a Bruker MSL-300 spectrometer using a high power broadband probe equipped with a solenoidal coil as described before [19]. Generally 1500 free induction decays were accumulated using a 2 μs (42°) pulse, 4K data points, a spectral width of 38.5 kHz and a 1-s repetition time. Prior to Fourier transformation an exponential multiplication was performed resulting in a 25 Hz line broadening. The isotropic peak of a lyso-PC suspension was chosen as the 0 ppm position. The fraction of lipids in a bilayer organization was determined either manually by estimating the line shapes of the components followed by cutting and weighing or computerized by subtracting of a pure bilayer component followed by integration. Both techniques yielded similar results with an estimated error of ±5%.

2.3. Lipid mixing assay

The ability of (acyl)gramicidin to cause lipid mixing was analyzed essentially as described by Tournois et al. [11]. This assay makes use of the fact that the contribution of monomers (M) and eximers (E) to the emission spectrum of pyrene-PC depends on the membrane concentration of this lipid. Large unilamellar donor vesicles (containing 90% DOPC and 10% pyrene-PC) and large unilamellar acceptor vesicles (100% DOPC) in 100 mM NaCl, 0.5 mM NaN_3, 0.2 mM EDTA, 10 mM Tris-HCl, pH 7.0 were prepared by extrusion through 400 nm filters. Donor and acceptor vesicles were added to the cuvet in a ratio of 1:70, respectively, with a total lipid concentration of 0.7 mM in a total volume of 1 ml buffer. Fluorescence emission spectra were recorded on an SLM Aminco SPF 500c spectrofluorometer from 360–550 nm with excitation at 343 nm. Gramicidin and oleoylgramicidin were added from DMSO stock solution. The amount of DMSO never exceeded 5% of the total volume.

The E/M ratio was determined before addition and one minute after addition of (acyl)gramicidin. The E/M

ratio was calculated from the fluorescence at 480 nm (excimer, F_{480}) and 377 nm (monomer, F_{377}) using:

$$E/M = (F_{480} - F_{t480})/F_{377}$$

in which F_{t480} is the fluorescence after addition of 20 μl 10% Triton X-100 to mimic a situation were only a monomeric contribution is expected in the fluorescence spectrum.

3. Results

3.1. Type-I lipids

^{31}P-NMR spectra of a micellar solution of lyso-PC show a characteristic sharp peak caused by rapid isotropic movement of the lipid molecules. The ^{31}P-NMR spectra of mixtures of palmitoylgramicidin and lyso-PC show in addition to the isotropic peak a second spectral component (Fig. 1a). The ^{31}P-NMR spectrum of this component is characterized by a low field shoulder and a high field peak with a residual chemical shift

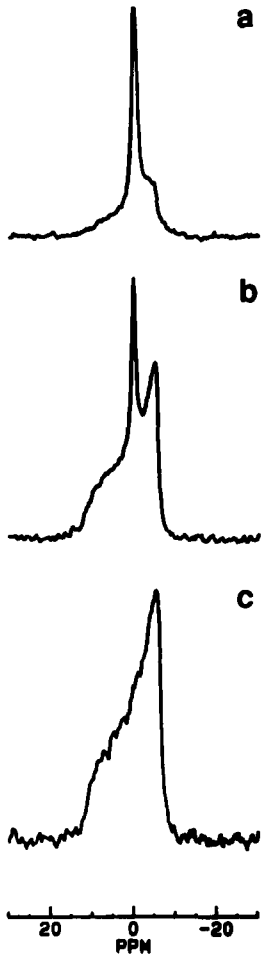

Fig. 1. ^{31}P-NMR spectra of aqueous 16:0 gramicidin/lyso-PC mixtures with a 1:10 (a), 1:7 (b) and a 1:4 (c) molar ratio of peptide to lipid recorded at 40°C.

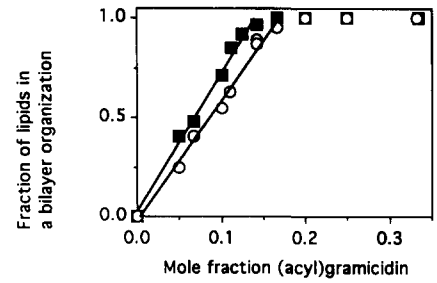

Fig. 2. Influence of the peptide/lipid ratio of aqueous mixtures of gramicidin/lyso-PC (■) and 16:0-gramicidin/lyso-PC (○) on the fraction of lyso-PC molecules in a bilayer organization as determined by ^{31}P-NMR.

anisotropy ($\Delta\sigma$) of 14.4 ppm and is ascribed to the formation of extended bilayers in analogy to previous studies on gramicidin/lyso-PC mixtures [7]. The line shape originates from motional averaging of the chemical shift anisotropy of the P-nucleus due to fast rotation of the lipid molecules around the long axis. The small value of $\Delta\sigma$ relative to diacylphospholipids arises from an increased motional freedom of the phosphate part of the lyso-PC molecule due to an increased motional freedom of the glycerol backbone [20,21]. The fraction of the molecules giving rise to this spectral component increases with the mole fraction of palmitoylgramicidin (Fig. 1b and c). At a molar ratio of 1:4 no isotropic component is present anymore. Fig. 2 quantifies this effect and shows that acylgramicidin is slightly less effective than gramicidin in the formation of extended bilayers. A linear relation was found between the fraction of lyso-PC organized in bilayers and the mole fraction of (acyl)gramicidin for mole fractions up till about 0.15. From this linear relationship it was calculated that one gramicidin molecule converts 7.1 ± 0.1 lyso-PC molecules from a micellar to a bilayer organization, in agreement with previous work [22]. Palmitoylgramicidin induces per molecule a bilayer organization in 6.1 ± 0.1 lyso-PC molecules. ^{31}P-NMR spectra of control samples containing equimolar amounts of free palmitic acid and gramicidin were difficult to interpret, possibly due to the complicating interactions between free palmitic acid and lyso-PC [23].

3.2. Type-II lipids

An aqueous dispersion of DEPE undergoes a transition from a lamellar liquid-crystalline state to a H_{II} phase around 55°C [24]. This is illustrated by the ^{31}P-NMR spectra shown in Fig. 3a–c, which change from a line shape with a high field peak and a low-field shoulder and a $\Delta\sigma$ of 36 ppm at 50°C to a line shape with a reversed asymmetry and a 2-fold reduced $\Delta\sigma$ at 60°C [25]. This is due to the additional averaging of the residual chemical shift anisotropy caused by rapid diffusion of the lipid molecules around the tubes of which

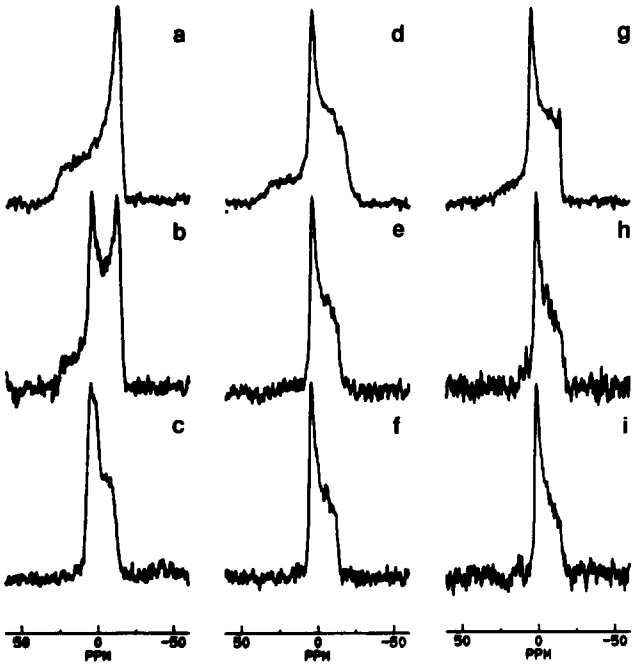

Fig. 3. ^{31}P-NMR spectra of aqueous dispersions of DEPE (a–c) and mixtures of gramicidin/DEPE (d–f) and oleoylgramicidin/DEPE (g–i) in a 1:50 molar ratio recorded at 50°C (a, d, g), 55°C (b, e, h) and 60°C (c, f, i).

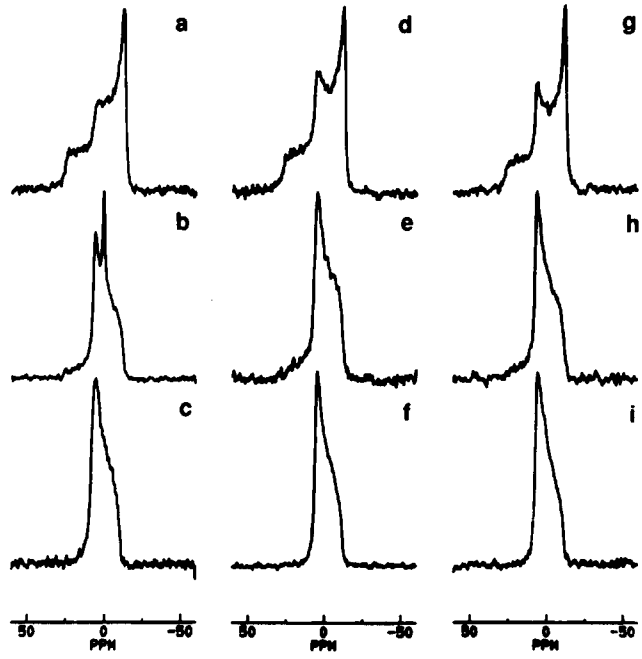

Fig. 5. ^{31}P-NMR spectra of aqueous dispersions of DOPC with gramicidin (a–c), 18:1$_c$-gramicidin (d–f) and gramicidin + equimolar amounts of oleic acid (g–i) with a 1:15 (a, d, g), 1:10 (b, e, h) and a 1:5 (c, f, i) molar ratio of peptide/DOPC recorded at 40°C.

the H_{II} phase consists. At 55°C the ^{31}P-NMR spectra reveal the coexistence of a lamellar and a H_{II} phase. Gramicidin promotes the formation of the H_{II} phase [8] as demonstrated by spectra 3d and 3e, recorded for samples containing one gramicidin per 50 DEPE molecules. Already at 50°C most lipids are present in an H_{II} phase. The ^{31}P-NMR spectra recorded from mixtures of oleoylgramicidin/DEPE (Fig. 3g–i) show a similar efficient promotion of H_{II}-phase. No significant changes in $\Delta\sigma$ in the L_a and H_{II} phase of DEPE were induced by either gramicidin or oleoylgramicidin. Fig. 4 compares the temperature dependency of the fraction of DEPE molecules in a bilayer organization for DEPE and mixtures with gramicidin, oleoylgramicidin and the control situation with non-covalently bonded oleic acid. In all cases ^{31}P-NMR spectra diagnostic for either the L_α, H_{II} phase or combinations of the two were observed. Gramicidin and oleoylgramicidin promote the H_{II} phase with similar efficiency by lowering the onset temperature of the transition which becomes broadened by the peptides. The presence of the free fatty acid in the control samples further promotes H_{II} phase formation. Lauroyl-, myristoyl-, palmitoyl-, and stearoylgramicidin had very similar effects as oleoylgramicidin on the L_α to H_{II} phase transition (data not shown).

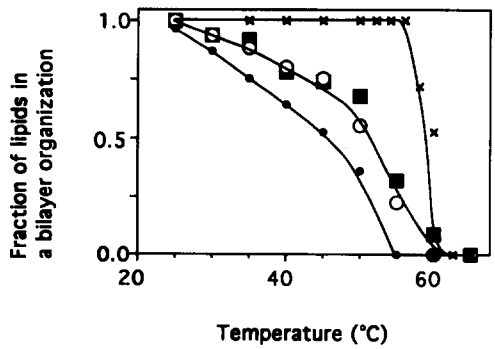

Fig. 4. Temperature dependence of the fraction of lipids organized in bilayers of aqueous dispersions of DEPE (×), mixtures of gramicidin/DEPE (■) (1:25 molar ratio), oleoylgramicidin/DEPE (○) (1:25 molar ratio) and gramicidin + equimolar amounts of oleic acid/DEPE (●) (1:1:25 molar ratio).

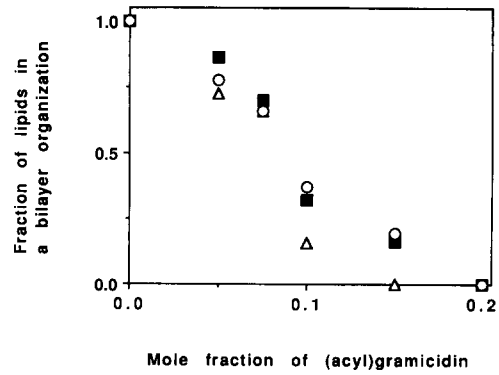

Fig. 6. Fraction of lipids in a bilayer organization in aqueous dispersions of mixtures of DOPC with gramicidin (■), 18:1$_c$-gramicidin (○) and gramicidin + equimolar amounts of oleic acid (△) as determined by ^{31}P-NMR at 40°C.

3.3. Bilayer forming lipids

Gramicidin induces an H_{II}-phase in dispersions of DOPC if the gramicidin/DOPC molar ratio exceeds 1:50 [10]. Fig. 5 illustrates this by ^{31}P-NMR spectra of gramicidin/DOPC mixtures at a 1/15 (a), 1/10 (b) and 1/5 (c) molar ratio. The coexistence of a lamellar ($\Delta\sigma \approx 40$ ppm) and H_{II} phase ($\Delta\sigma \approx 19$ ppm) is visible in Fig. 5a and b. At a 1:5 molar ratio (Fig. 5c) all the lipids are organized in an H_{II} phase. The small isotropic signal in spectrum b could arise from some smaller structures present in the preparation. The amount of lipid molecules organized in the H_{II} phase increases with an increasing mole fraction of gramicidin. Oleoylgramicidin also induces the H_{II} phase in DOPC (Fig. 5d–f). Similar to gramicidin, the amount of H_{II} phase increases with an increasing mole fraction of oleoylgramicidin. The control samples with gramicidin and free oleic acid also show similar ^{31}P-NMR spectra as the covalently bonded situation (Fig. 5g–i). Fig. 6 quantifies the amount of bilayer at different mole fractions of (oleoyl)gramicidin and shows that oleoylgramicidin is as effective as gramicidin in inducing the H_{II}-phase in DOPC. The ability of the acylgramicidin analogs lauroyl-, myristoyl-, palmitoyl-, and stearoylgramicidin to induce H_{II}-phase in mixtures of DOPC was similar to that of oleoylgramicidin (data not shown). The control samples containing DOPC, gramicidin and equimolar amounts of oleic acid showed a slight increased H_{II}-phase formation relative to the (acyl)gramicidin/DOPC mixtures, in accordance with the reported H_{II} phase promoting activity of free fatty acids in such systems [26].

3.4. Lipid mixing

Gramicidin causes lipid mixing and mixing of aqueous contents in vesicles of DOPC [11]. Together, the two events are a strong indication for fusion. Since these two events are closely paralleled, we have used a pyrene-PC based lipid mixing assay [11] as a an indication for fusion. Lipid mixing of a donor and acceptor population of lipids results in a decrease in E/M ratio as shown in Fig. 7 for fusion caused by gramicidin. Addition of gramicidin immediately results in a decrease of the E/M ratio (Fig. 7) such that in one minute an equilibrium E/M ratio was observed, in agreement with previous reports [11]. The value of E/M decreases with an increasing mole fraction of gramicidin. Addition of oleoylgramicidin also results in a decrease of the E/M ratio and thus causes lipid mixing of the two vesicle populations which is, in analogy to gramicidin, most probably due to fusion. However, oleoylgramicidin was found to be slightly less effective in the lipid mixing assays as compared to gramicidin. Interestingly, oleoylgramicidin is also less effective in inducing K^+ leakage from DOPC K^+ loaded LUVs (data not shown). Therefore we postulate that the decreased efficiency of oleoylgramicidin in the lipid mixing assay is a result of a decreased insertion of the acylpeptide. The fusogenic properties of the other acylgramicidin analogs were similar to that reported for oleoylgramicidin.

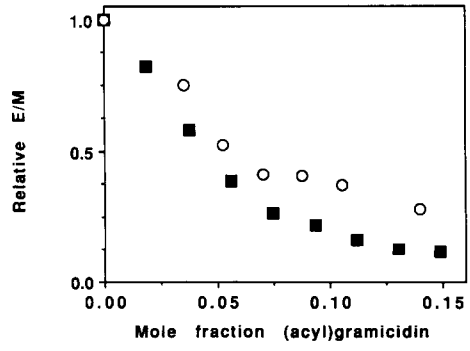

Fig. 7. Induction of lipid mixing between DOPC/pyrene-PC donor vesicles and DOPC acceptor vesicles by gramicidin (■) and oleoylgramicidin (○) at 25°C.

4. Discussion

This paper reports on the influence of acylated gramicidins on the aggregate structure of hydrated lipid dispersions.

Gramicidin and palmitoylgramicidin both induce the formation of extended bilayers in mixtures with lyso-PC. The only difference is that gramicidin is slightly more efficient than palmitoylgramicidin in promoting a lamellar organization in lyso-PC. The difference in the number of lyso-PC molecules that interact with gramicidin and palmitoylgramicidin suggest that the covalently coupled fatty acid occupies space that is otherwise reserved for the acyl chain of a lyso-PC molecule and that the efficiency of the peptide moiety itself is similar in both cases. Several models have been postulated to explain the strong effect of gramicidin on lipid structure [6]. The shape–structure concept [14] predicts that the lipid surface curvature is dependent on the ratio between the cross sectional areas of the polar head group (A_h) and the apolar tail (A_c). The strong effect of gramicidin on the lipid surface curvature is explained by an increase in the hydrophobic cross-sectional area (A_c) due to a preferential incorporation of gramicidin in the hydrophobic core of the membrane. Incorporation of gramicidin thus results in a decrease of the ratio A_h/A_c and accordingly a preference for gramicidin/lipid mixtures to organize in structures with a concave surface or like in case of lyso-PC to form lamellar organizations. In analogy to the shape-structure concept one might expect that a fatty acid cova-

lently coupled to a transmembrane polypeptide, like gramicidin, will further increase the hydrophobic cross-sectional area of the molecule which should result in a further decrease of A_h/A_c and consequently in an increased tendency to promote an organization in type-II structures. However, the striking finding in this paper is that acylgramicidins are as effective as gramicidin in modulating the lipid organization in the investigated PC and PE systems. It was shown that the ability of gramicidin to induce H_{II} phase in DEPE and DOPC and fusion between DOPC vesicles strongly depends on the chemical structure of gramicidin [6,11]. We have previously shown that the right-handed $\beta^{6.3}$ helix is the equilibrium conformation in a lipid environment for both acylgramicidins and gramicidin [27]. Therefore we must conclude that either the covalently coupled fatty acid does contribute little to the cross-sectional area of gramicidin or that an increased hydrophobic area alone is not sufficient and another mechanism is involved and dominates.

It was shown that the limiting molecular area of acylgramicidin at the air–water interface is similar to that of gramicidin [5]. This was explained by assuming a topology of the acylgramicidin molecule with the C-terminus towards the interface, similar to that found in a membrane environment, with the covalently attached acyl chain running inbetween the side chains parallel to the helix axis. Evidence for a similar structural organization of the acyl chain in a membrane environment was obtained from ^{2}H-NMR measurements on the acyl chain [28] and amino acid side chains [6]. Together with the present results this not only suggests that the topology of the acylgramicidin molecule and the gramicidin molecule in a bilayer is similar, but also that the hydrophobic area of acylgramicidin appears to be similar to that of gramicidin. However, not only the hydrophobic area of gramicidin but also intermolecular gramicidin interactions are believed to be important for the strong lipid structure modulating properties [6]. Evidence for gramicidin aggregation in mixtures with DOPC, in relation to H_{II} phase formation, was obtained from density gradient centrifugation experiments [29]. The loss of H_{II} phase specific lineshape in ^{31}P-NMR spectra of a gramicidin/DOPC mixtures in the H_{II} phase recorded at lower temperatures also indicated gramicidin aggregation [30]. The rate of lateral diffusion of phospholipids around the tubes of the H_{II} phase decreased due to the presence of gramicidin aggregates, consequently the additional averaging of the residual chemical shift anisotropy was reduced.This resulted in a transition to a "bilayer type" of lineshape at low temperatures [30]. A similar temperature dependent change in lineshape was observed in the ^{31}P-NMR spectra of an H_{II} phase of acylgramicidin/DOPC mixtures (Vogt, T.C.B., unpublished observations). This indicates that acylation also does not largely influence aggregation of gramicidin in the H_{II}-phase induced by the peptide in DOPC.

The lipid structure modulating property of gramicidin itself is apparently so strong that acylation does not significantly affect this property. This does not exclude, however, the possibility that in case of other membrane spanning polypeptides with less strong lipid structure modulating activities acylation can affect local lipid organization around such peptides which has to occur transiently in membrane processes as fusion [31,32] or budding [33].

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