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Synthesis of acylated gramicidins and the influence of acylation on the interfacial properties and conformational behavior of gramicidin A

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Five gramicidin A analogs were synthesized in which various acyl chains, differing in length and unsaturation, were covalently coupled to the C-terminal ethanolamine group. The analogs were characterized by various spectroscopic techniques and their molecular properties were investigated using monolayer techniques and circular dichroism. It is demonstrated that neither the interfacial properties nor the conformational behavior of gramicidin A at the air/water interface are seriously affected upon acylation. It is proposed that at the limiting area the gramicidin molecule is oriented with its C-terminus towards the subphase with the covalently coupled acylchain located parallel to the helical axis in between the protruding tryptophans. Circular dichroism experiments, in which gramicidin-containing vesicles were prepared from different organic solvents, indicate that the presence of a covalently coupled fatty acylchain tends to stabilize the $\beta^{6.3}$ helical conformation. It is demonstrated that, like for gramicidin A, also for the acylgramicidins the single-stranded $\beta^{6.3}$ helical conformation, or channel conformation, is the preferred conformation upon incorporation in bilayers.

Introduction

Over the last years many proteins have been found which are covalently modified by fatty acids (for review, see Ref. 1). Two major groups of acylproteins can be distinguished. The first group is formed by proteins with myristic acid linked via a peptide bond to the N-terminal glycine. The second group is formed by proteins where the fatty acid (predominantly palmitic or oleic acid) is coupled through a (thio)ester bond to a cysteine or serine residue. The palmitic acid residue is mainly located near a hydrophobic putative membrane spanning part of the protein. The function of protein acylation is not precisely known but probably diverse. It has been reported to include anchoring or targeting of proteins [2], activation or deactivation of enzymes [3] and involvement in processes like membrane fusion [4–6] and signal transduction [1]. Little is known about the structural and conformational consequences of fatty acylation of proteins.

Fatty acylation is not confined to proteins but also has been described for polypeptides [1], of which the linear polypeptide antibiotic gramicidin is an example. In the commercially available natural mixture of this polypeptide (gramicidin A'), Koeppe and co-workers [7] discovered gramicidin K (*O*-acylgramicidin A') in which predominantly palmitic or oleic acid is coupled through an ester bond to the C-terminal ethanolamine group of gramicidin A' [8]. The main structure of linear gramicidins is

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

where Y can be either Val or Ile and X is Trp (gramicidin A), Phe (gramicidin B) or Tyr (gramicidin C). Because of its hydrophobicity and its channel-forming properties gramicidin has been extensively used

Abbreviations: CD, circular dichroism; DCC, *N,N*-dicyclohexylcarbodiimide; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; PPY, 4-pyrolidinopyridine; SUVs, small unilamellar vesicles; TFE, trifluoroethanol.

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as a model for the hydrophobic part of membrane spanning proteins [11–14]. A detailed molecular understanding has been obtained of both the channel-forming characteristics of gramicidin [9,10] as well as its lipid structure modulating properties [11–14], which make acylgramicidins a promising model to get to an understanding of the structural and functional consequences of fatty acylation of proteins.

As a first step towards this goal we report here the synthesis, purification and characterization of a series of acylgramicidin A species with varying acyl chain length and unsaturation. From monolayer studies a first insight is obtained into the mode of organisation of the acylchain with respect to the polypeptide. The influence of acylation on the conformational properties of the polypeptide is addressed by circular dichroism in lipid vesicles.

Experimental

Materials

Gramicidin A' was obtained from Sigma (St. Louis, MO, U.S.A.), *N,N*-dicyclohexylcarbodiimide (DCC) and 4-pyrolidinopyridine (PPY) were from Aldrich Chemie (Steinheim, F.R.G.). The fatty acids lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1c) were obtained from Merck (Darmstadt, F.R.G.). All other reagents were of analytical grade. DCC, PPY and all fatty acids were lyophilized from benzene before use. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was either synthesized and purified according to standard procedures [15,16] or obtained from Avanti Polar-Lipids (Birmingham, AL, U.S.A.). Both preparations yielded identical results.

Isolation of gramicidin A

Gramicidin A was purified on a reversed phase HPLC column (50 × 5 cm) packed with SI-100 polyol from Serva (Heidelberg, F.R.G.) from the natural mixture as described by Killian et al. [17]. The inclusion of 2% (v/v) chloroform in a gradient of 75 to 80% of methanol in distilled water resulted in an improved resolution.

Preparation and purification of acylgramicidin

5 ml of benzene (stored on BaO) was added to a mixture of 250 mg gramicidin A (0.13 mmol), a five fold molar excess of PPY and 10-fold molar excesses of DCC and the appropriate fatty acid. This mixture was homogenized and subsequently stored in the dark under argon at room temperature. The reaction was followed by HPTLC on silica using chloroform/methanol/water (100:10:1, v/v) as eluens, in which the R_f values of gramicidin A and the acylgramicidin are 0.2 and 0.5, respectively. The spots were visualized by heating the HPTLC plates after spraying with 10% H_2SO_4 in water. After completion of the reaction, which usually took about 15 h and was judged by the complete disappearance of gramicidin A, 30 ml of benzene was added. The mixture was applied to a silica column (20 × 4 cm), packed with polygosil 60-4063 from Machery-Nagel (Düren, F.R.G.) and was eluted with chloroform/methanol/water (250:10:1, v/v) at a flow rate of 12 ml/min. The collected fractions were analyzed with HPTLC. The acylgramicidins eluted from the column in the 300–550 ml elution fraction. Traces of gramicidin A eluted after 600 ml. The combined acylgramicidin containing fractions were dried and redissolved in eluens at a final concentration of 60 mg/ml. Aliquots of 0.5 ml were applied to an HPLC silica column (60 × 2.2 cm), packed with Nucleosil 100–10 from Machery-Nagel and eluted with chloroform/methanol/water (250:10:1, v/v) at a flow rate of 9 ml/min. Elution was followed with a refractive index detector (Knauer). The acylgramicidins eluted after 340 ml in a total volume of 50 ml. The peptide was stored after lyophilization from dimethylsulfoxide (DMSO) as a powder at −20 °C or as a stock solution in ethanol at 4°C. The final yield was 70%.

Analytical HPLC

Samples of 20 μl of 0.5 mM (acyl)gramicidin in ethanol were injected on an analytical reversed phase octadecanoyl (lichrospher 100-5, Merck) column (25 × 0.4 cm) connected to a UV-detector (Knauer) set at 280 nm. The column was first eluted with a linear gradient of 75 to 100% methanol in distilled water in 30 min, followed by elution with 100% methanol for 20 min at a flow rate of 1 ml/min.

Absorbance and fluorescence spectroscopy

Absorbance spectra of solutions of (acyl)gramicidin in ethanol (5–40 μM) were recorded in the range of 210–320 nm using a Hitachi U-3200 spectrophotometer. The molar absorption coefficient at 280 nm was found to be 22300 M^{-1} cm^{-1} for gramicidin A as well as all acylgramicidins. This value was used for quantification and is comparable with the value previously reported for gramicidin A [17]. Fluorescence spectra of (acyl)gramicidins dissolved in ethanol were recorded from 295 to 500 nm using an SLM-Aminco spf-500 spectrofluorometer. The spectra were recorded using an excitation wavelength of 285 nm.

Gas chromatography

The fatty acids covalently coupled to gramicidin were identified and quantified by gas chromatography of the ethylesters. These were prepared by incubation of a known amount of acylgramicidin in 5% H_2SO_4 in ethanol in the absence and presence of known amounts of reference fatty acids.

NMR

^{1}H- and ^{13}C-NMR spectra were recorded at 30°C on a Bruker AM-500 and an MSL-300 spectrometer, respectively. The peptides were dissolved in DMSO-d_6 (99.8%, Merck) in the range of 7–15 mg/ml. ^{1}H-NMR spectra were recorded at 500.1 MHz with a 90° pulse of 12 μs and a spectral width of 6 kHz using a 16K memory. ^{13}C-NMR spectra were recorded at 75.5 MHz. The chemical shifts are related to those of DMSO-d_6, of which the ^{1}H- and ^{13}C-resonances are at 2.49 ppm and 39.5 ppm, respectively, downfield from tetramethylsilane.

Monolayer studies

Monolayer studies were performed as described in detail by Tournois et al. [19] using a Teflon Langmuir trough (620 cm^2) equipped with a movable barrier. The surface pressure was measured at 25°C using a paper plate connected to an electrobalance (Cary). A monomolecular layer was formed from 20 nmol (acyl)gramicidin dissolved in chloroform/ethanol (1:1, v/v) on a subphase of distilled water. The monolayer was compressed with a rate of 98 cm^2/min.

Preparation of small unilamellar vesicles

Dry mixed films of 0.25 μmol (acyl)gramicidin and 6.25 μmol DMPC were dissolved in 2 ml of either trifluoroethanol (TFE), chloroform/methanol (1:1, v/v) or ethanol and incubated for 1 h at room temperature. After evaporation of the solvent the film was dispersed by agitation in 1 ml of distilled water (pH = 5.0). Small unilamellar vesicles (SUVs) were prepared by sonication using a Branson B-12 tip sonicator for 10 times 0.5 min (duty cycle 50%) at 50 Watt while cooling on ice, followed by centrifugation (30 min, 25000 $\times g$, 4°C). No significant loss of either peptide or lipid was observed in the supernatant. Aliquots of 100 μl from the supernatant were used for subsequent circular dichroism (CD) measurements. As a control SUVs were also prepared from mixed films of gramicidin, DMPC and free fatty acid in a molar ratio of 1:25:1.

Circular dichroism

CD spectra of (acyl)gramicidin incorporated into DMPC SUVs were recorded at 25°C using a Jasco J-600 spectropolarimeter. The spectra were recorded from 185 to 260 nm in quartz cuvets with a path length of 0.2 mm, using a scan rate of 10 nm/min, a time constant of 1.0 s and a resolution of 0.2 nm. Ten accumulated spectra were averaged. Baseline corrections were made by substracting the CD spectrum from DMPC SUVs.

Results

Lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0) and oleic (18:1c) acid were covalently coupled to the hydroxyl group of ethanolamine at the C-terminus of gramicidin A. These fatty acids were selected because they include the fatty acids occurring in acylproteins and allow assessment of the influence of acylation and the effects of acylchain length and monounsaturation on the properties of the gramicidin molecule. Analytical HPLC showed that the subsequent purification of the acylgramicidins is adequate. This is illustrated in Fig. 1 for lauroyl- and myristoylgramicidin. The reference run of gramicidin A' (Fig. 1A) illustrates the resolution of the column and resolves gramicidin C (elution time 15 min), the dominant gramicidin A (elution time 18 min) and gramicidin B (elution time 21 min). No gramicidin A or any other impurities are detected in the lauroyl- (Fig. 1B) and the myristoylgramicidin (Fig. 1C) preparation. The expected increase in hydrophobicity of myristoylgramicidin as compared to lauroylgramicidin and gramicidin A is confirmed by the increased retention time on the reversed phase column. The elution times for lauroyl-, myristoyl-, palmitoyl-, stearoyl- and oleoylgramicidin were found to be 31, 33, 36, 38 and 36.3 min, respectively. Gas chromatography showed that no changes in

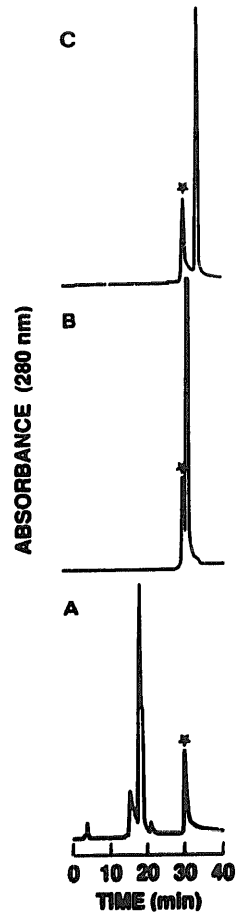


Fig. 1. Analytical HPLC runs of gramicidin (A), lauroylgramicidin (B) and myristoylgramicidin (C). The asterisk denotes an artifact due to the gradient.

the fatty acid composition had occurred during the synthesis. Moreover, an equimolar ratio (1.04 ± 0.05) of covalently coupled fatty acid to gramicidin A was found for all species. No differences were detected in absorbance or fluorescence spectra of gramicidin A and the acylgramicidins in ethanol. The fluorescence spectra were characterized by one peak with a maximum at 346 nm and a shoulder at 333 nm. The tryptophan quantum yield was determined relative to *N*-acetyl-L-tryptophanamide [18]. It was 0.55 ± 0.02 for gramicidin A and the acylgramicidins.

^{1}H- and ^{13}C-NMR spectra were recorded to confirm the covalent attachment of the fatty acyl chains via an ester bond at the C-terminal ethanolamine. The resonances of gramicidin A were assigned according to Hawkes et al. [20]. Table I summarizes the relevant chemical shifts of acylgramicidins and gramicidin A. The ethyl ester of palmitic acid was used as reference for covalently coupled fatty acids. Fig. 2 compares by way of example the ^{1}H-NMR spectra of palmitoylgramicidin (top) and gramicidin A (bottom). A complete disappearance of the resonance of the hydroxyl proton from ethanolamine, at 4.65 ppm, reveals a modification at this position (Fig. 2B, marked by ↓). In addition the resonance of the α-protons of the ethanolamine group shifts from 3.39 ppm for gramicidin A to a higher value for acylgramicidin and two peaks with resonances at 3.97 and 4.05 ppm are formed for these supposedly chemically equivalent protons (Fig. 2B, peaks marked by ○). The β-protons shift from 3.15 ppm for gramicidin A to 3.12 ppm for the acylgramicidin (Fig. 2B, marked by *). Additional changes in the resonance pattern of the acylgramicidins as compared to gramicidin A are observed both in the backbone amide region (7.6–8.3 ppm, Fig. 2C) and to a very small extent in the tryptophan indole region (6.8–7.6 ppm, Fig. 2C) and the tryptophan amide region, at about 10.8 ppm (data not shown). Resonances typical for esterified fatty acids are detected at 2.22 ppm for the α-protons and at 1.45 ppm for the β-protons (Fig. 2A). Additional fatty acid resonances were found at 1.2 ppm from $(CH_2)_n$ and at 0.8 ppm for the methyl group. All acylgramicidins exhibited these same spectral features and in all cases integration confirmed an equimolar ratio of covalently coupled fatty acid to gramicidin A. The esterification was also confirmed by ^{13}C-NMR (data not shown). Upon acylation the resonances of the α- and β-carbon atoms from the ethanolamine group were found to be shifted from 60.0 to 62.5 ppm and from 41.8 to 38.0 ppm, respectively, as expected upon coupling of a fatty acid via an ester bond (Table I). At 173.5 ppm, 33.6 and 22.2 ppm resonances were observed from the carbonyl, α- and β-carbons of the fatty acid, respectively, providing further evidence for the esterification. All additional fatty acid resonances were also observed (Table I).

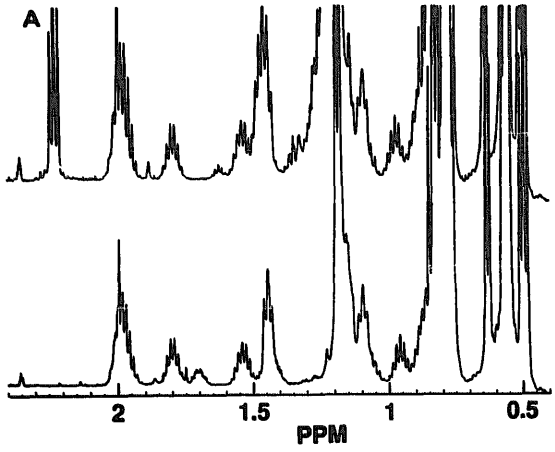
The interfacial behavior and molecular dimensions of the acylgramicidin were investigated by monolayer techniques at the air/water interface (Fig. 3 and Table II). The force(π)-area(A) compression curve of gramicidin A (Fig. 3, curve a) has a characteristic shape, as described previously [19,21–23]. At high molecular areas the curve is indicative for the presence of a highly compressible conformation. A deflection in the compression curve occurs at a surface pressure of 15 mN/m, corresponding to a molecular area of approx. 235 Å^2. At lower molecular areas the film has a low compressibility. As discussed in detail by Tournois et al. [19], the limiting molecular area of gramicidin A corresponds to the molecular area at a surface pressure of 19 mN/m. The π-A curves of all acylgramicidins showed similar characteristics, as illustrated for palmitoylgramicidin (curve b). As a control monolayers were also formed from equimolar amounts of grami-

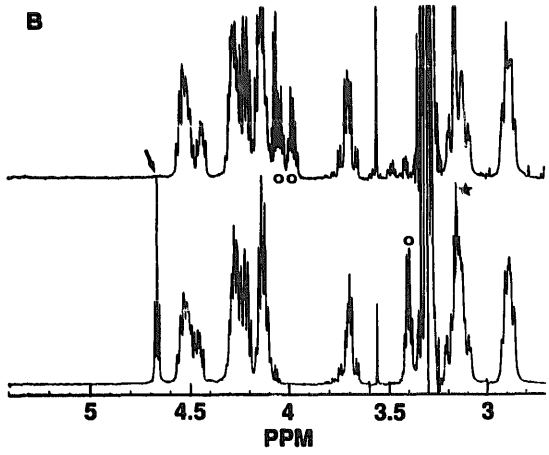
TABLE I

The relevant ^{1}H and ^{13}C chemical shifts of gramicidin A, the ethyl ester of palmitic acid and acylgramicidin

The chemical shifts are related to DMSO-d_6 of which the ^{1}H- and ^{13}C resonances are at 2.49 and 39.5 ppm, respectively, downfield from TMS.

	Resonances (ppm)					
	gramicidin A		ethyl ester of palmitic acid		acylgramicidin	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
-(CH$_2$)$_n$-CH$_2$-CH$_3$			14.2	0.8	14.2	0.8
-(CH$_2$)$_n$-CH$_2$-CH$_3$			21.1	1.2	21.1	1.2
-(CH$_2$)$_n$-CH$_2$-CH$_3$			28.5–29.5	1.2	28.5–29.5	1.2
-O-CO-CH$_2$-CH$_2$-			22.9	1.45	22.2	1.45
-O-CO-CH$_2$-CH$_2$-			34.4	2.22	33.6	2.22
-O-CO-CH$_2$-CH$_2$-			173.5		173.5	
NH-CH$_2$-CH$_2$-O-	41.8	3.15	15.9	1.30	38.0	3.12
NH-CH$_2$-CH$_2$-O-	60.0	3.39	59.0	4.15	62.5	3.97–4.05
NH-CH$_2$-CH$_2$-OH		4.65				





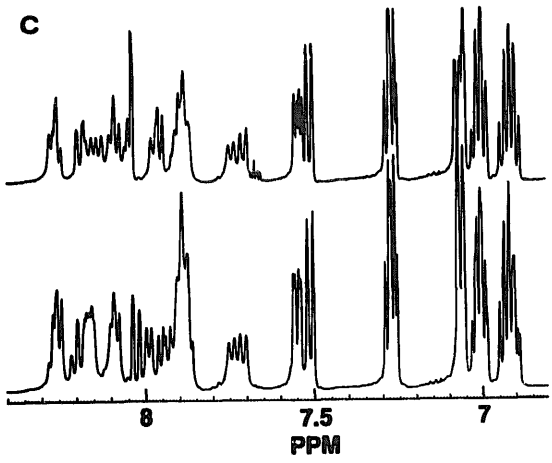


Fig. 2. 500 MHz ^{1}H-NMR spectra of palmitoylgramicidin (top) and gramicidin (bottom) recorded in DMSO-d_6 at 30°C. The ^{1}H-NMR spectrum of (acyl)gramicidin is divided in three sections, the first section (A) contains the aliphatic region (0.4–2.4 ppm), the second section (B) contains the resonances of the ethanolamine protons (3.0–5.0 ppm), and the third section (C) contains the backbone amide region (7.6–8.3 ppm) and the tryptophan indole region (6.8–7.6 ppm).

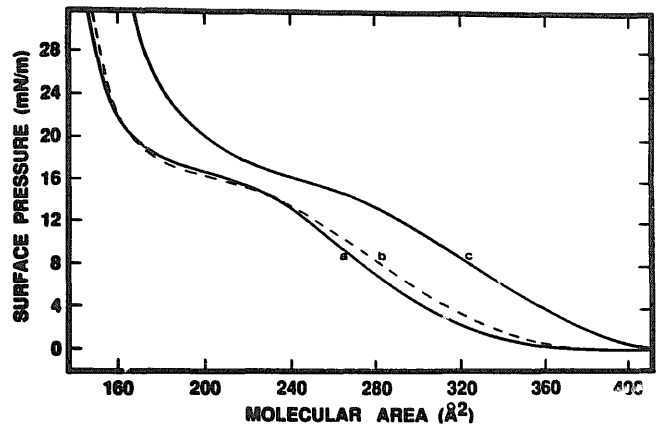


Fig. 3. Monolayer π-A curves recorded at 25°C of gramicidin A (a), palmitoylramicidin (b) and equimolar amounts of gramicidin A and palmitic acid (c).

cidin A and the free fatty acids. The force-area curves of these mixtures have a similar shape as those of gramicidin A and the acylgramicidins, but are shifted to a higher molecular area as examplified for palmitic acid (curve c). As shown in Table II this increase in molecular area at high surface pressure is 20–30 Å^2, corresponding to the molecular area expected for free fatty acids [24]. In contrast, the presence of a covalently coupled acyl chain does not alter the limiting molecular area of gramicidin. At lower surface pressure a different situation is encountered. Again, as shown at the deflection point the molecular areas of the mixtures of gramicidin with free fatty acid are considerably higher than those of gramicidin and the acylgramicidins. However, at this lower surface pres-

TABLE II

Average molecular areas of gramicidin A, different acylgramicidins and equimolar mixtures of gramicidin A and free fatty acid at the air / water interface, obtained from six monolayer experiments at 25°C

The calculated maximal errors are 3 Å^2 and 7 Å^2 for the molecular areas at 15 and 19 mN/m, respectively.

Peptides	Molecular area at the deflection point at 15 mN/m	Molecular area at the equilibrium surface pressure at 19 mN/m
Gramicidin A	235	179
Lauroylgramicidin A	227	179
Myristoylgramicidin A	237	177
Palmitoylgramicidin A	242	170
Stearoylgramicidin A	256	180
Oleoylgramicidin A	258	171
Gram A + myristic acid	263	203
Gram A + palmitic acid	279	209
Gram A + stearic acid	268	203

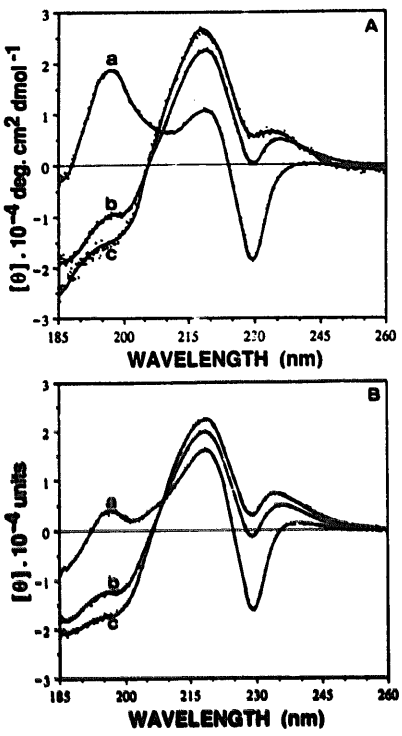


Fig. 4. Circular dichroism spectra of gramicidin A (A) and palmitoylgramicidin (B) incorporated in DMPC SUVs, in a molar ratio of 1:25 (peptide/lipid), from either ethanol (a), chloroform/methanol (1:1, v/v) (b) or TFE (c).

sure, covalent coupling of an acyl chain does affect the molecular area, resulting in an increase with increasing chain length.

Circular dichroism has extensively been used to study the conformation of gramicidin. Although an exact interpretation of the CD-spectrum is not yet possible because of the complexities arising from alternating D- and L-amino acids and the contribution of the four tryptophans, it is possible to use CD to obtain a fingerprint of the conformational state of gramicidin [10]. As shown in Fig. 4 the CD spectrum of gramicidin upon incorporation in lipid bilayers is dependent upon the organic solvent used for sample preparation, in agreement with previous observations [25–27]. When gramicidin containing SUVs are prepared from TFE the CD pattern is characteristic of a single-stranded $\beta^{6.3}$ helical conformation (curve c), which in the head-to-head dimerized form constitutes the gramicidin channel [9,10]. When SUVs are prepared from ethanol, however, the CD pattern is characteristic for a non-channel conformation (curve a) and is believed to represent an equilibrium between different conformations with the antiparallel double helix being the dominant one [25,26]. For chloroform/methanol (1:1, v/v) an intermediate situation is encountered (curve b), with the CD pattern more closely resembling that of the $\beta^{6.3}$ helical conformation. Similar CD patterns were obtained when, as a control, gramicidin-containing SUVs were prepared in these solvents in the presence of equimolar amounts of free fatty acid (not shown). Interestingly, covalent attachment of an acylchain does not appear to significantly alter the conformational behaviour of the peptide. The CD patterns of gramicidin A and acylgramicidin incorporated into SUVs prepared from either TFE or chloroform/methanol (1:1, v/v) are almost identical, as illustrated in Fig. 4B for palmitoylgramicidin. Upon preparation of the samples from ethanolic solution, however, significant differences between the CD patterns of gramicidin and acylgramicidin were detected (compare curves a in Figs. 4A and 4B). These spectral changes, which were observed for all acylgramicidins, can be interpreted as a shift in conformational equilibrium towards the $\beta^{6.3}$ helical conformation upon acylation. Previously it was demonstrated for samples containing gramicidin in a non-channel conformation, that incubation at elevated temperatures results in a conversion of this conformation to a $\beta^{6.3}$ helical conformation [25]. Importantly, a similar conversion as for gramicidin was observed for all acylgramicidins when samples prepared from ethanol were incubated at 65°C (not shown). These data demonstrate that for gramicidin as well as for acylgramicidin the single-stranded $\beta^{6.3}$ helix is the thermodynamically preferred conformation in a lipid bilayer.

Discussion

As a first step in investigating the effect of acylation of gramicidin on the functional properties of this peptide we synthesized, purified and characterized five gramicidin analogs. Fatty acids which differ in length and unsaturation were covalently coupled to the C-terminal ethanolamine group of gramicidin. The synthesis and subsequent purification were successful as shown by HPTLC and HPLC analysis. Special care was taken to establish the intactness of the tryptophans, because these amino acids are important for the single-channel conductivity of gramicidin [28,29] as well as for its influence on lipid polymorphism [30] and its interaction with RNA polymerase [31]. No differences between acylgramicidin and gramicidin A were found upon comparing either the tryptophan fluorescence or the absorbance spectra, both of which are very sensitive to changes in the chemical structure of the tryptophans.

NMR techniques confirmed the covalent coupling of fatty acids through an esterbond at the C-terminal ethanolamine. ^{1}H-NMR measurements on acylgramicidin in DMSO revealed some unexpected features. For the α-protons of the ethanolamine two resonances

were observed of equal intensity, separated by 0.08 ppm. Since the corresponding protons from neither gramicidin A or ethyl oleate form such a doublet, this suggests that the ethanolamine group of acylgramicidin adopts a relatively rigid structure in which the α-protons are chemically inequivalent. Furthermore, upon acylation small changes were observed in the resonance pattern of the amide backbone and the tryptophan indole protons. We propose that these result from a local perturbance due to alignment of the acyl chain along the gramicidin molecule and close to or in between the tryptophans. Despite this local perturbance no changes in tryptophan fluorescence were observed upon acylation. Since not all tryptophans contribute to the fluorescence of gramicidin [32], a possible explanation for this apparent discrepancy is that the tryptophan next to the fatty acid does not contribute to the fluorescence of gramicidin.

The very low solubility of gramicidin in water offers the possibility to determine the behavior of this peptide at the air/water interface. Compression of a monomolecular layer of (acyl)gramicidin results in characteristic π-A curves, with a deflection at a surface pressure of 15 mN/m. Several mechanisms have been proposed to explain this deflection, among which a transition from a single-stranded monomeric to a double-stranded dimeric conformation upon increasing the surface pressure [33] and a reorientation of the gramicidin molecule with its long axis from parallel to perpendicular to the air/water interface [22,23]. At the equilibrium surface pressure of 19 mN/m the limiting molecular area of gramicidin was increased by the presence of equimolar amounts of free fatty acid. In contrast, covalently coupled acylchains did not affect the limiting molecular area, suggesting a shielding of the fatty acid in the acylgramicidins. This could occur for instance when the fatty acid is located on top of the monomolecular gramicidin layer. Since at high surface pressures gramicidin is believed to be oriented with its long axis perpendicular to the air-water interface [22,23], this would imply an orientation of the gramicidin molecule with its N-terminus towards the subphase, as proposed by Brasseur et al. [34]. Another possibility is that the fatty acid is shielded by alignment parallel to the gramicidin axis in between the protruding tryptophans. In view of the hydrophobicity of the acyl chain this would indicate an orientation of gramicidin with its C-terminus towards the subphase, in line with recent energy calculations [35]. The latter possibility is favored by the authors, because it would correspond to the topology of gramicidin A in lipid bilayers [36]. Unlike the situation at high surface pressure the molecular area of gramicidin at the deflection point is affected by the presence of a covalently coupled acylchain and increases with increasing fatty acid chain length. Since addition of free fatty acid, which is oriented perpendicular to the air/water interface, does not show such a chain length dependence, we propose that, at surface pressures below 15 mN/m, gramicidin and its covalently coupled fatty acid are oriented with their long axis parallel to the air/water interface. This would support the notion that the deflection in the π-A curves represents a reorientation of the gramicidin helix from parallel to perpendicular to the air/water interface upon increasing the surface pressure.

The influence of acylation on the conformational properties of gramicidin was investigated with CD. The CD spectrum obtained from gramicidin incorporated into DMPC SUVs has been described as a superposition of two spectra corresponding to two different conformations, the single-stranded $\beta^{6.3}$ or channel conformation and the anti-parallel double-stranded dimer [25,26]. The relative amounts of these conformations is believed to reflect the ratio of single-stranded monomer to double-stranded dimer in the organic solvent used to cosolubilize the peptide and the lipid [26]. Like gramicidin A all acylgramicidins appear to adopt the $\beta^{6.3}$ helical conformation upon incorporation in DMPC SUVs from TFE. This is consistent with the notion that acylgramicidins can form transmembrane channels comparable to those of gramicidin A, as shown for gramicidin K [7,37] and for all acylgramicidins used in this study (manuscript in preparation). As compared to gramicidin A, incorporation of acylgramicidin in DMPC SUVs from ethanol results in a CD pattern that is more representative of the $\beta^{6.3}$ helical conformation. This suggests that acylation mitigates against the formation of double-stranded dimers in ethanol. Also the presence of lipids can increase the monomer-dimer ratio in organic solvents, as reported for egg phosphatidylcholine [26]. Therefore it can be tentatively concluded that the covalently coupled fatty acid has a similar effect on the conformational properties of gramicidin as the presence of lipids and that it promotes incorporation of the peptide in the $\beta^{6.3}$ helical conformation. Furthermore, for gramicidin A as well as for acylgramicidin this single-stranded $\beta^{6.3}$ helix or channel conformation is the thermodynamically preferred conformation in DMPC SUVs, as was demonstrated by incubation experiments at elevated temperatures.

In conclusion, we have shown that the interfacial properties and conformational characteristics of gramicidin are not much affected by the presence of a covalently coupled acylchain at the C-terminal ethanolamine and that overall the acylgramicidins display a very similar behavior, independent of the length and unsaturation of the covalently coupled fatty acylchain. Future studies with these analogs will enable us to investigate the consequences of the different types of acylation for the functional properties of gramicidin in lipid bilayers.

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