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GRAMICIDIN PROMOTES FORMATION OF THE HEXAGONAL H_{II} PHASE IN AQUEOUS DISPERSIONS OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLCHOLINE

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It is shown by ^{31}P-NMR and electron microscopy that gramicidin promotes the formation of the hexogonal H_{II} phase in aqueous dispersions of dielaidoylphosphatidylethanolamine and dioleoylphosphatidylethanolamine, when present in molar ratios of 1 : 200 and higher. In addition gramicidin also induces the hexogonal H_{II} phase in aqueous dispersions of dioleoylphosphatidylcholine, when present in molar ratios of 1 : 25 and higher.

Most biological membranes contain lipids that are able to adopt non-bilayer phases [1,2], of which the hexagonal H_{II} phase is the predominant one. The possible temporary occurrence of non-bilayer structures in biological membranes may serve to explain various functional abilities of those membranes such as fusion and transport processes. Therefore it is essential to understand the various factors that can modulate the occurrence of these non-bilayer structures. It has been shown that temperature, lipid composition, pH and divalent cations may be important parameters [1,2]. From the different phase behaviour of intact membranes and extracted lipids [3–6] it can be inferred that membrane proteins can also influence the structural organization of the lipids. Most clearly this is demonstrated for the apparent bilayer stabilizing role of the intrinsic membrane protein rhodopsin [6]. Using model membranes it was observed that the extrinsic membrane protein cytochrome *c* specifically induced non-bilayer structures in cariolipin containing membranes [7], whereas another basic polypeptide, poly-L-lysine, inhibits the formation of such structures [8]. However, little is known about the influence of reconstituted intrinsic membrane proteins on lipid polymorphism. Only recently it has been found that glycophorin from human erythrocytes exerts a strong bilayer stabilizing influence when reconstituted with dioleoylphosphatidylethanolamine [9]. Gramicidin, a hydrophobic pentadecapeptide, may serve as a model for the hydrophobic segment of intrinsic membrane proteins or mimic signal peptides. It has been shown that the NH_2-terminal to NH_2-terminal Π_6 (L, D) helical dimer model [10,11] for the organization of the aqueous pore forming gramicidin is the major conformation occurring in phosphatidylcholine bilayers [12,13]. As this peptide has been used before as a model for hydrophobic segments of membrane proteins [14], we thought it of interest to study the effect of gramicidin incorporation on lipid polymorphism. It will be shown that gramicidin promotes the formation of the hexogonal H_{II} phase when incorporated in dielaidoylphosphatidylethanolamine, dioleoylphosphatidylethanolamine and dioleoylphosphatidylcholine.

Gramicidin from *Bacillus brevis* which is a mixture of gramicidins A, B and C, was obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_c$, $18:1_c$-phosphatidylcholine) was synthesized as described before [15]. 1,2-Dioleoyl-*sn*-

glycero-3-phosphoethanolamine (18 : 1_c, 18 : 1_c-phosphatidylethanolamine) and 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine) were obtained from the corresponding phosphatidylcholines by the phospholipase D catalyzed base exchange reaction [16]. All the lipids were purified by HPLC [17]. Gramicidin was dissolved in chloroform/methanol (1 : 1, v/v) and mixed with chloroform solutions of the lipids, evaporated to dryness under nitrogen and stored overnight under vacuum. Dispersions were prepared by adding 1.0 or 1.3 ml of a 25% (v/v) ^2H_2O buffer (100 mM NaCl, 25 mM Tris acetate, 0.2 mM EDTA, pH 7.0) to the dry film, which contained approx. 60 μmol phospholipid, and subsequently vigorously vortexed above the gel to liquid crystalline transition temperature.

^{31}P-NMR spectra of an aqueous dispersion of 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine are shown in Fig. 1A for various temperatures. At 25 and 30°C broad asymmetric spectra are obtained which are typical for gel state phospholipid [18,19] organized in extended bilayers, the broadening at the edges caused by some residual dipolar coupling. A transition to the liquid crystalline state can be seen to occur around 35°C, in agreement with calorimetric data [20]. Above 40°C, spectra are obtained with a residual chemical shift anisotropy ($\Delta\sigma$) of approx. 40 ppm, typical for liquid crystalline phospholipids in extended bilayers [1,21], until 55°C where the transition to a hexagonal phase [22] is clearly apparent and completed at 60°C in agreement with previous results [23]. The hexagonal phase is characterized by an asymmetrical ^{31}P-NMR spectrum with a low field peak and a reduced $\Delta\sigma$ of approx. 20 ppm due to diffusion of the lipid molecules around the aqueous channels of the hexagonal H_{II} phase cylinders [22]. When gramicidin is present in an aqueous 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine dispersion in a 1 : 200 molar ratio, it is clear from Fig. 1B that the onset of the formation of a hexagonal H_{II} phase is shifted to much lower temperatures and that even a small amount of phospholipid molecules remain in the hexagonal H_{II} phase below the gel to liquid crystalline transition temperature. This transition temperature itself does not seem to be affected by the presence of gramicidin, as was also found for dipalmitoylphosphatidylcholine/gramicidin mixtures

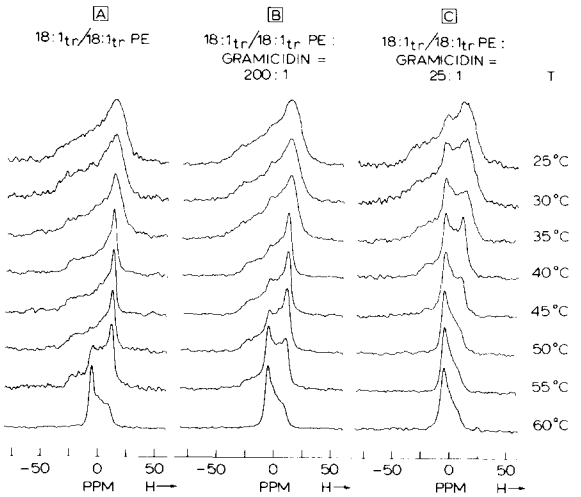

Fig. 1. Proton noise decoupled 36.4 MHz ^{31}P-NMR spectra recorded on a Bruker WH 90 spectrometer, using a 12 kHz spectral width, a 45° pulse angle and a 0.17 s interpulse time. Accumulated free induction decays were obtained from 10 000 to 20 000 transients on 1 ml samples in 10 mm tubes containing approx. 60 μmol of phospholipid in a 25% (v/v) ^2H_2O buffer, and exponentially multiplied, resulting in a 50 Hz line broadening. 0 ppm corresponds with the chemical shift of sonicated egg phosphatidylcholine vesicles. Composition of the samples (molar ratios) and temperature (*T*) are indicated in the figure.

[14]. Increasing the gramicidin content to a ratio of 1 : 25 shows the hexogonal H_{II} phase promoting ability even more pronounced (Fig. 1C), such that the transition to this phase is already completed at 50°C. The transitions were found to be reversible, although a hysteresis was present ranging from 1°C for the lowest gramicidin concentration to 5°C for the highest one. In Fig. 2 a quantitative interpretation is given of the spectral data of Fig. 1 together with some additional data. The bilayer destabilizing properties of gramicidin are shown to be clearly concentration dependent. From the curves in Fig. 2 the temperatures can be obtained at which the amount of bilayer organization equals the amount of hexagonal H_{II} phase. When these are plotted versus the gramicidin concentration, a linear relationship emerges as shown in the insert of Fig. 2.

Similar results were obtained for 18 : 1_c, 18 : 1_c-phosphatidylethanolamine (data not shown). Although the low bilayer to hexagonal H_{II} transi-

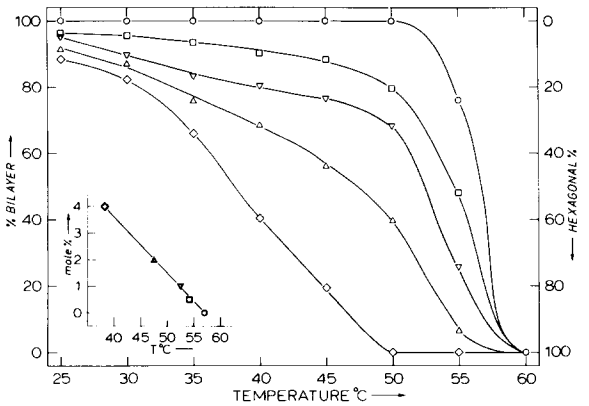

Fig. 2. Amount of bilayer and hexagonal H_{II} phase in aqueous dispersions of 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine with various gramicidin concentrations at different temperatures as derived from 36.4 MHz ^{31}P-NMR spectra presented in Fig. 1 and additional spectra. Data points represent mean values of heating and cooling sequences. Concentrations expressed as molar ratios. ○, pure 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine; □, 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine : gramicidin = 200 : 1; ▽, 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine : gramicidin = 100 : 1; △, 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine : gramicidin = 50 : 1; ◇, 18 : 1_{tr}, 18 : 1_{tr}-phosphatidylethanolamine : gramicidin = 25 : 1. The amount of bilayer and hexagonal H_{II} phase have been determined by computer subtraction with an estimated accuracy of 5%. The insert shows the relationship between gramicidin concentration (mol%) and the temperature at which 50% of the lipid is organized in the hexagonal H_{II} phase.

tion temperature of this lipid (5°C) makes it impossible to follow the complete transition at high gramicidin concentrations, the hexagonal H_{II} phase promoting effect of gramicidin was clearly noticeable.

A straightforward implication of these results is the association of gramicidin with phospholipid molecules within the hexagonal H_{II} phase. The most likely organization is that of gramicidin present in the hydrophobic core and oriented with the long axis of the molecule parallel to the fatty acid chains, or as dimers spanning the hydrophobic domain between adjacent water cylinders.

In view of the strong bilayer destabilizing effect of gramicidin we thought it of interest to investigate the effect of gramicidin on lipids that are known to preeminently organize themselves in bilayers, such as 18 : 1_c, 18 : 1_c-phosphatidylcholine. Gramicidin present in a molar ratio of 1 : 200 in an aqueous dispersion of 18 : 1_c, 18 : 1_c-phosphatidylcholine, introduces a small isotropic component in the ^{31}P-NMR spectrum at 25°C and causes a slight reduction in $\Delta\sigma$ (Fig. 3A), compared with pure dioleoylphosphatidylcholine (not shown). Going to higher temperatures, the intensity of the isotropic peak increases and $\Delta\sigma$ decreases further. When cooling down to 25°C the original situation is restored, indicating the reversibility of the system. Freeze-fracturing of this sample shows a liposomal preparation very heterogenous in size (Fig. 4A). The diameters of the structures range from 500 Å to >10 000 Å. The isotropic peak in the ^{31}P-NMR spectra, the reduced $\Delta\sigma$ and their temperature dependence are fully compatible with the heterogeneity of the preparation and the occurrence of small vesicles in it [24].

Increasing the gramicidin ratio from 1 : 200 to 1 : 25 gives a sample with the same ^{31}P-NMR characteristics as found with the 1 : 200 preparation. Most interestingly, however, an additional small spectral

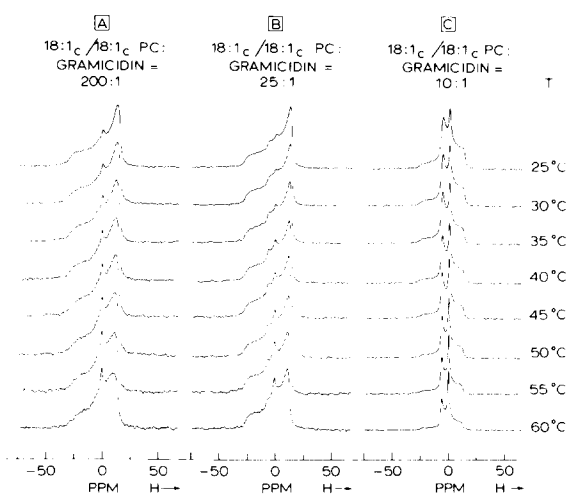

Fig. 3. Proton noise decoupled 81.0 MHz ^{31}P-NMR spectra recorded on a Bruker WP 200 spectrometer using a 25 kHz spectral width, a 90° pulse angle and a 1.0 s interpulse time. Accumulated free induction decays were obtained from 2000–4000 transients on 1.3-ml samples in 10-mm tubes containing approx. 60 μmol phospholipid in a 25% (v/v) ^2H_2O buffer and exponentially multiplied, resulting in 50 Hz line broadening. 0 ppm corresponds with the chemical shift of sonicated egg phosphatidylcholine vesicles. Composition of the samples (molar ratios) and temperature (T) are indicated in the figure.

component indicating 18 : 1_c, 18 : 1_c-phosphatidylcholine molecules organized in a hexagonal H_{II} phase is observed (Fig. 3B). A further increase of the gramicidin concentration (molar ratio 1 : 10) increases the amount of 18 : 1_c, 18 : 1_c-phosphatidylcholine organized in a hexagonal phase, as can be seen in Fig. 3C. The amount of 18 : 1_c, 18 : 1_c-phosphatidylcholine molecules in these preparations with gramicidin, that are organized in a hexagonal H_{II} phase, is apparently independent on temperature. The occurrence of the hexagonal H_{II} phase in the gramicidin/18 : 1_c, 18 : 1_c-phosphatidylcholine mixtures was confirmed by freeze-fracture electron microscopy. Fig. 4B shows the striated fracture pattern typical for the hexagonal H_{II} phase. The diameter of the cylinders closely resembles that of the corresponding phosphatidylethanolamine.

The present study clearly demonstrates that gramicidin has a strong hexagonal H_{II} phase promoting capability in various lipid systems. The tendency of a lipid to adopt a particular phase can be understood in terms of the molecular shape of the lipid molecule [1]. In this concept cylindrical shaped molecules will be organized in bilayers, whereas cone shaped molecules, with the polar head group at the smaller end of the cone, will favour an organization in the hexagonal H_{II} or other inverted phase. Concerning the mechanism of the bilayer destabilization of gramicidin at least two alternatives should be considered. Firstly, this could indicate that the gramicidin monomer itself has an overall cone shape, which promotes the hexagonal H_{II} phase formation. As the

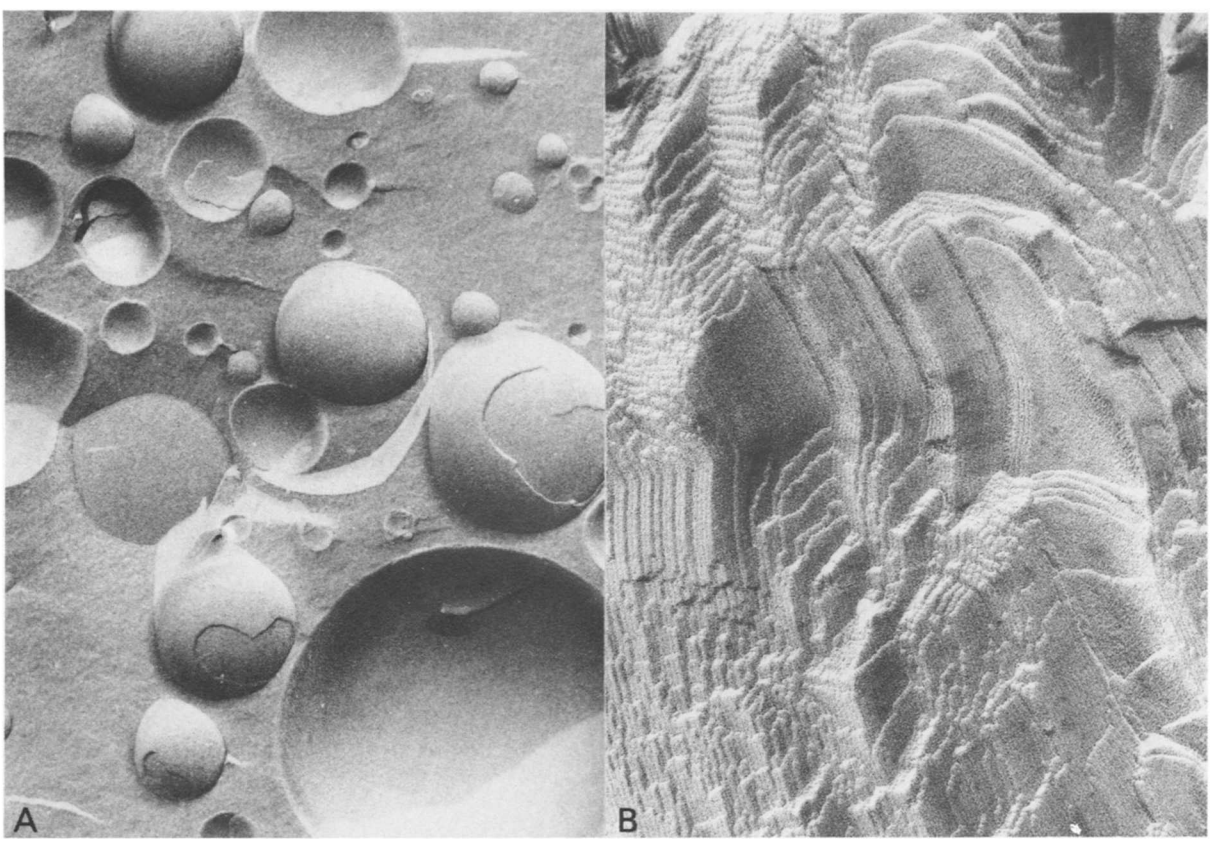

Fig. 4. Freeze-fracture electron micrographs of aqueous dispersions of: (A) 18 : 1_c, 18 : 1_c-phosphatidylcholine : gramicidin = 200 : 1; (B) 18 : 1_c, 18 : 1_c-phosphatidylcholine : gramicidin = 10 : 1. Freeze-fracture electron microscopy was performed as outlined before [25]. 25% (v/v) glycerol was added to the samples to prevent freeze damage. Samples were quenched from 25°C. Magnification: (A) 50 000X, (B) 100 000X.

NH_2-terminal is thought to be located [10–13] in the centre of the bilayer this would imply that the COOH-terminal side would be the smaller end of the cone. Alternatively, the entity of gramicidin with surrounding lipids could have a conical shape. This for instance could be the result of a mismatch of the length of the gramicidin dimer (25–30 Å [11]), with the thickness of the hydrophobic part of the lipid bilayer which in the first instance may lead to meniscus formation at the peptide/lipid interface, thereby promoting hexagonal H_{II} phase formation. In this latter mechanism there will be a strong dependency of the hexagonal H_{II} phase promoting ability of gramicidin on the length of the acyl chains. Preliminary experiments indeed demonstrate such a dependency (Van Echteld, C.J.A., unpublished data). These findings suggest that the length of the hydrophobic part of intrinsic membrane proteins has to match with the bilayer thickness to properly accommodate the protein in the membrane, as has been proposed by Israelachvili [26]. Alternatively, they may indicate an active role of lipid molecules during protein insertion processes.

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