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External addition of gramicidin induces the hexagonal H_{II} phase in dioleoylphosphatidylcholine model membranes

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^{31}P-NMR, small angle X-ray diffraction and freeze-fracture electron microscopy show that dioleoylphosphatidylcholine liposomes undergo a transition from the lamellar to the hexagonal H_{II} phase upon injection of an ethanolic solution of gramicidin in the aqueous medium, when the molar ratio of peptide to lipid is 1 to 20 or higher.

Introduction

Biological membranes contain a large variety of lipids, differing in size and charge of the polar headgroup and in length and degree of saturation of the acyl chains. When pure lipids are dispersed in an aqueous solution they may organize in several ways. Main structures are the bilayer organization, the hexagonal H_{II} phase and the micellar organization.

In models of biological membranes a bilayer organization of the lipids has been widely accepted [1]. However, functional abilities such as membrane fusion, transbilayer movement of lipids and transport across membranes are inconsistent with such an organization. In these and related membrane events non-bilayer structures might be involved. To regulate lipid structure and to trigger bilayer/non-bilayer transitions lipid-protein interactions are obvious candidates.

Gramicidin, a hydrophobic linear pentadecapeptide, has been widely used as a model for the hydrophobic part of intrinsic membrane proteins [2,3]. It is believed to be present as a membrane spanning helical dimer in which an aqueous channel is present [4,5].

It has been shown that gramicidin is able to modulate lipid structure. When gramicidin is incorporated in dielaidoylphosphatidylethanolamine it lowers the bilayer to hexagonal H_{II} phase transition temperature [6]. In dioleoylphosphatidylcholine, which is a typical bilayer-forming lipid, gramicidin was shown to induce the hexagonal H_{II} phase [6]. Lysophosphatidylcholine, which on its own prefers a micellar organization upon aqueous dispersion, forms lamellar structures with gramicidin [7].

In all these experiments, however, samples were prepared by hydration of mixed lipid-peptide films. If lipid polymorphism is important in biological processes, such as fusion, and if in these processes modulation of lipid structure is triggered by lipid-protein interactions, the important question arises whether proteins will be able to induce changes in lipid phase behaviour upon entering the membrane through an aqueous phase.

This question is intriguing in the light of the insertion of hydrophobic peptides into membranes and the mode of action of fusion inducing hydrophobic virus spike proteins. Since it is well established that addition of an ethanolic solution of gramicidin results in insertion of the peptide into

model membranes, as can be inferred from its channel activity [8], this peptide could be an attractive model to investigate this possibility. Therefore we compare in this paper by ^{31}P-NMR, small angle X-ray diffraction and freeze-fracture electron microscopy the effects of external addition and mixed film incorporation of gramicidin on the phospholipid organization of dioleoylphosphatidylcholine model membranes.

Materials and Methods

Gramicidin from *Bacillus brevis*, which is a mixture of gramicidins A, B and C, was obtained from Sigma (St. Louis, MO, U.S.A.). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (dioleoylphosphatidylcholine) was synthesized as described elsewhere [9] and purified by HPLC [10].

For the preparation of mixed peptide-lipid films, samples of 60 μmoles of lipid in 2 ml chloroform/methanol (1 : 1, v/v) and the appropriate amount of gramicidin in a volume of 3 ml ethanol were dried on a rotavapor. The samples then were quantitatively transferred with chloroform/methanol (1 : 1, v/v) to 10-mm NMR tubes, after which they were dried under a stream of nitrogen and stored overnight under high vacuum. To the dry lipid-peptide film 1.2 ml of a 150 mM NaCl, 10 mM Tris/acetate, 0.2 mM EDTA buffer (pH 7.0), containing 25% (v/v) ^2H_2O was added. The films were allowed to swell for several hours at room temperature, after which the samples were vigorously vortexed. Dispersion of the gramicidin containing samples was difficult, especially at higher gramicidin to dioleoylphosphatidylcholine ratios.

The samples in which gramicidin was added externally were prepared as follows. Dry lipid films of 60 μmoles of dioleoylphosphatidylcholine were dispersed at room temperature in 10 ml of a 150 mM NaCl, 10 mM Tris acetate, 0.2 mM EDTA buffer (pH 7.0). The lipid dispersions were transferred to erlenmeyer flasks, equipped with a magnetic stirrer, and buffer was added to a final volume of 200 ml. While drawing a vortex the appropriate amount of gramicidin in a volume of 3 ml ethanol was added dropwise. It could be clearly seen that addition of gramicidin resulted locally in an instantaneous increase in turbidity, suggesting aggregation and/or fusion of the multilayered liposomes, which effect was more pronounced at higher amounts of gramicidin. In case of the pure dioleoylphosphatidylcholine sample 3 ml of ethanol was added and no visible changes in turbidity occurred. ^{31}P-NMR and X-ray diffraction characteristics of these liposomes prepared in the absence of ethanol were found to be identical. Also virtually no changes in turbidity were observed when gramicidin was added in the absence of lipids. After 5 min of incubation at room temperature the samples were spun for 15 min at 16000 $\times$ *g* at 4°C. Phosphorus analysis [11] and ultraviolet absorbance measurements showed that in samples with a peptide to lipid molar ratio of 1 : 10 approximately 80% of the initial amount of lipid and 75% to 80% of the gramicidin was associated with the pellet. The pellets were washed once in the same volume of buffer. Finally they were dispersed in 1.2 ml of a 150 mM NaCl, 10 mM Tris acetate, 0.2 mM EDTA buffer (pH 7.0), containing 25% (v/v) ^2H_2O and transferred to 10-mm NMR tubes. ^{31}P-NMR measurements were carried out within 2 h after the addition of gramicidin. No spectral changes occurred upon storing the samples for up to 24 h.

For both the small angle X-ray and the freeze-fracture electron microscopy experiments pelleted NMR samples were used. The combined use of these three techniques was necessary to obtain a clear picture of the structure of both lipid-peptide systems.

Results and Discussion

In Fig. 1, ^{31}P-NMR spectra are shown of dioleoylphosphatidylcholine with various amounts of gramicidin. Samples of hydrated mixed films are compared with samples in which gramicidin is added externally to dioleoylphosphatidylcholine liposomes. In both cases the pure dioleoylphosphatidylcholine samples give rise to a lineshape with a low-field shoulder and a high-field peak, which is typical for lipids organized in a lamellar liquid crystalline phase. The chemical shift anisotropy (csa) is approximately 44 ppm, in agreement with previous data on diacylphosphatidylcholines [12,13]. When gramicidin is incorporated via hydration of a mixed peptide-lipid film

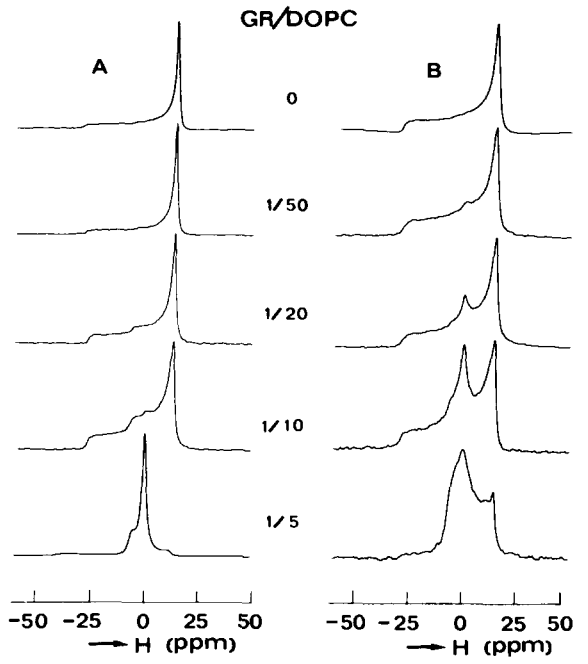

Fig. 1. Proton noise decoupled 81.0 MHz ^{31}P-NMR spectra of dioleoylphosphatidylcholine/gramicidin mixtures, prepared by mixed film incorporation (A) or external addition (B) of gramicidin. The molar ratios of peptide to lipid are indicated in the figure. The spectra were recorded on a Bruker WP-200 spectrometer at 25°C, using 4 K data points with an interpulse time of 1 s, a 90° pulse and a 25 kHz spectral width. A gated decoupling method was used with an input power of 5 W during 10% of the interpulse time. 3000–10000 free induction decays were accumulated and exponential multiplication was applied, resulting in a 50 Hz linebroadening. 0 ppm corresponds to the resonance position of dioleoylphosphatidylcholine undergoing isotropic motion.

up to a molar ratio of 1 : 50 no change in lineshape is observed. Upon further increasing the gramicidin content to molar ratios of 1 : 20 and 1 : 10 a small shoulder appears at a resonance position of −7 ppm, which is characteristic for lipids organized in a hexagonal H_{II} phase. The organization of lipids in the tubes of which this phase is composed gives rise to an NMR spectrum with a reversed asymmetry and with a reduced csa due to diffusion of the phospholipids around the aqueous channels [13]. In the absence of changes in local order this will result in a low field peak at −7 ppm. By subtracting the bilayer component, the amount of lipids organized in the hexagonal H_{II} phase at a molar peptide to lipid ratio of 1 : 10 was estimated to be approx. 15%. When the gramicidin-lipid ratio is further increased to 1 : 5 a hexagonal H_{II} type of spectrum remains with a large isotropic component. Incorporation of increasing amounts of gramicidin furthermore causes a slight decrease of the csa, such that at a molar ratio of peptide to lipid of 1 : 10 the csa is reduced by 8%. As was suggested before [6] the decrease in csa and the appearance of an isotropic ^{31}P-NMR signal could be the result of the formation of smaller structures, leading to motional averaging of the csa due to particle tumbling and/or lateral diffusion of the lipids.

When gramicidin is added externally at low molar ratios the ^{31}P-NMR spectrum shows superposed on the bilayer type of spectrum a small broad isotropic component, which increases with increasing gramicidin content. At a molar ratio of peptide to lipid of 1 : 10 a small shoulder appears at the resonance position of the hexagonal H_{II} phase peak. The amount of lipids giving rise to the hexagonal and the isotropic component in the spectrum of this sample was estimated to be 30%. At a gramicidin to lipid ratio of 1 : 5 the spectrum seems to be a superposition of a broad isotropic component and lineshapes of lipids organized in extended bilayers and in the hexagonal H_{II} phase. Addition of increasing amounts of gramicidin to dioleoylphosphatidylcholine dispersions does not change the csa of the phospholipid phosphates, suggesting that the size of the lipid aggregates is not decreased by the addition of gramicidin to the liposomes. Since isotropic ^{31}P-NMR signals cannot be unambiguously interpreted in terms of lipid structure both gramicidin/dioleoylphosphatidylcholine mixtures were investigated by freeze-fracture electron microscopy.

Previous freeze-fracture electron microscopy experiments on dioleoylphosphatidylcholine-gramicidin samples, made by mixed film incorporation of gramicidin, showed a bilayer organization of the lipids at a low molar ratio of peptide to lipid of 1 : 200 and a hexagonal H_{II} phase at a molar ratio of 1 : 10 [6]. Fig. 2 shows an electron micrograph of the dioleoylphosphatidylcholine dispersion to which gramicidin is added externally in a molar ratio of 1 : 5. Besides lipids organized in extended bilayers, short hexagonal H_{II} tubes are visible, which are highly curved. In addition unde-

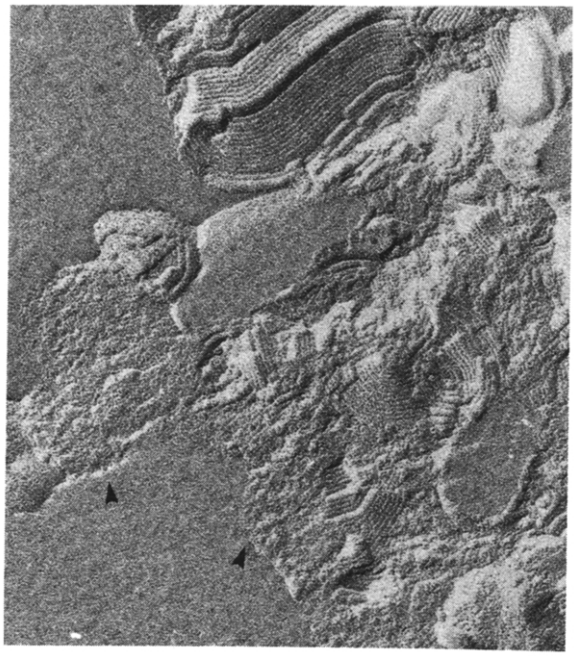

Fig. 2. Freeze-fracture electron micrograph of a gramicidin/dioleoylphosphatidylcholine dispersion prepared by external addition of gramicidin in a molar ratio of 1:5. Next to hexagonal cylinders, undefined granular material is present (arrow). Freeze-fracture electron microscopy was performed as outlined before [13]. 25% (v/v) glycerol was added to prevent freeze damage. Without the use of cryoprotectants similar results were obtained. The sample was quenched from 25°C. To determine the repeat distance of the hexagonal H_{II} phase the replicas were photographed in an Icol 200C electron microscope equipped with a centric side-entry tilting stage. A series of tilting experiments were carried out to be sure that the rows of the tubes were perpendicular to the electron beam. With the aid of a stereo-viewer, flat parts with tubes were selected whereafter with an optical diffractometer the intertube spacing was determined. Magnification: 100000×.

fined particulate structures are present. The tube to tube distance in the hexagonal H_{II} phase is calculated to be approx. 68 Å. The broad isotropic component, present in the ^{31}P-NMR spectrum of this sample could result either from lipids organized in these short and curved hexagonal H_{II} tubes, or from the particulate structures.

The formation of a hexagonal H_{II} phase in dioleoylphosphatidylcholine systems by either mixed film incorporation of gramicidin or external addition of the peptide, was further demonstrated by small angle X-ray diffraction. Lipids, organized in multilamellar structures give rise to a series of reflections with repeat distances which relate as $1:1/2:1/3\ldots$ etc., while a hexagonal H_{II} organization of lipids shows additional reflections at distances which relate to the first-order reflection as $1/\sqrt{3}$, $1/\sqrt{7}$, ... etc. A pure dioleoylphosphatidylcholine dispersion shows two reflections with repeat distances which relate as $1:1/2$, indicating a lamellar organization of the lipids (Fig. 3). In the gramicidin/ dioleoylphosphatidylcholine (1 : 10, molar ratio) systems, prepared either via the mixed film method or via external addition of the peptide, an additional reflection can be seen at a distance which relates as $1/\sqrt{3}$ to the repeat distance of the first-order reflection, indicating the presence of lipids organized in a hexagonal phase. Because the first order repeat distances of the bilayer and hexagonal phase appear to be identical, it is not possible to distinguish the contribution of the bilayer component to the diffraction patterns of the gramicidin containing samples. It is theoretically possible that the reflection at 35–37 Å in case of the sample in which gramicidin was added externally arises from the isotropic phase, as visualized by ^{31}P-NMR, which may represent structures other than the suggested short hexagonal tubes. However, the similarity in tube to tube distances ob-

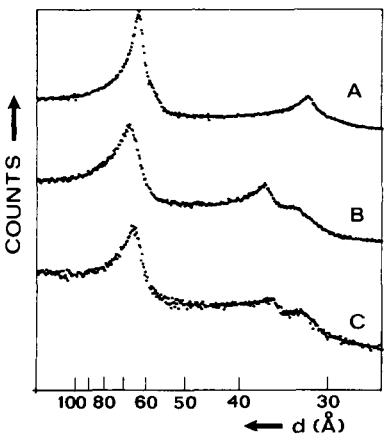

Fig. 3. Small angle X-ray diffraction patterns of dispersions of dioleoylphosphatidylcholine (A) and a mixture of gramicidin and dioleoylphosphatidylcholine in a molar ratio of 1:10, prepared by the mixed film method (B) or by external addition of gramicidin (C). The experiments were performed at 25°C, using a Kratky camera with a Cu-K_α beam (40 kV, 20 mA) and a position sensitive detector. Samples were mounted in a slit in a steel cuvette and both sides were covered by a sheet of cellophane. Exposure times were 10 to 15 min.

served by X-ray diffraction and freeze-fracture electron microscopy indicates that this reflection is related to the hexagonal H_{II} phase. In Table I the reflections of the various samples are summarized. At low gramicidin concentrations in both systems only the first- and second-order reflections of a lamellar organization are observed, in agreement with the NMR data. At higher gramicidin to dioleoylphosphatidylcholine ratios starting from 1 : 20 in case of the mixed films and from 1 : 10 in the samples with externally added gramicidin, a $1/\sqrt{3}$ reflection is present, demonstrating the presence of a hexagonal phase. The interbilayer repeat distance appears to be independent on the gramicidin concentration. From the repeating distance of 64 Å in the sample with externally added gramicidin in a molar ratio of 1 : 5, the tube to tube distance in the hexagonal H_{II} phase can be calculated to be 74 Å, which is in fair agreement with a tube to tube distance of 68 Å as was measured from electron micrographs.

While the hexagonal H_{II} phase-inducing ability of gramicidin in hydrated mixed gramicidin/dioleoylphosphatidylcholine systems was demonstrated earlier [6] the present results clearly show a similar hexagonal H_{II} phase formation upon external addition of the peptide as an ethanolic solution to dioleoylphosphatidylcholine model membranes. In principle it could be argued that the combined action of ethanol and gramicidin is responsible for the induction of a hexagonal H_{II} phase in this latter case. Since in the mixed film approach no ethanol is present in the lipid samples and qualitatively similar results are obtained, we consider this rather unlikely. Thus gramicidin, when added in the aqueous medium, is able to induce changes in lipid structure upon insertion into the membrane. Several mechanisms are proposed for the hexagonal H_{II} phase-inducing ability of gramicidin, in which alternatively the shape of the gramicidin molecule [6], the length of the gramicidin dimer as compared to the thickness of the lipid bilayer [15] and lipid packing [15] are suggested to play a role. An additional important parameter for the formation of the hexagonal H_{II} phase in general is lipid headgroup hydration. Since the bilayer to hexagonal H_{II} phase transition appears to be an interbilayer fusion event [16] it can be suggested, that prior to the hexagonal H_{II} phase formation, the opposing bilayers have to come into close proximity, which, among other factors, can be achieved by dehydration of the phospholipid headgroups. Whereas hexagonal H_{II} type of lipids all have a low headgroup hydration, the bilayer forming lipid dioleoylphosphatidylcholine hydrates easily. When gramicidin is incorporated in dioleoylphosphatidylcholine systems dispersion becomes increasingly difficult, indicating headgroup dehydration of the lipid molecules. Gramicidin is very hydrophobic and therefore it could very well cause this dehydration by either direct interaction with the surrounding phospholipid headgroups or by attractive forces between gramicidin molecules in adjacent bilayers. The X-ray data give some insight in this matter. In the case of phosphatidylethanolamine dispersions, typically a 10–15 Å increase in repeat distance occurs upon going from the multilamellar to the hexagonal H_{II} phase [17,18]. From the geometry of these structures it can be estimated that such a change is required to maintain similar water contents of both phases. However, in the case of the gramicidin/dioleoylphosphatidylcholine system the repeat distance of the multilamellar and the hexagonal H_{II} phase is very similar, indicating dehydration of the lipids upon incorporation of gramicidin.

It has been suggested, that there is a relationship between hexagonal H_{II} phase formation and vesicle fusion [19]. Indeed, in accordance with this suggestion preliminary experiments showed, that when gramicidin is added to large unilamellar

TABLE I

SMALL ANGLE X-RAY DIFFRACTION OF DIOLEOYLPHOSPHATIDYLCHOLINE/GRAMICIDIN SYSTEMS, PREPARED BY MIXED FILM INCORPORATION OR BY EXTERNAL ADDITION OF GRAMICIDIN (SEE LEGEND FIG. 3 FOR DETAILS)

Molar ratio gramicidin to lipid	Reflections (Å)					
	Mixed film incorporation			External addition		
0	64		32			
1 : 100	65		32	65		32
1 : 50	66		33	65		32
1 : 20	66	36	33	65		32
1 : 10	68	37	33	64	36	32
1 : 5	61	35		64	36	

vesicles at a low peptide to lipid molar ratio of 1 : 100 fusion occurs (Killian, J.A., unpublished observation). Thus gramicidin might serve as a useful model to investigate the interaction of hydrophobic peptides, such as present in virus spike proteins, with biological or model membranes.

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