UNIFORMLY ORIENTED GRAMICIDIN CHANNELS EMBEDDED IN THICK MONODOMAIN LECITHIN MULTILAYERS

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ABSTRACT Phosphatidylcholine multilayers, containing 20% water by total sample weight and gramicidin/lipid molar ratios up to 1:40 were aligned by low temperature annealing (<60°C) and mechanical stressing. We were able to obtain large (>80 μm thick × 40 mm^2 area) monodomain defect-free multilayers containing ~10^{17} uniformly oriented gramicidin channels. The alignment of lipid multilayers was monitored by conoscopy and polarized microscopy. The smectic defects, which appeared during the alignment process, were identified and dissolved. The incorporation of gramicidin into the multilayers in the form of transmembrane channels was indicated by its circular dichroic (CD) spectrum. A well-defined CD spectrum of uniformly oriented gramicidin channels was obtained. The oriented samples will allow spectroscopic studies of the ion channel in its conducting state and diffraction studies of the channel–channel organization in the membrane.

INTRODUCTION

We report preparation of large (80 μm thick × 40 mm^2 area) samples of monodomain lecithin multilayers with gramicidin channels embedded in them. Consequently there are 10^{16}–10^{17} uniformly oriented gramicidin channels in each sample available for new types of experiments. Gramicidin is perhaps the best characterized ion channel–forming protein (1). It has been used as a model for studying the mechanism of transmembrane channel transport such as gating and ion selectivity (2). Also, because of its known structure and properties, gramicidin can be used as a probe to study the properties of bilayer membranes (3). So far the experiments on gramicidin channels were primarily performed with two types of samples. The gramicidin-mediated ion conductions were usually measured by using black lipid membranes, whereas the spectroscopic studies such as nuclear magnetic resonance (NMR), Raman, dielectric relaxation, circular dichroism, and infrared absorption were mostly performed with micellar or vesicular suspensions (see references 1, 2, and 4 for reviews). Because it was difficult to couple the spectroscopic measurements with the conduction experiments, the molecular dynamics of the gramicidin channel have not been fully examined. The oriented samples now open the possibilities of studying the ion movement in the channel by dielectric relaxation (5) and performing other spectroscopic measurements with an electric field applied along the channel. Furthermore, these samples will allow diffraction studies of the channel–channel organization in membrane (6, 7). In this paper we describe the method of preparing oriented samples, the characterization of aligned multilayers by conoscopy and polarized microscopy, and the characterization of gramicidin channels embedded in oriented multilayers by circular dichroism (CD).

Pershan and his colleagues (8–10) pioneered techniques for preparing large monodomain phospholipid bilayer arrays. They developed an annealing technique and a compression/dilation technique for aligning phosphatidylcholine with various water concentrations. Powers and Pershan (9) also used the annealing technique to study the incorporation of gramicidin and other membrane-associated molecules into aligned multilayers. For example, they measured the changes in the birefringence of the multilayers due to gramicidin. However, we know of no report of a systematic study of the incorporation of gramicidin in aligned multilayers without using high temperature (>70°C) annealing. We used the method of Asher and Pershan (10), i.e., a combination of mechanical stressing and low temperature annealing to align lecithin multilayers containing various concentrations of gramicidin. The process of alignment, monitored by conoscopy and polarized microscopy, was usually accompanied by the formation of various defect structures. To some extent, these smectic defects are understood (10–13). We found that multilayers containing gramicidin have the same defect structures as pure multilayers. However, the gramicidin containing multilayers are more difficult to align and the degree of difficulty increases with the gramicidin

concentration. Nonetheless we found a procedure by which we have routinely produced defect-free samples with areas >40 mm^2, thicknesses from 2 to 80 μm, and protein/lipid molar ratios up to 1:40.

Evidence that gramicidin was incorporated in the channel form was obtained by CD. The different conformations that gramicidin adopts under different solvent environments are distinguishable by CD spectra (14). By means of ^{13}C and ^{23}Na NMR a unique CD spectrum for vesicle-incorporated gramicidin in the transmembrane channel form has been identified (15, 16). Gramicidin incorporated in oriented multilayers has a similar yet slightly different CD compared with the vesicle incorporated; both are distinctly different from the CD of gramicidin not in the channel form. Also, we did not detect evidence that gramicidin aggregates to the smectic defects.

MATERIALS AND METHODS

Dilauryl-, dimyristoyl-, and dipalmitoyl- phosphatidylcholine (DLPC, DMPC, and DPPC, respectively) and gramicidin were purchased from Sigma Chemical Co. (St. Louis, MO). Lipids indicating a purity better than 99.5% by thin-layer chromatography were used without further purification. Amino-acid analysis on gramicidin showed it to be 70% gramicidin A, 8% gramicidin B, and 22% gramicidin C; it was used without further purification.

Gramicidin and lipid (typically 200 mg), in molar ratios of 1:40 to 1:300, were dissolved in $CHCl_3$, to thoroughly mix the components. The mixture solution was rotary-evaporated so as to remove the solvent, and the solutes were spread as a thin layer onto the wall of a 100-ml, round-bottom flask and then dried under vacuum (<10 μm) for 2 h. The flask was flushed with dry nitrogen and a small amount (~1 ml/g) of benzene was added to the flask. The solution was incubated at 37°C until all the solute dissolved. If the lipid was not completely dry, then a suspension is formed in the benzene, not a solution. In this case, a higher incubation temperature (not to exceed 70°C) may help or a small amount of methanol can be added. The solution was quickly frozen using dry ice and isopropanol and placed in a vacuum (<10 μm) for 24 h. After lyophilization, the mixture was allowed to reach room temperature and it was further left under vacuum for another 24 h to ensure total removal of the benzene solvent.

The samples were aligned between two 1-mm thick fused silica plates separated by a spacer made of mylar. A circular hole of 8 mm in diameter was made in the spacer to provide a cavity for holding the sample. The sample thickness was controlled by the thickness of the spacer. Various thicknesses between 2 and 80 μm were experimented with. The silica plates were cleaned with hot sulfuric/chromic acid and then rinsed thoroughly with distilled water. The samples were prepared by weighing the appropriate amount of water (~20% by weight) into an amalgamator capsule containing ~200 mg of the dry, lyophilized gramicidin–lipid mixture. Nitrogen was lightly blown into the capsule and it was sealed. A dental amalgamator was used to mix the contents of the capsule. After mixing the mixture in the sealed capsule, the sample was further allowed to equilibrate for 2 d in the dark at 30°C. After equilibration, an appropriate amount of the gramicidin–lipid–water mixture was quickly scraped out of the amalgamator capsule and placed into the cavity formed by the spacer and one silica plate. The second plate was immediately clamped onto the sample. Also, the remaining mixture in the amalgamator capsule was resealed and stored for future use. The time that the sample was exposed to air was kept as short as possible so that the water content remained approximately constant. (Many of our samples were made in a glove box filled with dry nitrogen. However, if a sample was only briefly exposed to air [<20 s], no ill effects had been detected.) The sample assembly was held in a holder, similar to the construction used by Asher and Pershan, which clamped the two silica plates tightly together and allowed microscopic and conoscopic inspection of the sample. Also, a small volume of the gramicidin–lipid–water mixture was taken from the amalgamator capsule and dissolved in methanol to determine the protein and lipid concentration. The protein concentration was determined spectrophotometrically by using a molecular extinction coefficient of 22,500 $mol^{-1}cm^{-1}$ at 281 nm. A modified Fiske-SubbaRow method (17) was used for the lipid concentration measurement.

Although we aligned gramicidin-containing multilayers with DLPC, DMPC, and DPPC, we shall present only the data of DLPC, which has the lowest gel transition temperature (<20°C depending on both the H_2O concentration and the gramicidin concentration) among the three. We started the aligning process by heating the sample until the gramicidin–lipid–water mixture appeared to flow easily under compression and dilation of the silica plates. The lowest temperature for this condition to appear depended on the gramicidin/lipid molar ratio, R(G/L), as well as on the water concentration. At ~20% water by total sample weight, it is ~55°C for R(G/L) ≃1:40 and ~24°C for pure DLPC. The majority of lipid appeared to align rather quickly when a small amount of pressure was applied to the silica plates at the sample area and released cyclically. This mechanical process tended to push the oily streak defects toward the sample periphery. For 20% H_2O by weight and 80-μm-thick DLPC without gramicidin, an oily streak defect-free area of 40 mm^2 could be obtained in ~1 h after the mechanical stressing was started. Under similar conditions, but with a molar ratio R(G/L) ≃ 1:40, sample alignment took ~4 h. The aligned samples were slowly cooled down to room temperature (~0.1°C/min). Mechanical stressing can cause some types of smectic defects to form, such as the polygonal array defects; however, the slow cooling process readily anneals these defects. If the polygonal array defects did pose a problem, then the sample was either warmed to a higher temperature and cooled at an even slower rate, or it was simply left alone at room temperature for several days, in which case the defects usually annealed away. We also experimented with several other different alignment procedures with various degrees of success (18).

The gramicidin-containing vesicle suspensions were prepared in a manner similar to that described by Weinstein et al. (19). This procedure is at first similar to the multilayer preparation, for which we first dissolved the gramicidin and lipid in $CHCl_3$ and then evaporated off the solvent. However, instead of adding a small amount of benzene to the solutes, we added H_2O (10 ml) to the flask and incubated the flask at 30°C to hydrate the sample. The sample was then transferred to a cup horn accessory attached to a sonifier (model 185; Branson Sonic Power Co., Danbury, CT) The sample was flushed with nitrogen and then sonicated at power 5 for 0.5 h at 42°C. It was removed from the cup horn and diluted to a final lipid concentration of 4 mg/ml. Finally, to ensure mimimal light scattering during the CD measurements, the vesicle suspensions were further extruded through polycarbonate membrane filters in decreasing pore sizes of 0.4, 0.2, 0.08, and 0.05 μm. The peptide concentrations were determined by UV absorption and the lipid concentrations were determined by using a modified Fiske-SubbaRow method.

CD spectra were measured with a spectropolarimeter (model J-500A; Jasco Inc., Easton, MD). For vesicle samples, the measurement was taken at two positions in order to check the effect of light scattering. One position was 1 in. from the photodetector with a 1¼ in. window corresponding to an acceptance angle of 0.4π and the other was 8 in. away corresponding to an acceptance angle of 0.006π. All accepted spectra had the two measurements agree within 5%. For multilayer samples, the defect-free regions were transparent; therefore, light scattering was not a problem. Also, the appropriate backgrounds of either vesicles in water or multilayers (lipid + H_2O) were subtracted from the measured spectra to obtain the CD spectra of the gramicidin. The multilayer CD spectra presented below were taken from the defect-free region. However, the spectra practically remained the same when the detected areas were enlarged to include oily streak or polygonal array defects. We detected no evidence that gramicidin aggregates to the defects.

RESULTS AND DISCUSSIONS

The homeotropic alignment of lecithin multilayers was proven by the interference pattern produced by a conoscope (Fig. 1), which indicated that the sample was uniaxial (20). The figure was produced by a cone of divergent polarized light with its central ray perpendicular to the silica plates. This light first passed through the sample and then through a crossed analyzer. If the sample is isotropic, the image would be completely dark. However, since aligned multilayers are uniaxially birefringent, a dark cross and concentric rings are expected as shown in Fig. 1 (8, 9). Our measurements of the birefringence of pure lecithin multilayers ($\Delta n \simeq 0.031$ for a 20% H_2O by total weight DLPC sample at 23°C) and multilayers containing gramicidin ($\Delta n \simeq 0.024$ for a 24% H_2O by total weight gramicidin-DLPC sample at 23°C) agreed with previous results (8).

Previous CD studies of vesicle-incorporated gramicidin were mostly performed with samples of R(G/L) > 1:50 and a more or less "standard" spectrum was identified for gramicidin in the transmembrane channel state (14, 15). We reproduced this "standard" spectrum from a vesicle sample with R(G/L) ≃1:40 (see Fig. 2). However, we found that lower values of R(G/L), e.g., 1:100, 1:200, and

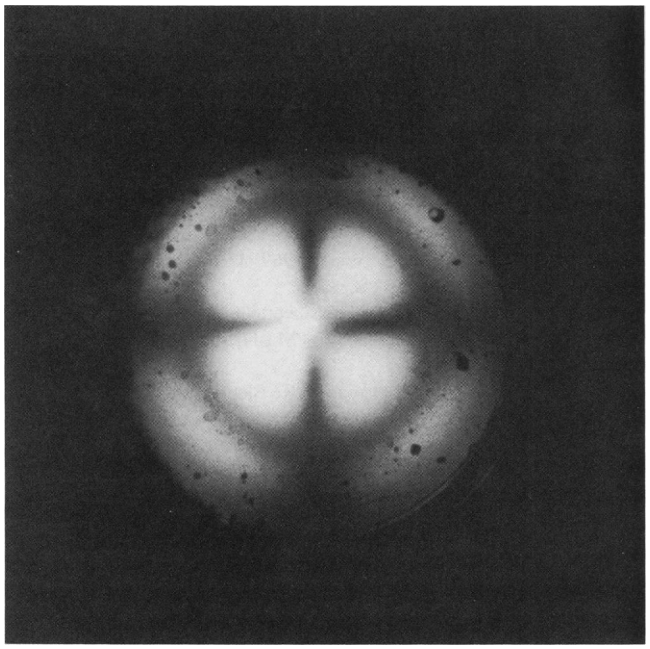

FIGURE 1 Conoscopic interference pattern observed for a 50-μm-thick aligned gramicidin-DLPC sample with a molar ratio of 1:40 and 24% H_2O by total sample weight. The polarizers were crossed under conoscopic white light illumination. Orientation of the polarizers was the same as the dark cross in the pattern. If it was a color photograph, the rings would be colored with red inside and blue outside. The average index of refraction of the sample was measured (independently) to be 1.443 at λ = 632.8 nm. From the radii of the rings, the birefringence Δn was calculated to be 0.024 at λ = 632.8 nm (20). The center should be dark if the polarizers are perfect. The bubbles (appearing as dark spots) are on the screen and not in the sample.

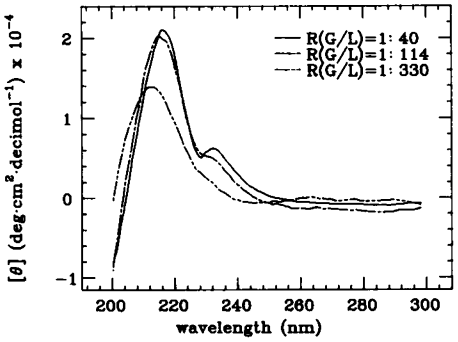

FIGURE 2 CD spectra of gramicidin incorporated in DLPC vesicles at three different protein/lipid molar ratios R(G/L). The lipid concentration for each suspension was ~4 mg/ml. The spectra at high molar ratios, R(G/L) > 1:50, have been reported in many publications (1, 6, 14, 15); they were considered the "standard" spectrum for gramicidin in the transmembrane channel conformation. A mean molecular weight per residue of 124.5 was used to calculate the ellipticities.

1:300, the two main peaks, located at 217 nm and 236 nm for R(G/L) > 1:40, are progressively blue-shifted and broadened, so that at R(G/L) ≃ 1:300 the second peak becomes a shoulder of the first peak (Fig. 2). Concentration dependence of CD spectra for vesicle-incorporated gramicidin was previously noted by Ivanov and Sychev (21) and by Wallace (22). The dependence shown in Fig. 2 is similar to that of Wallace but rather different from that of Ivanov and Sychev. In contrast to such concentration dependence, gramicidin incorporated in aligned multilayers shows a consistent CD spectrum for all values of R(G/L) from 1:40 to 1:300 (Fig. 3). (We were not able to obtain a reliable CD at λ < 220 nm for R(G/L) ≃ 1:40 due to strong UV absorption, even for a sample as thin as 2 μm; above 225 nm the spectrum for R(G/L) ≃ 1:40 is identical to that of more dilute samples.) In comparing the spectra in Fig. 3 with the "standard" spectrum in Fig. 2, we note the following: (*a*) the spectra of multilayers are better

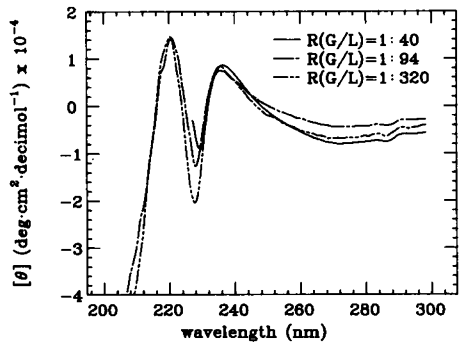

FIGURE 3 CD spectra of gramicidin incorporated in monodomain DLPC multilayers with 20% H_2O by total sample weight. At R(G/L) ≃ 1:40, strong UV absorption made the spectrum below 225 nm extremely noisy. (This was not a problem for the vesicle suspensions since the UV absorption can be minimized by making the vesicle concentration low.) A mean molecular weight per residue of 124.5 was used to calculate the ellipticities.

defined because their peaks are narrower; (*b*) the peaks of the vesicle CD are blue-shifted relative to the multilayer CD by ~3 nm; (*c*) the relative peak amplitudes are $\Delta[\theta]_{217}/\Delta[\theta]_{236} \simeq 3$ for vesicle suspensions and $\Delta[\theta]_{220}/\Delta[\theta]_{239} \simeq 2$ for multilayers; (*d*) all spectra in Fig. 3 have a negative ripple at $\lambda \simeq 280$ nm, whereas the spectra for the vesicle suspensions go to zero. The differences described above are small if one compares these spectra with that of gramicidin in other solvent environments (14). We can conclude with good certainty that the spectra in Figs. 2 and 3 represent the same molecular conformation of gramicidin: Fig. 2 for randomly oriented channels and Fig. 3 for uniformly oriented channels. The small differences may be due to the orientational effect. That chiral-optical effects may depend on the propagation direction of the measuring light with respect to the orientation of the molecular axes has been anticipated theoretically (23). However, until the CD spectra (for example either those in Fig. 2 or in Fig. 3) are understood in terms of the molecular structure, theoretical interpretation of the orientation effect is difficult.

In summary, we have demonstrated a procedure for incorporating gramicidin into thick aligned multilayers of lecithin without raising the temperature of the sample above 60°C. For the first time, the CD spectrum of uniformly oriented gramicidin channels was obtained. We expect that the same or a similar technique can be used to incorporate other model ion channels in aligned multilayers, in particular voltage-gated models such as melittin and alamethicin. With an applied electric field, such a sample will allow spectroscopic and scattering studies of ion channels in their voltage-dependent conducting states.

This research was supported in part by Office of Naval Research contract N00014-86-K-0087, National Institutes of Health grant HL 32593, and The Robert A. Welch Foundation.

Received for publication 7 October 1986 in final form 13 February 1987.

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