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SUPRAMOLECULAR ORGANIZATION OF LYSOPHOSPHATIDYLCHOLINE-PACKAGED GRAMICIDIN A'

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Heat derived gramicidin A'/L-α-lysophosphatidylcholine complexes were separated on a sucrose gradient to form two fractions: Fraction A which had an approximately constant Gramicidin A' to phospholipid ratio of 8 to 10 lipid molecules per Gramicidin A' molecule and Fraction B which had a larger but variable ratio. Fluorescence and circular dichroism studies confirmed Fraction A to be a lipid-incorporated channel state. Electron microscopic studies, using uranyl acetate negative staining, showed fraction A to be a membranous state with the formation of bilayer vesicles, that is, the interaction of peptide and phospholipid micelles causes the lipid to reorganize into a bilayer structure. Freeze-fracture replicas of the channel incorporated state demonstrated the presence of a supramolecular organization of particles exhibiting a tendency to form rows with a 50–60 Å periodicity along the row and with 70–80 Å distance between rows. An idealized working model for the incorporated state is presented.

Introduction

Transmembrane ion transport is a fundamental process in all living organisms, and channel transport is, of course, a key element in the function of nerve and muscle cells [1–4]. During the past decade channel mechanisms have been extensively and productively studied [5–10]; however, the molecular mechanisms of gating and ion selectivity of channels have yet to be thoroughly detailed and less is known concerning the effects of channel-channel interactions on, for example, single-channel conductances.

Previous work [11,12] has shown that Gramicidin A and its analogues, incorporated into phospholipid structures, can be used as a suitable model for ion channels studies. Once incorporated in a lipid system, Gramicidin A shows selectivity, conductances and gating mechanisms of relevance to those of physiological channels [9]. Utilizing either Gramicidin A or a synthetic malonyl dimer, incorporated into lysophosphatidylcholine-containing phospholipid structures, several occupancy models for Na^+ transport through the channel have been considered using rate constants and binding constants derived from ^{23}Na-NMR studies [13,14]. Further studies, using ^{13}C-enriched carbonyls in synthetic Gramicidin A, have located two symmetrically related binding sites near the mouths of the channel and have demonstrated the absence of ion occupancy midway through the channel [15]. The

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left-handedness of the helix has been confirmed by ^{13}C-NMR and the location of the ion binding sites have been further delineated with the results indicating two sites separated by approx. 20 Å [16]. By means of dielectric relaxation measurements over the frequency range of 5 kHz to 900 MHz, a relaxation process has been identified which correlates with the rate of jumping between sites (i.e. over the central barrier) for the two site mechanisms with possible association of channels and correlated ion movement [17]. Also when single-channel currents obtained by direct observation, are compared with those obtained in the presence of many channels using noise fluctuation analysis, there are differences which imply effects arising from association of channels [18]. Thus, as the detailed ionic mechanisms of transport are becoming clarified, the question of association of channels within the phospholipid structure becomes of particular interest.

Accordingly an important and as yet poorly characterized aspect of ion transport by Gramicidin channels is the study of their localization and organization within the phospholipid structure. Indeed, such a problem is relevant to ion transport because the intermolecular interactions between channels could modify channel conductance by altering the surrounding dielectric constant considered to be important in determining, for example, the magnitude of the central barrier [20]. Moreover intermolecular interactions could be a factor in inducing and stabilizing particular side-chain conformations of the channel which could be a factor in the dispersity of single-channel conductances (Refs. 21, 22, and Busath, D., and Szabo, C., unpublished data).

Some years ago Chapman and coworkers [23] considered the possibility of different arrangements of the Gramicidin A channels within lipid bilayers of dimyristoyl-(DMPC) and dipalmitoylphosphatidylcholine (DPPC). They concluded for their system that a random arrangement of channels within the plane of the bilayer would be most likely.

In the present paper, data are reported which demonstrate the existence of a supramolecular organization of Gramicidin A channels which have been heat incorporated into dispersions of lysophosphatidylcholine. Of particular interest is the observation of a thermodynamically preferred Gramicidin A/lipid molar ratio indicative of a relatively stable, aggregated structure. Also the induction by Gramicidin of a bilayer structure in the normally micellar lysophosphatidylcholine is noted.

Materials and Methods

Gramicidin was purchased from ICN Pharmaceuticals, Inc., Irvine, CA as a mixture of 80% Gramicidin A, 6% Gramicidin B, 14% Gramicidin C and was used without further purification. This mixture will be referred to as Gramicidin A′. Egg yolk L-α-lysophosphatidylcholine (Lot. No. 50F-8370) was obtained from Sigma Co., St. Louis, MO, and was found to be essentially free of L-β-lysophosphatidylcholine and of phosphatidylcholine as determined by the absence of unsatured carbons in the ^{13}C-NMR spectra. All the other standard chemicals were of reagent grade.

Fraction separation. The lipid Gramicidin A′ mixture containing 25 mg/ml lipids and 6 mg/ml Gramicidin A′ in 1 mM NaCl, after incubation for 20 h at 70°C, was centrifuged at $3000 \times g$ for 15 min. The supernatant was then loaded on a 10–30% continuous sucrose gradient and centrifuged at $126000 \times g$ with a SW-27.1 Beckman rotor for 20 h at 30°C. The gradient tubes were then eluted and the fractions collected with an LKB 2112 Redirac Fraction Collector. A continuous recording at 280 nm of the elution profile was obtained with a JASCO Uvidec 100-III spectrophotometer equipped with a continuous flow, 1 mm pathlength cell. The fractions corresponding to each peak were subsequently pooled. Determination of the lipid content was achieved following a modification of the Fiske-SubbaRow method [24]. The purity of the lipid after heat-treatment was demonstrated by thin-layer chromatography (chloroform/methanol/water, 65 : 25 : 4, v/v) and by ^{13}C-NMR. The Gramicidin A′ content was determined spectrophotometrically by measuring the absorbance at 280 nm of aliquots of the pooled fractions dissolved in methanol. A molar extinction coefficient of $2.25 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ was used.

Spectroscopic measurements. The Perkin-Elmer 576 ST spectrophotometer was utilized to obtain absorption spectra of Gramicidin A′ incorporated

in lipid systems or dissolved in organic solvents. The corresponding absolute fluorescence spectra were obtained by using a Perkin-Elmer MPF 44A spectrofluorimeter equipped with a microprocessor unit. The samples were diluted with NaCl (1.0 mM) or with methanol for control measurements.

Circular dichroism measurements were performed on a JASCO J-500 Spectropolarimeter equipped with a microprocessor unit for spectra accumulation. The samples were diluted with NaCl (1 mM) and run using cuvettes with 0.2 or 0.1 mm pathlength. The ellipticities were calculated using a mean molecular weight per residue of 124.5.

Electron microscopy. Phospholipid suspensions, both in the presence and in the absence of Gramicidin A′ and at the concentration specified in the result section, were equilibrated at 21°C and diluted with 1.0 mM NaCl to a final concentration of 1.0 mg/ml.

Negative staining. A small drop of suspensions was spread on a 400 mesh copper grid covered by carbon film and washed with 7 to 10 drops of 1% uranyl acetate. The excess of the stain was removed with filter paper and the grid left to air dry. All operations were performed at 21°C.

Rotary shadowing. Diluted suspensions of the specimens were spread on freshly cleaved sheets of mica, air dried and rotary shadowed with platinum/carbon from 8°. A 20 nm thick film of carbon was evaporated from 90° on the specimens and the replicas were floated on doubly distilled water and collected on 400 mesh copper grids.

Freeze-fracture. Lysophosphatidylcholine suspensions, obtained as described above and equilibrated at 21°C without any dilution, were quickly frozen in Freon 22 or in liquid propane and stored in liquid nitrogen. Freeze-fracture replicas were obtained in a Balzers freeze-etching device BAF 301, by fracturing the specimens at −100°C, shadowing with platinum/carbon from an angle of 42° to the fracture surface and then by covering the specimen with a 30 nm thick carbon film. The biological material was digested with a 25% solution of sodium hypochlorite followed by treatment with doubly distilled water, 80% acetone and exhaustive washing in doubly distilled water. The replicas were collected on 400 mesh copper grids and observed in the microscope. Siemens Elmiskop IA and Philips 410 electron microscopes, whose magnification were calibrated by optical diffraction of catalase crystals, were used.

Results

Incorporation studies

Previous studies [12,24] have shown that Gramicidin A′ incorporates into L-α-lysophosphatidylcholine micelles when heated for hours at elevated temperatures. Centrifugation of the samples, prepared according to the procedure described elsewhere [12], yielded a supernatant and a

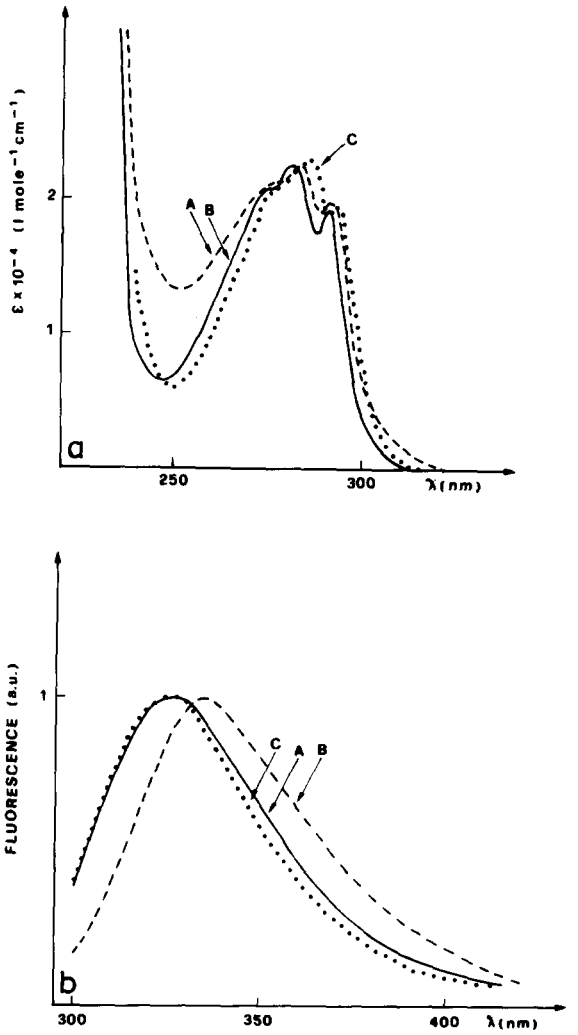


Fig. 1. Absorption spectra (a) and fluorescence emission spectra (b) of Gramicidin A′ in different media: (A) lysophosphatidylcholine, (B) methanol, (C) dioxane.

pellet. The composition of these two fractions showed that the percentages of Gramicidin A′ and lipids found in the supernatant decreased with increasing initial Gramicidin A′ concentration (see Table I).

Absorption and fluorescence spectra

In Fig. 1(a and b) are presented the typical absorption and fluorescence spectra of Gramicidin A′ in association with lysophosphatidylcholine and in organic solvents such as methanol and dioxane. With excitation of the 280 nm band of tryptophan, the shift of the maximum of fluorescence emission from 335 to 327 nm on going from methanol to dioxane is due to the difference in the dielectric constant of the two solvents, and the emission maximum of 328 nm for Gramicidin A′ associated with lysophosphatidylcholine is indicative of a nonpolar environment for the tryptophans. Gramicidin A′ does not give a spectrum in water since it is insoluble in such a solvent; the pellet also when resuspended in water does not give any spectroscopic signal. The supernatant, on the other hand, yields fluorescence and absorption spectra; it contains Gramicidin A′ interacting with the lipid in a dispersed form. Attention is therefore focused on the supernatant.

Centrifugation on sucrose gradient

Fig. 2 shows a typical elution pattern of a 10–30% (w/v) continuous sucrose gradient loaded with the supernatant. Two main populations could be detected at a sucrose concentration of 22.3% ± 0.7 (w/v) and 13.8% ± 1.2 (w/v) corresponding to a density of 1.09 g/cm^3 and 1.05 g/cm^3, respectively.

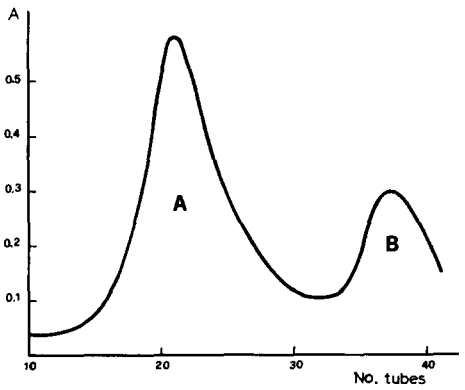


Fig. 2. Elution profile recorded at 280 nm of the sucrose density gradient of the Gramicidin A′/lysophosphatidylcholine mixture after incubation for 20 h at 70°C. See text for discussion.

The analysis of the lipids and polypeptide content of the two fractions showed markedly different compositions. Table I reports the quantitative analysis of fraction A obtained from different preparations in which the initial Gramicidin A′ concentration was varied while the phospholipid concentration was kept constant.

Regardless of the initial concentration of Gramicidin A′, one of the two fractions was characterized by a relatively constant lipid/Gramicidin A′ molar ratio while the other contained a lipid/Gramicidin A′ mixture characterized by a molar ratio widely ranging from 40 to several hundred. What is apparent on comparison of the initial Gramicidin A′ concentration with the concentration of Gramicidin A′ incorporated into fraction A is a saturation process with a preference for 8 to 10 molecules of lysophosphatidylcholine per Gramicidin A′ molecule.

Circular dichroism spectra

Fig. 3 shows the circular dichroism (CD) spectra obtained from the two fractions, A and B. Only fraction A shows a CD pattern that has been taken to be indicative of the single-stranded β-helical structure [25–27] while the spectral pattern exhibited by fraction B is due to polypeptide in a different aggregated state.

Electron microscopy

Negative staining. Fig. 4A illustrates pure lysophosphatidylcholine suspended in buffer, prepared as described in Methods and negatively stained with uranyl acetate on the grid. The lipid was organized as micelles, with a mean diameter of 80.0 ± 0.8 Å (500 measurements). The micelles tended to aggregate and seldom formed stacks. Lysophosphatidylcholine/Gramicidin A′ mixtures, at each molar ratio assayed and when equilibrated at 21°C and observed by negative staining, consisted of a very heterogeneous population of particles (Fig. 4B). Among these particles are readily observed roundish flat vesicles and isolated or stacked micelles. As demonstrated in Fig. 4C at

TABLE I

INCORPORATION OF GRAMICIDIN A′ (GA′) INTO LYSOPHOSPHATIDYLCHOLINE STRUCTURES (PL), FRACTION A

In all the experiments, the initial amount of lysophosphatidylcholine was 25 mg/ml. The molecular weight of lysophosphatidylcholine used for the calculation was 508.

Initial GA′ (mg/ml)	Fraction A				Molar ratio PL/GA′
	GA′		PL		
	mg/ml	mM	mg/ml	mM	
1.20	0.14	0.074	0.36	0.7	9.5 : 1
3.00	0.27	0.14	0.63	1.25	8.1 : 1
6.00	0.34	0.182	0.87	1.72	9.4 : 1
40.00	0.400	0.21	0.860	1.69	8.0 : 1

higher concentrations on the grid where vesicle-like structures are stacked, folded sheets are often observed with dimensions typical of bilayers, i.e., the vesicles are commonly unsealed.

As noted above, this mixture could be separated into two fractions on a sucrose gradient. Fraction A contained, almost exclusively, vesicles with diameters ranging from 40 up to 300 nm (Figs. 5A and 7). A few huge multilamellar vesicles were also present. The great majority of the vesicles, however, appeared to be made of a single or a few lamellae (Figs. 5B and 5C), and exhibited great tendency to aggregate and fuse with time at 21°C. Fraction B (see Fig. 5D) consisted of micelles quite similar to those of pure lysophosphatidylcholine but with a tendency to align in rows. The mean diameter of the micelles in fraction B was 9.9 ± 0.7 nm (850 measurements).

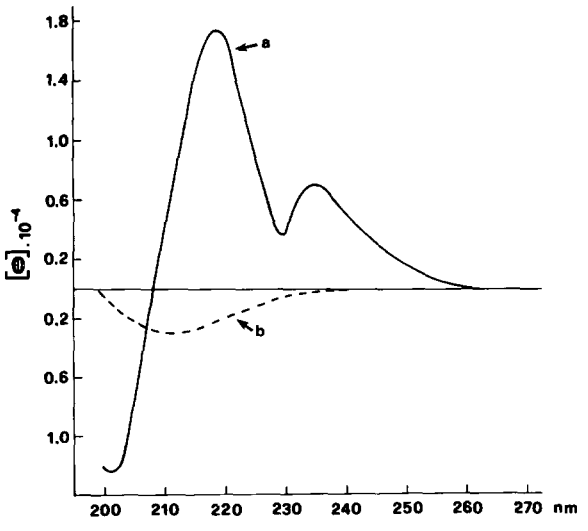


Fig. 3. Circular dichroism of fraction A and fraction B obtained by sucrose density gradient centrifugation. Curve a (fraction A) is the channel state (see text).

Freeze-fracture. Freeze-fracture allowed observation of the effects at the molecular level of Gramicidin A′ on lysophosphatidylcholine in an aqueous medium. Concentrated pure lysophosphatidylcholine suspensions, equilibrated and quenched from 21°C, were shown to consist of heterogeneous polymorphous globules, very often connected by long and thin strands of lipid material. The large globules, when fractured, exhibited smooth fracture surfaces, as shown in Fig. 6A.

Lysophosphatidylcholine/Gramicidin A′ mixtures, at the same lipid concentration and under identical experimental conditions as described above for fraction A, consisted of a heterogeneous population of 30–80 nm wide discrete particles and very large globular structures. The particles, at high magnification, revealed either a monolamellar vesicle structure or a compact homogeneous organization. The large globules, on the contrary, were organized as complex multilamellar liposomes (Fig. 6B). The lamellar thickness, measured by optical diffraction, was 5.5 ± 0.5 nm, which is typical for membrane bilayers.

When the fracture plane runs through the middle of a bilayer, as shown in Fig. 6C, the fracture

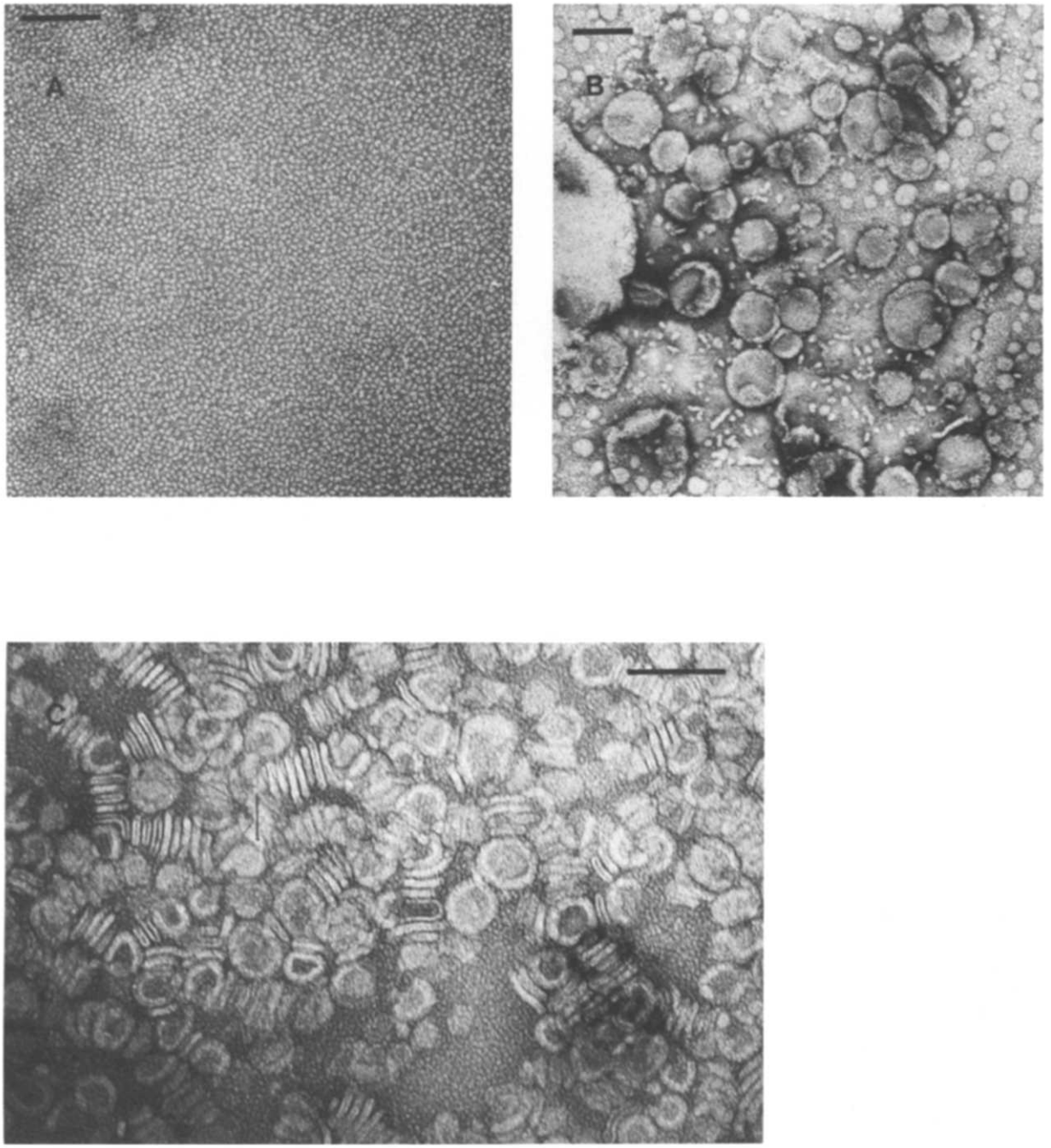


Fig. 4. (A) L-α-Lysophosphatidylcholine in water. The lipid suspension was prepared as described in Methods. The specimen was stained on the grid with 1% uranyl acetate. The suspension consisted of a homogeneous population of particles. 120000×. (B) Lysophosphatidylcholine/Gramicidin A′ mixture obtained as described in Methods. The suspension consisted of micelles, with a tendency to aggregate into rows, and vesicles. Staining on the grid with 1% uranyl acetate. 90000×. (C) At higher concentrations of phospholipid structures on the grid the vesicles tend to align au rouleau, but in a number of places the vesicles are unsealed exhibiting single bilayer sheets, as most obviously exemplified by the U-shaped structure near the center of the print. 150000×. All the bars are 100 nm.

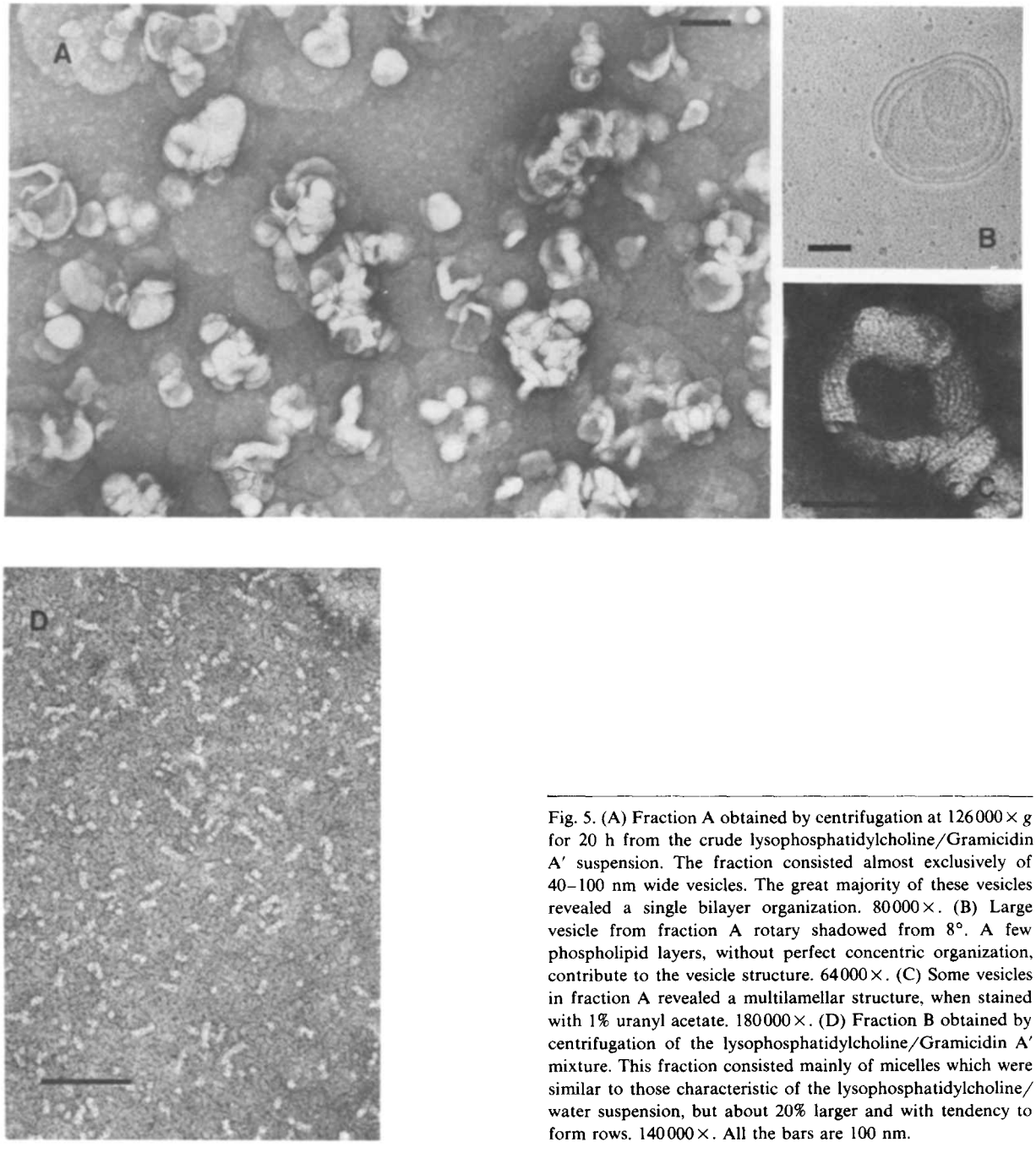
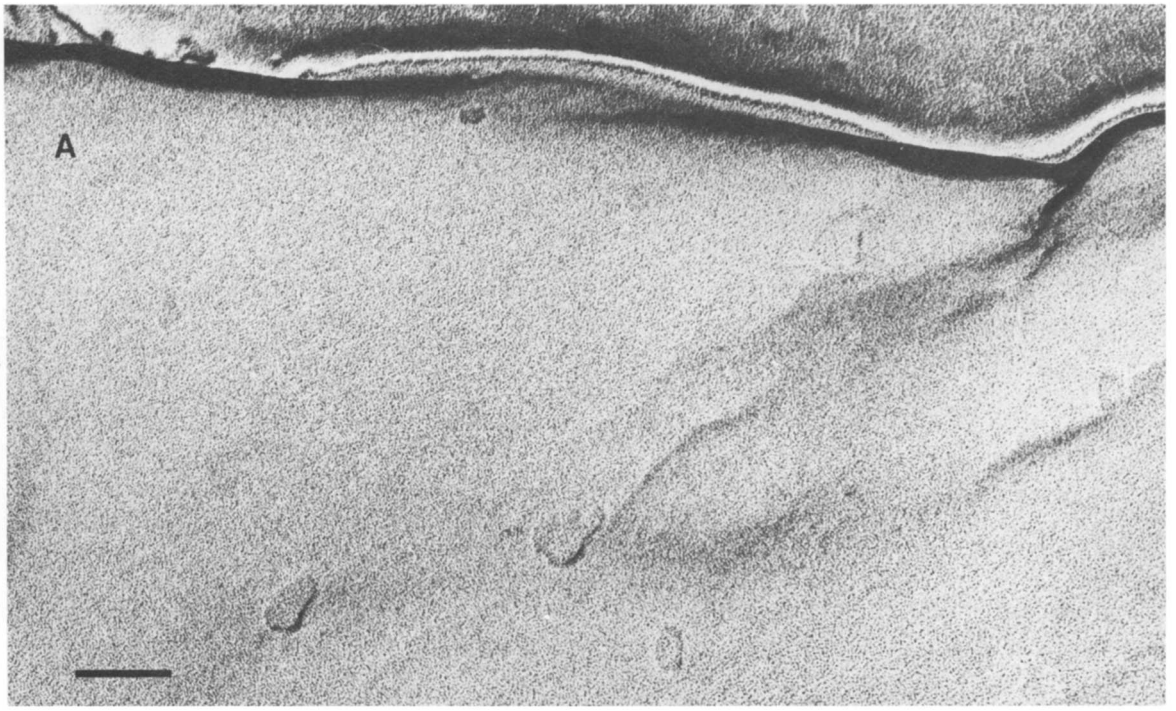


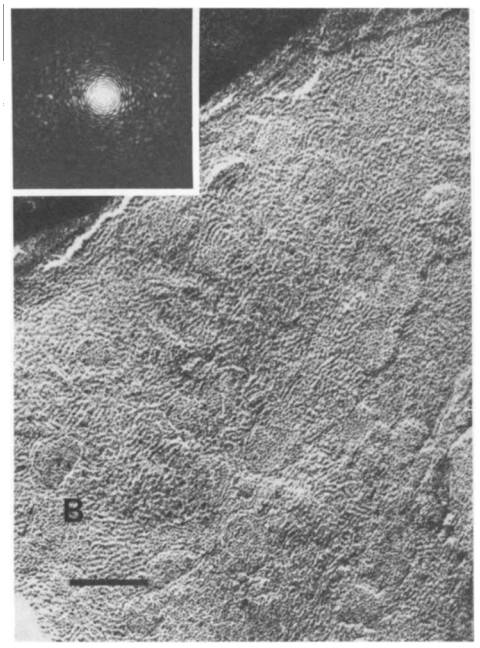
Fig. 5. (A) Fraction A obtained by centrifugation at 126000 × *g* for 20 h from the crude lysophosphatidylcholine/Gramicidin A′ suspension. The fraction consisted almost exclusively of 40–100 nm wide vesicles. The great majority of these vesicles revealed a single bilayer organization. 80000×. (B) Large vesicle from fraction A rotary shadowed from 8°. A few phospholipid layers, without perfect concentric organization, contribute to the vesicle structure. 64000×. (C) Some vesicles in fraction A revealed a multilamellar structure, when stained with 1% uranyl acetate. 180000×. (D) Fraction B obtained by centrifugation of the lysophosphatidylcholine/Gramicidin A′ mixture. This fraction consisted mainly of micelles which were similar to those characteristic of the lysophosphatidylcholine/water suspension, but about 20% larger and with tendency to form rows. 140000×. All the bars are 100 nm.

surface was rough and exhibited areas of rows and patches of particles. By optical diffraction of the micrographs, the most regular areas revealed periodicities of 7.7 nm, that very likely represent the distances between adjacent rows.

Discussion

The heat incorporation of Gramicidin A′ into lysophosphatidylcholine phospholipid structures has been demonstrated to result in two popu-





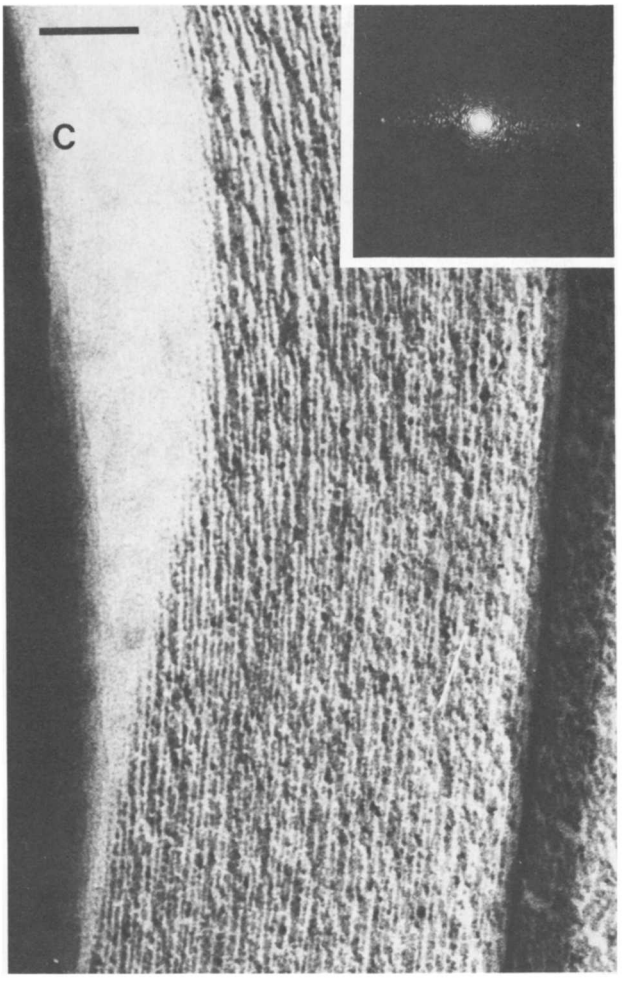


Fig. 6. (A) Freeze-fracture of pure lysophosphatidylcholine in water, when quenched from 21°C. The fracture surfaces were always very smooth. 120000×. (B) Freeze-fracture surface of lysophosphatidylcholine/Gramicidin A′ in water quenched from 21°C. The fracture surfaces were always corrugated by depressions which tended to run parallel to each other at a distance of 7.7 nm, as revealed by optical diffraction (inset). 105000×. (C) Multilamellar organization of a large vesicle in freeze-fracture suspensions of lysophosphatidylcholine/Gramicidin A′ mixtures quenched from 21°C. 130000×. Optical diffraction reveals a 5.1 nm periodicity. All the bars are 100 nm.

lations of lipid/polypeptide states (see Fig. 2). These states are separable by means of differing densities on a sucrose gradient. The less dense band, fraction B, exhibits a variable lipid/peptide molar ratio varying between 300 : 1 and 40 : 1 which depends on the initial mix of lipid and peptide and on the time of incubation [28]. The CD spectrum of fraction B is quite non-descript showing only a weak, broad negative band; and the tryptophan fluorescence emission spectrum has a maximum near 340 nm [28] which indicates a relatively more polar environment for the tryptophans. As seen in Fig. 5D, fraction B remains micellar albeit with enlarged diameters going from about 8 nm for pure lysophosphatidylcholine to 10 nm for lysophosphatidylcholine plus Gramicidin A′. Accordingly fraction B is considered to be a metastable state resulting from an initial association of aggregates of Gramicidin A′ molecules with lysophosphatidylcholine micelles [28].

Fraction A, on the other hand, is a more dense and stable state resulting from the association of peptide and lipid. The greater density is due to the lower lipid to peptide molar ratio, 9 ± 1.0, which occurs regardless of the initial incubation mix of lipid and peptide. The tryptophan fluorescence maximum at 328 nm (see Fig. 1), as well as the limited quenching due to I^- [28] indicates that the tryptophans are in the lipid matrix. ^{13}C-NMR studies also have indicated peptides within the lipid as shown by decreased lipid mobility on heat incorporation [11,12,25]. More specifically, the circular dichroism spectrum of fraction A seen in Fig. 4 has become identified with the left-handed, head-to-head hydrogen bonded, single-stranded $\beta_{3.3}^{6.3}$-helical structure of the Gramicidin channel [16,26,27]. For the numerous reasons given in the Introduction and for additional arguments of ion competition and energies of activation for exchange and transport [11,12,14], the evidence is substantial that the channel state of Gramicidin A shown in Fig. 7 has been obtained in the present phospholipid system. Having established this, the concern becomes one of achieving a description of the supramolecular structure of lysophosphatidylcholine packaged Gramicidin channels.

On heating, the presence of Gramicidin A′ converts micelles to rather well-defined vesicle-like

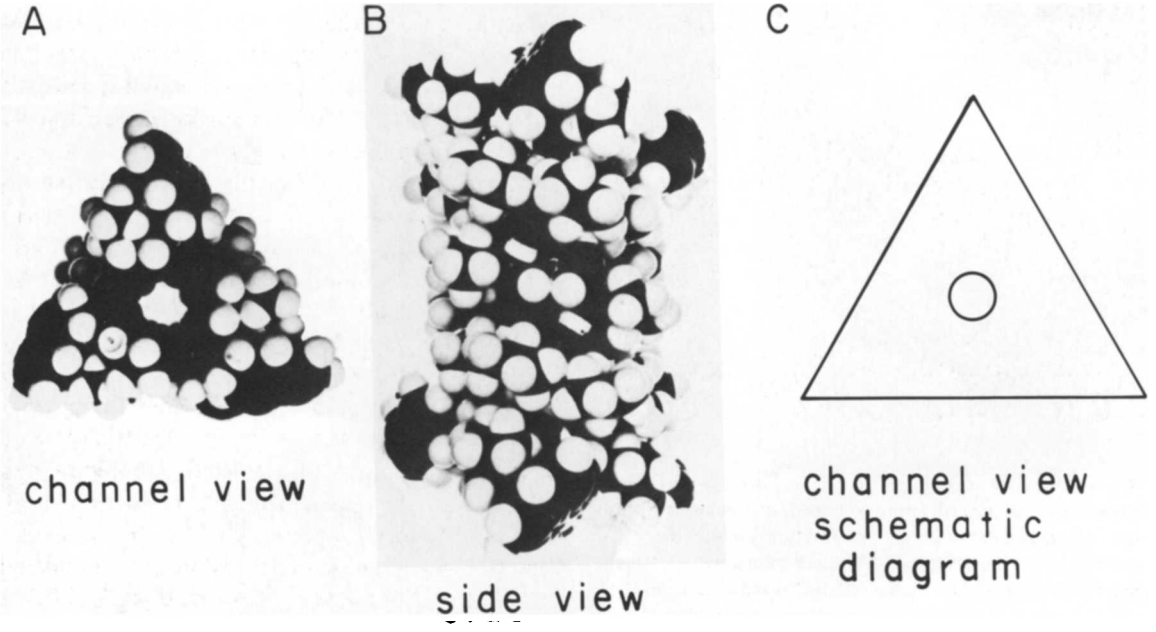


Fig. 7. The channel structure of Gramicidin A. (A) View along channel axis. (B) Side view of channel showing formyl (head-to-head) function. (C) Triangular representation of channel view perspective. This triangular shape and the differences in the apices in terms of numbers of tryptophans can provide a basis for limited aggregation, for example the formation of a hexamer as shown in Fig. 8.

structures; and while the vesicle-like structures may not be well sealed (that is, they are often seen as the folding of bilayer sheets, see Fig. 4C), it is apparent that the presence of Gramicidin A′ has effected the stabilization of a regular lipid bilayer structure (see Fig. 5 A, B and C). Freeze-fracture of the lysophosphatidylcholine and of the vesicle-like Gramicidin A′/lysophosphatidylcholine phospholipid bilayer structure demonstrates the effect of Gramicidin A′ to be one of introducing particles which have a tendency to align in rows separated by 70–80 Å and which tend to recur along the row with a periodicity of 50–60 Å. Our concern, therefore, is to consider aggregational modes of the Gramicidin A′ channel which could give rise to this periodicity.

By means of fluorescence studies, it has been demonstrated that incorporation of Gramicidin A′ into these lysophosphatidylcholine bilayer structures occurs utilizing trytophan-tryptophan contacts as deduced from dramatic quenching of tryptophan fluorescence as fraction A is formed [28]. With the triangular positioning of tryptophans in the channel structure, this argues that the quenching is between tryptophans from different channels, that is, that the aggregation of channels is characterized by trytophan-tryptophan intermolecular interactions. With the tryptophan side chains occurring at the apices of the triangular structure and particularly with Trp_{11} and Trp_{13} each occurring at an apex but with Trp_9 and Trp_{15} sharing the third apex, there is a structural basis for the development of clusters of limited size. This would be the hexagonal packing shown in Fig. 8 which was early considered to be a possible state of aggregation [11]. Interestingly the hexagonal cluster would have a dimeter of from 40–50 Å. Accordingly the implication from the freeze-fracture data is one of a tendency of these hexagonally packaged clusters of Gramicidin channels to form linear arrays. A perfect set of these arrays separated by 77 Å with the hexameric unit recurring at 55 Å and with a lipid headgroup occupying about 50 $Å^2$ [29] would give a ratio of 10–12 lipid molecules per Gramicidin A′ molecule. This is close to the observed ratio (see Table I). While yet a simplistic picture, Fig. 8 becomes a working model for the supramolecular organization and it is sufficiently intriguing to provide optimism that variations in channel structure (primary structure) and the choice of lipid or detergent could well result in a regular two-dimensional packing suitable for more detailed analyses.

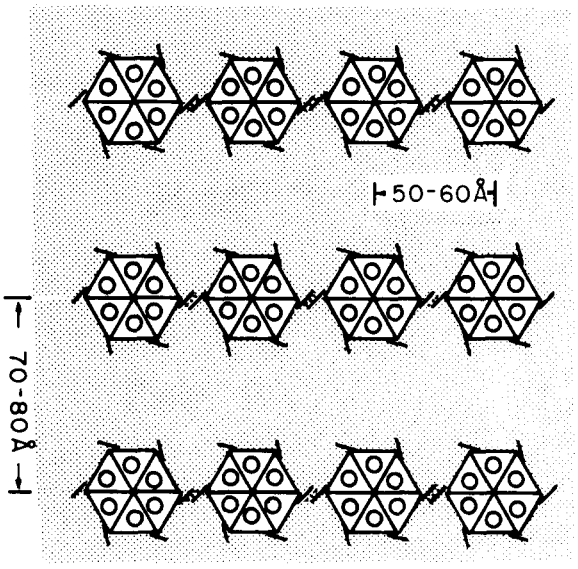


Fig. 8. Working model for the supramolecular organization of Gramicidin A′ in lysophosphatidylcholine membranes. Hexamers are aligned in rows with a periodicity of 50–60 Å and the rows are separated by 70–80 Å. The diameter of the hexamer is 40–50 Å and it is suggested that the tryptophan side chains interact to provide a basis for alignment and additional spacing. The exact number of channels in the aggregate and the size of the aggregate will depend on preferred side chain orientations.

The result of lysophosphatidylcholine packaging of Gramicidin A′ may also be considered in terms of points of possible relevance to the more general study of biomembranes. It is well-accepted that the aggregation of membrane proteins is induced by lowering the temperature below the transition temperature of the membrane phospholipid [30], but here it has been shown that the aggregation of an ion transporting polypeptide can be induced by heating, i.e., by hydrophobic associations when the lipids are yet in a fluid state. Moreover lysophosphatidylcholines are known to play a role in stabilizing membrane structure and in inducing the process of cell fusion [31,32]. The present results raise the possibility that lysophosphatidylcholine structures might also act as carriers of polypeptides during the process of

membrane assembly or during protein replacement in established membranes. Additionally, the perspective is raised that polypeptide itself may facilitate structural changes in lipid organization.

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