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INTERMOLECULAR INTERACTIONS OF GRAMICIDIN A′ TRANSMEMBRANE CHANNELS INCORPORATED INTO LYSOPHOSPHATIDYLCHOLINE LIPID SYSTEMS

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Fluorescence studies are reported on gramicidin A′ incorporated into lysophosphatidylcholine phospholipid structures. The shift in the emission maximum during incorporation and the quenching of fluorescence by I^- and by acrylamide of the incorporated state obtained after prolonged heating are consistent with the presence of the channel state comprised of two single-stranded β^6-helices associated head-to-head (formyl end-to-formyl end). The quantum yield for the incorporated state, when gramicidin A′ is within the lipid matrix, is very low and indicates the occurrence of intermolecular Trp-Trp interactions. Possible interactions between channels within the lipid matrix are discussed utilizing Trp-Trp contacts.

Introduction

Studies on model channels, their conformations and mechanisms, are fundamental to understanding selective permeation of cations through biological membranes. The first conformationally described monovalent cation selective channel is that of gramicidin A′: it has a 4 Å diameter channel with an approximate length of 26 Å [1,2], and derives from head-to-head hydrogen bonded dimerization of two single-stranded β-helices, with 6.3 residues per turn [3–5].

Conformational studies of gramicidin A′ incorporated into lysophosphatidylcholine micelles and phosphatidylcholine liposomes [6] showed that the channel assumes a stable conformation with a circular dichroism pattern that is characteristic of left-handed helices. ^{23}Na- and ^{13}C-nuclear magnetic resonance studies have provided arguments that the stable heat-incorporated state is that of the functional transmembrane channel [7]. A working model for the packaging of channels in micelles has been put forward [7] and rate constants capable of calculating single-channel currents have been experimentally determined [8]. Further knowledge of channel-channel and channel-lipid interactions is required for understanding mechanism in greater detail and the supramolecular organization of the transport system is an important element in enhancing that understanding.

This paper reports steady state fluorescence studies on the quenching of tryptophan fluorescence for the system of gramicidin A′ incorporated into lysophosphatidylcholine phospholipid structures and the studies follow the time course of incorporation. Working models are considered for the packaging of channels in the lipid system.

Materials and Methods

Samples preparation

Gramicidin A′ was purchased from ICN Pharmaceuticals, Inc.-Life Sciences Group, Cleve-

land, OH and was used without further purification; it is a mixture of gramicidin A, B and C at ratios of 7:1:2, respectively. L-α-Lysophosphatidylcholine (Lot No. 50F-8370) was obtained from Sigma Chemical Co., St. Louis, MO, and CH_3OH from Merck and Co., Inc., Rahway, NJ (spectrograde product). All other reagents were of analytical grade.

Samples with different gramicidin A′ to phospholipids ratios were prepared. Micelles of L-α-lysophosphatidylcholine were obtained by suspending 25 mg of phospholipids in 1 ml of 1 mM NaCl. Variable amounts of gramicidin A′, as a dry powder, were then added. Samples were mixed by vortexing, sonified by means of a Braun Labsonic 1510 Sonifier for 3 min at a power of 100 W, incubated for 20 h in a water-bath at 70°C and then centrifuged at 4000 $g \cdot$ min at room temperature. A quantity of 1.2 ml of the supernatant was centrifuged on a 10% to 30% continuous sucrose gradient at 82600 $g \cdot$ min at room temperature, using a Beckman SW 27 rotor. The gradients were eluted in fractions of 0.3 ml by means of a fraction collector. Of the two fractions collected, A and B, the fraction exhibiting the highest absorbance at 280 nm (fraction A) was used for the fluorescence studies [9].

A molar extinction coefficient of 22500 $mol^{-1} \cdot cm^{-1}$ at 281 nm was determined for gramicidin A′ in CH_3OH for the concentration range near 0.5 to 1 mM. The polypeptide concentrations were determined spectrophotometrically. Lysophosphatidylcholine concentrations were determined according to a modified Fiske-SubbaRow method [10].

Fluorescence measurements

Fluorescence measurements were carried out in a Perkin-Elmer MPF 44A spectrofluorimeter equipped with a thermostatically controlled cuvette holder at a temperature of 30 ± 0.5°C. All fluorescence spectra were corrected for monochromator and phototube response by means of a DCSU2 unit. Each measurement was corrected for the light scattering contribution to the signal due to the micelles alone. In the experimental conditions used this contribution was rather small and the only blank signal originated from Raman spectra of water. Source intensity fluctuations were eliminated by recording in the ratio mode.

Quantum efficiency measurements were performed at an excitation wavelength of 280 nm and band widths of 2 nm were used in order to minimize photodecomposition of tryptophan in water [11]. The quantum efficiency values were calculated on the basis of a quantum efficiency of 0.14 for tryptophan in water [11] according to the following equation [12]

$$\Phi_n = \Phi_o \cdot \frac{F_n}{F_o} \cdot \frac{A_o}{A_n} \tag{1}$$

where Φ is the quantum yield, the subscripts 'n' and 'o' denote the unknown and reference, respectively. F is the integrated area of the emission band and A the absorption intensity at the excitation wavelength. The absorbance at the excitation wavelength was < 0.05.

Polarization measurements were performed by means of a Perkin-Elmer polarization accessory. Solutions of approx. 0.2 absorbance units were used in order to increase the fluorescence signal. The intensities of the horizontal (I_H) and vertical (I_V) component of the emitted light were corrected for light scattering contributions by determining them for a reference solution (I_H^S and I_V^S). Polarization (p) was then calculated according to the following equation [13]:

$$p = \frac{(I_V - I_V^S) - G(I_H - I_H^S)}{(I_V - I_V^S) + G(I_H - I_H^S)} \tag{2}$$

where G is the grating correction factor.

In a typical experiment which followed with time the process of gramicidin incorporation into lysophosphatidylcholine phospholipid structures at 70°C, a few microliters of the gramicidin-lipid mixture were diluted to the desired concentration directly in the fluorescence cuvette. The temperature drop from 70°C to 30°C virtually stopped the incorporation process and corrected emission spectra and polarization measurements were obtained.

Experimental details of fluorescence quenching measurements are presented in the legend to Fig. 2. Absorption measurements were carried out in the turbid sample compartment of a Perkin-Elmer 576 spectrophotometer.

Results and Discussion

Fluorescence studies on gramicidin A′ in solution and incorporated into lysophosphatidylcholine

Experiments carried out at different initial phospholipid:gramicidin A′ molar ratios show that the fraction exhibiting the highest absorbance at 280 nm (fraction A) is characterized by a relatively constant phospholipid:gramicidin A′ molar ratio ranging from 8 to 10 [9]. Fluorescence measurements have been carried out on this fraction.

Gramicidin A′, heat incorporated into lysophosphatidylcholine, shows a fluorescence spectrum typical of tryptophan in an apolar solvent (Fig. 1). The emission maximum is at 328 nm close to that in *p*-dioxane (327 nm) but blue shifted with respect to that in methanol (335 nm). The fluorescence polarization value of gramicidin A′ in micelles (λ_{ex} 280 nm, λ_{em} 330 nm) is 0.1, higher than in methanol (0.03), indicating less freedom of rotation of the tryptophan residues of gramicidin A′ within phospholipids. This is to be expected for the channel state incorporated into lipids. In the head-to-head dimerized, single-stranded β-helical structure, the tryptophans would be largely within the lipid in the outer turns of the helical channel [1,2,14] near the lipid-polar interface. In this channel structure, the Trp^{15} residue would be the most accessible from the polar phase with tryptophans 13, 11 and 9 becoming increasingly buried within the lipid. This is, of course, a result of the primary structure of gramicidin A, HCO-LVal^1-Gly^2-LAla^3-DLeu^4-LAla^5-DVal^6-LVal^7-DVal^8-LTrp^9-DLeu^{10}-LTrp^{11}-DLeu^{12}-LTrp^{13}-DLeu^{14}-LTrp^{15}-$HNCH_2CH_2OH$ [15,16] and the head-to-head (amino end-to-amino end) hydrogen bonded dimerization of the β-helical structures [2].

In order to have a better understanding of the orientation of the channel within the lipid structure and the disposition of the tryptophans, fluorescence quenching measurements have been carried out using I^-, a collisional charged quencher of tryptophan fluorescence [17] that does not penetrate the lipid matrix, and acrylamide, a neutral quencher capable of selectively quenching tryptophan fluorescence in proteins [18] and micelles [19]. I^- quenching of gramicidin A′ fluorescence is shown in fig. 2. The Stern-Volmer plot presents a noticeable downward curvature indica-

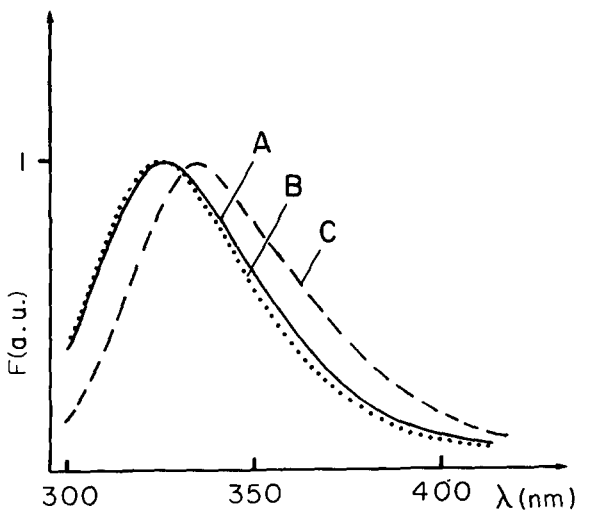

Fig. 1. Fluorescence emission spectra of gramicidin A′ in different media: A, lysophosphatidylcholine; B, *p*-dioxane; C, methanol. λ_{ex} 280 nm; temp. 30°C.

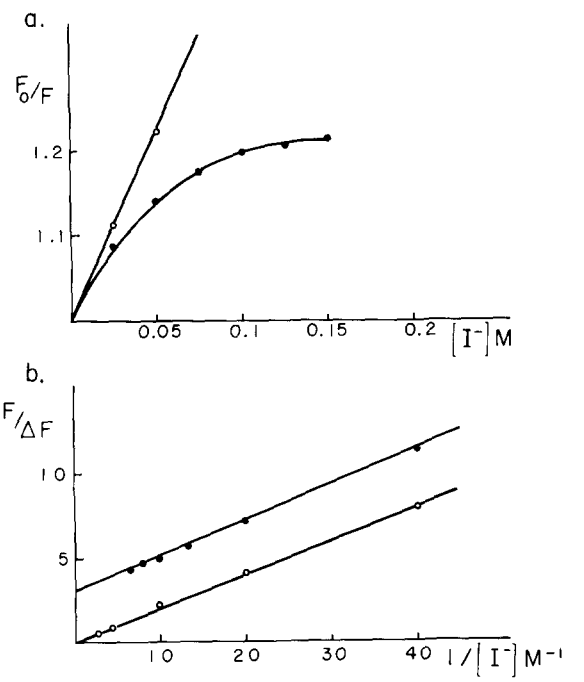

Fig. 2. (a) Stern-Volmer plots of the quenching of gramicidin A′ fluorescence by I^- in methanol (○——○) and in lysophosphatidylcholine (Fraction A) (●——●). (b) Inverse Stern-Volmer plots of the quenching of gramicidin A′ fluorescence by I^- in methanol (○——○) and in lysophosphatidylcholine (Fraction A) (●——●). λ_{ex} 280 nm; λ_{em} 340 nm; temp. 30°C.

tive of tryptophans embedded in different environments [20]. Conversely the acrylamide quenching Stern-Volmer plot (Fig. 3a) is virtually a straight line; only at low quencher concentration is a small downward curvature suggested. These results can be explained by assuming that incorporated gramicidin A′, on the average, exposes about one tryptophan residue to quenching by I^- from the aqueous environment, since I^- in the absence of I_2 cannot enter the lipid. Acrylamide, on the other hand, penetrates the lipid structure and shows a higher quenching capability. The inverse Stern-Volmer plots (Figs. 2b and 3b) support such an interpretation. The maximum accessible fluorescence for I^- is about 30%, while for acrylamide it is greater than 70% suggesting that about one tryptophan per molecule of gramicidin A′ is quenched by I^- while three-fourths are quenched by acrylamide which, though a neutral quencher, cannot penetrate deeply into the lipid layer. These results are in reasonable agreement with those previously reported [21] on fluorescence quenching of a series of *N*-(9-anthroyloxy) fatty acid probes by gramicidin A′ incorporated into dimyristoylphosphatidylcholine liposomes.

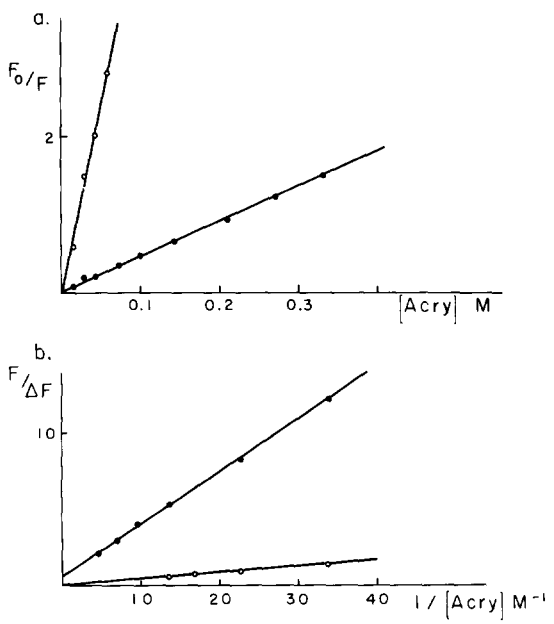

Fig. 3. (a) Stern-Volmer plots of the quenching of gramicidin A′ fluorescence by acrylamide in methanol (○——○) and in lysophosphatidylcholine (●——●). (b) Inverse Stern-Volmer plots of the quenching of gramicidin A′ fluorescence by acrylamide in methanol (○——○) and in lysophosphatidylcholine (●——●).

Fluorescence measurements of gramicidin A′ during incorporation

Elevated temperatures are utilized to combine the gramicidin A′ molecules with lysophosphatidylcholine micelles and to form phospholipid-packaged channels. It is known that high temperatures can induce hydrophobic association in peptides [22] and it has been proposed [23] that the mechanism of incorporation begins with formation of aggregates of the polypeptide at the interface of the lipid layer and proceed to the formation of active channels. Interest here is on understanding the mechanism whereby temperature drives the incorporation of the polypeptide into lipid structures and particularly on the interactions occurring between the incorporated gramicidin A′ molecules. Monitoring absorbance at 280 nm, the dependence on incubation time of the extent of incorporation of gramicidin A′ at an initial concentration of 3 mg/ml is reported in Fig. 4. The absorbance increases with time as the gramicidin A′ slowly incorporates into the lipid matrix. The experiments have been performed in the turbid sample compartment such that the light scattering effects are minimized. Interestingly, concomitant fluorescence measurements show that the emission maximum shifts from 340 nm to 328 nm, clearly demonstrating that the tryptophans are moving into a more apolar environment.

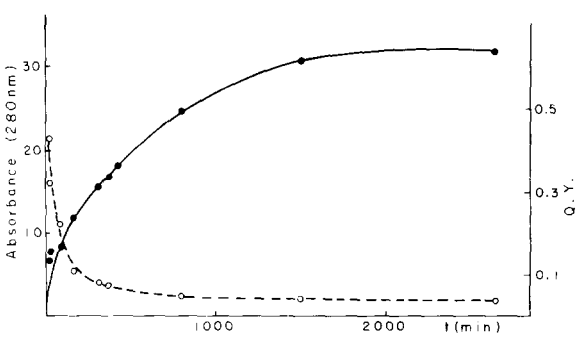

Fig. 4. Absorbance (●——●) and fluorescence quantum yield (Q.Y.) (λ_{ex} 280 nm) (○------○) of gramicidin A′ in lysophosphatidylcholine measured during the incorporation at 70°C.

Also in Fig. 4 the dependence of fluorescence quantum yield on incubation time is reported. Particularly noticeable is the high value of the quantum yield, 0.4, exhibited by gramicidin A′ immediately after sonication but prior to heating, which is to say before the process of incorporation has begun. Such a value is close to that of gramicidin A′ in TMP *, the highest value found in organic solvents (Cavatorta, P., unpublished resuts). At the same stage, the emission maximum is observed at 340 nm, which is the fluorescence maximum previously found in lipid bilayers for *N*-stearoyltryptophan by Haigh et al. [21]. Experiments before heating on tryptophan fluorescence quenching by I^- and acrylamide showed that no quenching occurs, even at very high quencher concentration. Presumably when gramicidin A′ is clustered on the surface of the micelle, the tryptophans are neither accessible from the lipid nor from solution. These results suggest that prior to heat incubation, gramicidin A′ is aggregated on the surface of the micelles with only a few molecules of the antibiotic embedded in the lipid phase. The initial high quantum efficiency and the absence of quenching followed after incorporation by a low quantum efficiency and high acrylamide quenching indicate that the aggregated molecules on the surface do not allow quenchers to penetrate, and that the incorporated polypeptide molecules are located with tryptophan in contact with the lipid matrix. That the decrease in quantum yield is caused neither by photodestruction nor by thermodestruction is shown by dissolving the sample in CH_3OH which gives rise to an immediate increase of fluorescence intensity and a quantum yield similar to that of gramicidin A′ in the same solvent. In order to understand if the decrease in quantum yield is caused by quenching occurring when polypeptide interacts with the lipids, quenching experiments have been carried out with tryptophan and choline or phosphocholine. The absence of quenching by these moieties indicates that the polar heads of the phospholipids are not responsible for the decrease in quantum yield reported in Fig. 4. In Fig. 5 is reported the quenching of *N*-acetyltryptophan methyl ester in order to test if the acyl chains of the phospholipid could decrease

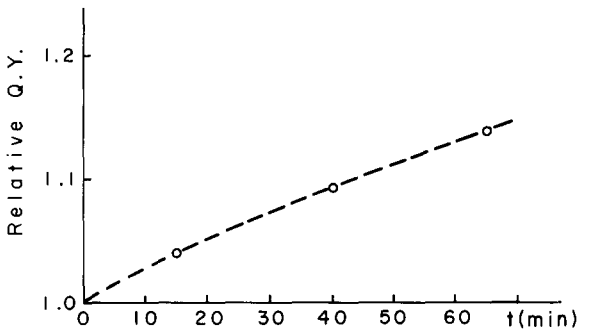

Fig. 5. Time dependence of the fluorescence quantum yield of *N*-acetyltryptophan methyl ester in presence of lysophosphatidylcholine micelles.

the quantum efficiency of tryptophan. Not only is there an absence of any quenching effect but rather there is a slight increase in the fluorescence parameter.

Such an increase of tryptophan fluorescence, which has also been observed when glucagon or myelin protein interact with lysophosphatidylcholine [24–26], characteristicaly occurs when tryptophan becomes buried in a lipid matrix. Thus the decrease in quantum yield seen in Fig. 4 indicates a process unique to the incorporated state of gramicidin A′. Tryptophan fluorescence quenching reactions occuring in proteins have been previously identified by means of suitable model systems [27]. Such studies include quenching by vicinal disulphide links, the imidazolium ion, the α-ammonium group of the N-terminus, vicinal ϵ-amino groups and vicinal sulphydryl groups. However no such groups are present in gramicidin A′ and no fluorescence variation is detected when pH is changed from 3 to 9. Since tryptophan interactions with phospholipids and the other amino acid side chains can be excluded as a possible source of quenching, the apparent mechanism would seem to arise from Trp-Trp interactions. Such quenching has been reported by Konev [28] to occur in solution at high tryptophan concentrations. The quenching can be collisional between a molecule in the excited state and one in the ground state, or it can be static or both. In any case, the results are indicative of association of gramicidin A′ molecules utilizing Trp-Trp contacts. Interestingly, gramicidin A′ fluorescence quenching has been

* TMP, trimethyl phosphate.

observed in organic solvents and has also been attributed to molecular aggregation [29]. Thus it is reasonable to consider that there are associations of gramicidin A′ channels which involve close interactions between tryptophan side chains.

On the association of channels within the phospholipid matrix

In this effort to characterize further the structure and process of lysophosphatidylcholine packaging of gramicidin A channels, the fluorescence studies have supported general structural features of the channel and have added the significant element of intermolecular Trp-Trp interactions. In considering these interchannel interactions, the disposition of the tryptophans in the channel structure is noted in Fig. 6. In particular, in Fig. 6A it is seen that the tryptophans constitute the corners of a triangular structure. Using the schematic representation given in Fig. 6C, possible modes of association which emphasize the Trp-Trp contacts are given in Fig. 7. In Fig. 7A a network of channels is shown in which the interchannel contacts are only at the apices i.e. utilizing only Trp-Trp contacts. In this structure the lipid to gramicidin A molar ratio is very large about 45 : 1, whereas the experimental values are 8 or 10 to one [9]. The hexagonal packing of channels seen in Fig. 7B was previously considered as a possible state of aggregation within the lysophosphatidylcholine micelle [7]. This structure has the advantage of structurally discriminating the apices because a single tryptophan occurs at two apices and both the tryptophans 15 and 9 occur at the third apex. A micelle of lysophosphatidylcholine contains about 180 lipid molecules such that the molar ratio for the structure Fig. 7B in a micelle would be of the order of 15 lipid molecules per gramicidin A molecule. A structure, which on the basis of a micelle, would have about the correct ratio is seen in Fig. 7C where there would be 24 gramicidin A molecules per 180 lysophosphatidylcholine molecules.

It, of course, stretches credibility that the micelle could simply expand to accommodate larger and larger clusters of channels. The association of channels within a micelle, however, could be the initial process before micelles themselves associate to form sheets or even vesicles as has been observed by electron microscopy using negative

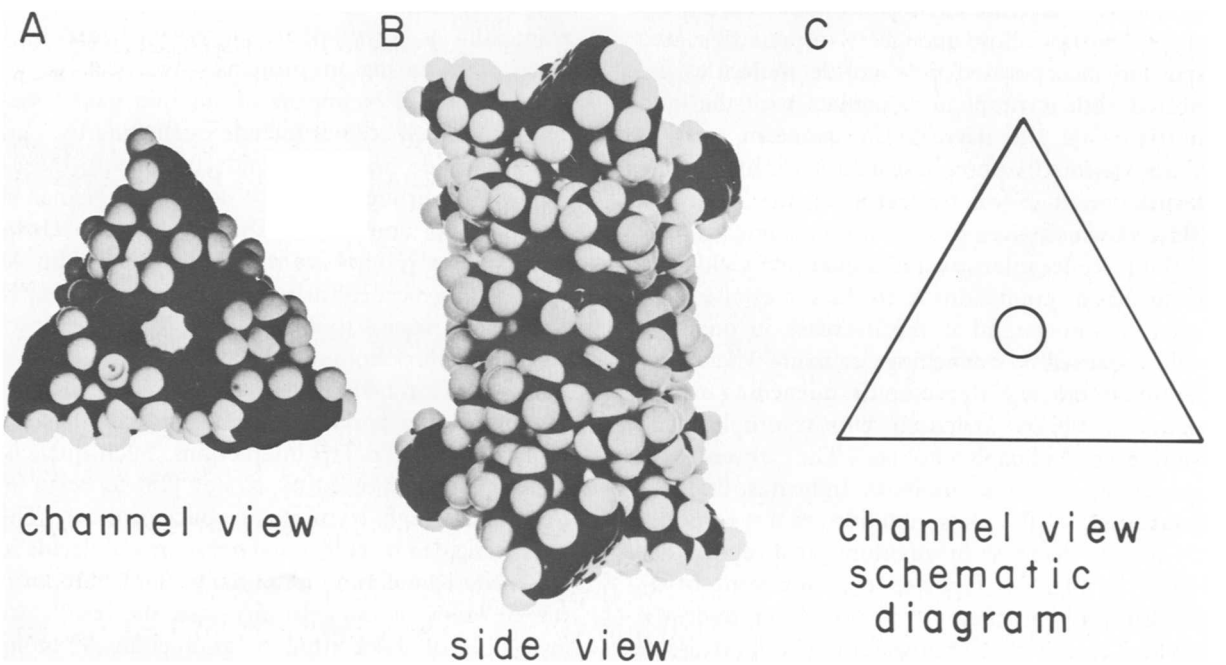

Fig. 6. The channel structure of gramicidin A′ represented by space filling models. (A) Channel view. (B) Side view (from Ref. 1). (C) Schematic representation of channel view.

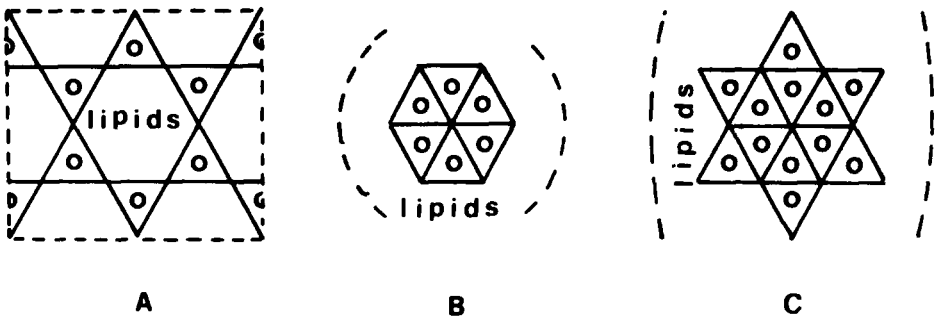

Fig. 7. Models considered for the modes of gramicidin A′ packaging in lipid structures.

staining techniques [9]. Our analysis, here, for considering the association of channels is based on the fluorescence data arguing for quenching of tryptophan fluorescence by means of Trp-Trp interactions. The importance of tryptophan in the lysophosphatidylcholine packaging of channels has been demonstrated in an entirely independent way. It has been found that the Phe^{9}, Phe^{11} and $Phe^{9,11,13,15}$ gramicidin analogues do not incorporate into lysophosphatidylcholine phospholipid structures (Urry, D.W., Prasad, K.U. and Trapane, R.J., unpublished data). Thus the importance of Trp-Trp intermolecular interactions is further emphasized for this particular process of phospholipid packaging of gramicidin channels. Further characterization of the lipid-channel product using cryofracture and negative staining electron microscopy techniques can be used to delineate among the structures considered in Fig. 7 or indeed to provide consideration of other channel-channel arrangements [9].

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