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Conformational Transitions of Gramicidin A in Phospholipid Model Membranes. A High-Performance Liquid Chromatography Assessment†

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ABSTRACT: We have investigated the conformation of gramicidin A reconstituted in different phospholipid environments, small unilamellar vesicles, extensive bilayers, and micelles, by exploiting a recently proposed experimental approach based on high-performance liquid chromatography [Bañó et al. (1988) *J. Chromatogr. 458*, 105; Bañó et al. (1989) *FEBS Lett. 250*, 67]. The method allows the separation of conformational species of the peptide, namely, antiparallel double-stranded (APDS) dimers and $\beta^{6.3}$-helical monomers, and quantitation of their proportions in the lipid environment. Various experimental parameters (e.g., nature of organic solvent, time of incubation in organic solvent, lipid-to-peptide mole ratio, time of sonication, and temperature) commonly involved in sample preparation protocols have been analyzed independently. The results show how the peptide conformation in model membranes is exquisitely dictated by the particular nature of the reconstitution protocol. In addition, we have elucidated the nature of the slow conformational transition of gramicidin toward the channel configuration that takes place upon incubation of the model membranes. This transition has been characterized as a temperature-dependent conversion from APDS dimeric to $\beta^{6.3}$-helical monomeric forms. Analysis of kinetic data permits an accurate calculation of the rate constant for this process at different temperatures in phospholipid vesicles and micelles. Finally, an explanation is proposed for the laboratory-to-laboratory variation in the observed spectral patterns of inserted gramicidin. Evidence is presented that a given circular dichroism spectrum can be attributed mainly to the contributions of two well-defined conformational species, APDS dimers and $\beta^{6.3}$-helical monomers, coexisting in the lipid environment in a proportion which is in turn determined by the specific protocol followed for sample preparation.

The conformation of the transmembrane channel forming hydrophobic pentadecapeptide gramicidin A, in organic solvent and especially in phospholipid model membranes, has been the subject of extensive study in a number of laboratories for the last 2 decades. As a result, a variety of different models have been suggested for its structure, essentially inferred from spectroscopic techniques such as circular dichroism (CD),[1] nuclear magnetic resonance (NMR), and infrared spectroscopy (IR) [for reviews, see Urry (1985), Wallace (1986, 1990), and Cornell (1987)], diffraction studies (Wallace & Ravikumar, 1988; Langs, 1988, 1989), and theoretical considerations (Venkatachalam & Urry, 1983; Pullman & Etchebest, 1987). Among the most relevant, parallel (PDS) and antiparallel double-stranded (APDS) helical dimers and head-to-head (HH), head-to-tail (HT), and tail-to-tail (TT) β-helical dimers have been proposed. It has been thoroughly demonstrated and seems at present well established that the HH dimer (N-terminus-to-N-terminus dimer) is actually the predominant configuration of the active transmembrane cation channel (Urry et al., 1983a; Andersen, 1984; Wallace, 1986). PDS, HT, and TT dimers appear to be much less stable than APDS and HH dimers on the basis of their diminished ability for hydrogen bonding (Sung & Jordan, 1989), and, moreover, available evidence has not supported so far their presence in reconstituted model membranes. As concerning APDS dimers, however, recent experimental work by Durkin et al. (1987) and theoretical molecular mechanics studies (Sung & Jordan, 1988, 1989) have led to a reconsideration of the possible significance of this configuration by suggesting that, if formed in the membrane, it might also behave as an ionic channel.

CD spectroscopy (and to a lesser extent NMR) has demonstrated in the past to be a very sensitive technique to show that gramicidin can adopt different conformations in different lipid environments (Urry et al., 1979; Masotti et al., 1980; Wallace et al., 1981) and that the spectra can also vary considerably depending on the conditions used for reconstitution (Urry et al., 1982, 1983a,b). In this context, samples that were prepared by cosolubilization of gramicidin and phospholipid exhibited different CD patterns depending on the peptide:lipid

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[1] Abbreviations: APDS, antiparallel double stranded; CD, circular dichroism; DMSO, dimethyl sulfoxide; HH, head-to-head; HPLC, high-performance liquid chromatography; IR, infrared; LPC, egg yolk lysophosphatidylcholine; MLV, multilamellar vesicle(s); NMR, nuclear magnetic resonance; PC, egg yolk phosphatidylcholine; SUV, small unilamellar vesicle(s); TFE, trifluoroethanol; THF, tetrahydrofuran.

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mole ratio and/or the heat treatment conditions (Sychev & Ivanov, 1982; Shungu et al., 1986; Wallace, 1986). Other laboratories have prepared samples by adding gramicidin either as a dry powder or from organic solution to preformed micelles or vesicles. Such preparations require extensive incubation at elevated temperatures (around 70 °C) to achieve the characteristic spectral features of the gramicidin HH dimer channel state (Urry et al., 1979, 1985, 1986; Spinsi et al., 1979; Masotti et al., 1980). On the basis of the above observations, it has been suggested that a mixture of gramicidin conformers may be present in different proportions in different reconstitution experiments, the double-helical dimer being a possible intermediate in the membrane insertion process (Wallace, 1986).

In the last years, interest has been particularly spurred by the observation in several laboratories that the conformational versatility of gramicidin inserted in a lipid environment is further complicated by a remarkable dependence on the "history" of the sample preparation protocol (Killian et al., 1988; LoGrasso et al., 1988; Bañó et al., 1988, 1989). A recent demonstration of how the gramicidin folding motif varies with the reconstitution protocol is seen in CD and NMR studies carried out with hydrated lipid bilayers (Killian et al., 1988; LoGrasso et al., 1988) and micelles (Killian & Urry, 1988). In particular, CD data revealed that it is the conformational behavior of gramicidin in the organic solvent which determines the peptide conformation in the model membrane (Killian et al., 1988). Although a great variety of solvent systems have been used for gramicidin incorporation (Shungu et al., 1986; Tournois et al., 1987; Takeuchi et al., 1990), it seems that only with TFE or DMSO it is possible to directly incorporate the peptide in the $\beta^{6.3}$-helical (channel) configuration (Masotti et al., 1980; Tournois et al., 1987; Killian et al., 1988; LoGrasso et al., 1988). However, if gramicidin is inserted from less polar solvents as a "nonchannel state", a conformational transition can be induced in the lipid environment by sonication and heating of the samples which leads to a final spectrum indicative of the functional conformation (LoGrasso et al., 1988). Due to the fact that this conversion also seems to occur at room temperature (though extremely slowly), it has been concluded that the thermodynamically preferred membrane conformation is the $\beta^{6.3}$-helical one (Killian et al., 1988). It is important to emphasize that, although a great deal of spectroscopic information on the conformational behavior of gramicidin in model membranes is at present available, the exact nature of the observed spectral changes often remains uncertain at a molecular level. On the other hand, in relation to the effect of the organic solvent, it has been recently reported that the single-channel properties of gramicidin in planar lipid bilayers show no solvent history dependence (Sawyer et al., 1990), although this discrepancy relative to spectroscopic data seems to be only apparent and is likely due to the much lower peptide:lipid ratios used in the conductivity studies (Sawyer et al., 1990).

Recently, we have described a novel methodology based on high-performance liquid chromatography (HPLC) which allows, for the first time, the direct visualization of monomers and dimers of gramicidin incorporated in model membranes (Bañó et al., 1988). Briefly, the strategy permits a complete resolution of APDS dimers and β-helical monomers after eluent disruption of the phospholipid assemblies, whose proportion reflects the actual composition in the original membrane. By using HPLC and CD in combination and taking into account our previous knowledge of the dimer–monomer conformational equilibrium of gramicidin in organic solvent (Braco et al., 1986a, 1987, 1988a,b), it was possible to quantitatively account for the reported solvent dependence of the inserted peptide conformation (Killian et al., 1988; Killian & Urry, 1988; LoGrasso et al., 1988). It is now clear from HPLC data that it is the dimer-to-monomer ratio in the organic solvent which determines the conformation observed in the resulting fresh phospholipid model system (Bañó et al., 1989). When APDS dimers predominate in the organic solution (benzene, THF, chloroform, etc.), this is basically the conformation in the membrane, the corresponding CD spectra displaying pronounced nonchannel characteristics. On the contrary, when gramicidin is incorporated from solvents such as methanol or TFE where >95% of monomeric forms are present, HPLC reveals a similar percentage of monomers in the vesicles which corresponds to a typical CD $\beta^{6.3}$-helical conformation (Bañó et al., 1989).

With such an HPLC approach, we show in the present paper that it is now possible to obtain a more detailed molecular picture of gramicidin incorporated in a phospholipid model membrane and to monitor further time-dependent conformational transitions of the peptide while it is in the lipid environment. This allows us, for the first time, to accurately determine rate constants for the process of dissociation of APDS dimers in different lipid environments, small unilamellar vesicles (SUV), micelles, and multilamellar vesicles (MLV). In addition, a systematic analysis, as independent as possible, has been carried out of each one of the commonly used experimental variables involved in sample preparation protocols (e.g., organic solvent, sonication time, lipid-to-peptide mole ratio, incubation temperature, etc.). As a result, a quantitative explanation based on a simple equilibrium model can now be proposed, accounting for gramicidin spectroscopic observations in terms of concrete individual conformational species of the peptide, i.e., APDS dimers and $\beta^{6.3}$-helical monomers.

Materials and Methods

Materials. Gramicidin (natural mixture) was supplied by Koch Light Labs. (Buckinghamshire, U.K.) and was used without further purification. Egg yolk lecithin (PC) was purchased from Merck (Darmstadt, FRG) and purified according to Singleton et al. (1965). Egg yolk L-α-lysophosphatidylcholine (LPC) was from Sigma Chemical Co. (St. Louis, MO). Tetrahydrofuran (THF) and all other organic solvents were either HPLC or spectroscopic grade. THF was passed through a 0.45-μm regenerated cellulose filter (Micro Filtration Systems, Dublin, CA) before use.

Sample Preparation. Gramicidin-containing liposomes were prepared as in Bañó et al. (1988): typically, PC and gramicidin were codissolved in a given organic solvent by mixing identical volumes (100 μL of each unless otherwise stated) of stock solutions. The solvent was rapidly evaporated under a nitrogen stream and later under high vacuum overnight to ensure complete removal of traces. Milli-Q-grade water (1 mL) was then added, and the lipid was hydrated under vortexing for 10 min at room temperature. The opaque suspension of multilamellar vesicles was next sonicated for 10 min on ice by using an ultrasonic generator with a microtip probe (Vibra cell, Sonics and Materials, Inc., Daubury, CT) at power setting 4 and 50% duty cycle. After sonication, the samples were centrifuged for 15 min at 35000g to remove probe particles and the remaining multilamellar aggregates. The lipid content in the resulting SUV was determined by a phosphorus assay (Dittmer & Wells, 1968), and the gramicidin content was evaluated by measuring the absorbance at 280 nm after dilution of the sample in methanol using a molar extinction coefficient of 20700 $cm^{-1} \cdot M^{-1}$ (Killian et al., 1988). The final

gramicidin concentration was 0.074 mg/mL. The lipid: gramicidin mole ratio was 50, practically equal to that of the initial mixture. For kinetic measurements, particularly at room temperature or lower, the samples were deliberately incubated for a long time to ensure an appropriate monitoring of the conformational transitions, enabling a further reliable determination of rate constants. The integrity of SUV preparations was controlled by negative-stain electron microscopy and size-exclusion HPLC using TSK G6000PW and G5000PW columns in series (Ollivon et al., 1986).

Gramicidin-containing micelles were prepared according to Masotti et al. (1980) with some modifications. In all cases, 10 μL of a 7.4 mg/mL stock solution of gramicidin in a given organic solvent was added to 1 mL of preformed LPC micelles. After 5-min vortexing at room temperature, the samples were sonicated for 10 min (power setting 4). Insoluble, nonincorporated gramicidin was centrifuged into a pellet for 15 min at 15000*g*. The final peptide and lipid content was determined as described above. Alternatively, gramicidin concentration was checked by HPLC. In other experiments, micelles were also prepared by cosolubilization in chloroform in a similar way as for preparation of PC vesicles.

Any other details of experimental conditions are given in the corresponding figure legends.

High-Performance Liquid Chromatography. The liquid chromatograph (from Waters Chromatography Division, Millipore, Milford, MA) and the general experimental conditions for elution were as previously described (Bañó et al., 1988). Briefly, the method consists of the direct injection of a few microliters of an aqueous suspension of the gramicidin-containing model membrane onto an Ultrastyragel 1000-Å column, which is eluted with a nonpolar solvent such as THF. The phospholipid assemblies are immediately disrupted on top of the column by the equilibrating organic solvent, releasing the peptide conformational species and lipid molecules to the eluent stream. Since the dimer–monomer transition in THF is extremely slow as compared to the elution time (Braco et al., 1986a; Bañó et al., 1988), the percentage of APDS dimers and β-helical monomers in a given chromatogram can be considered as a very reliable, accurate measure of their actual proportion in the original phospholipid model system. All chromatographic measurements were made in triplicate, and the standard deviation was always <3%.

Circular Dichroism. CD measurements were performed at 25 °C with a Jobin Yvon Mark III spectropolarimeter using a 0.5-mm optical path-length cell. Blank runs of vesicles or micelles in water were subtracted from the measured spectra of gramicidin-reconstituted samples. CD results were expressed as mean residue weight ellipticities, in units of degrees centimeter squared per decimole. The reported spectra are the average of three scans from two independent preparations of each sample. For a strict comparison of CD and HPLC results, aliquots of the same SUV suspension were simultaneously measured by both techniques after different incubation times at the selected temperature.

Results

Dependence of the Gramicidin Conformation in SUV on the Organic Solvent. As a number of groups have recently reported, the conformational state of gramicidin incorporated into freshly prepared liposomes can vary over a wide range depending, among other factors, upon the nature of the solvent(s) used for cosolubilization (LoGrasso et al., 1988; Killian et al., 1988; Killian & Urry, 1988). This has also been recently verified by HPLC (Bañó et al., 1989), as summarized in the first column of Table I. The trend observed by chromatography is quite clear: an increase in solvent polarity results in a decrease in the percentage of APDS dimers concomitant with an increase in monomeric forms ($\beta^{6.3}$-helical configuration). Thus, the conformation of the polypeptide inserted in the bilayer somewhat reflects the situation in the starting organic solution before evaporation to form the lipid film. Interestingly, when these SUV prepared from different solvents were incubated at room temperature for several hours, HPLC analysis revealed in all cases a progressive dissociation of APDS dimeric forms regardless of their initial proportion, which is a clear indication of a slow transition toward the thermodynamically favored $\beta^{6.3}$-helical conformation (see Table I).

Table I: Mass Fraction of Gramicidin APDS Dimers Determined by HPLC in SUV Prepared by Cosolubilization from Different Organic Solvents[a]

solvent	vesicle incubation time (h)			
	0	6	24	48
benzene	0.82	0.79	0.70	0.58
THF	0.75	0.72	0.63	0.53
benzene/methanol (95:5)	0.73	0.70	0.61	0.52
chloroform	0.70	0.67	0.59	0.50
ethanol	0.55	0.53	0.46	0.39
chloroform/methanol (2:1)	0.18	0.17	0.15	0.13
methanol	0.08	0.07	0.07	0.06
TFE	<0.02	<0.02	<0.02	<0.02

[a] Vesicles were incubated in all cases at room temperature.

Among the solvents listed in Table I, THF was chosen for cosolubilization in most of the present work because (i) it is the chromatographic mobile phase (Bañó et al., 1988), (ii) it has a polarity similar to other solvents (chloroform, benzene/methanol 95:5 etc.) widely used in the preparation of gramicidin-containing liposomes (Lee et al., 1984; Massari & Colonna, 1986; Cornell et al., 1988), and is a fairly good lipid and gramicidin solvent, and (iii) we had previous accurate information on the gramicidin self-association equilibrium in THF (Braco et al., 1986a,b). Since HPLC has proved to be an excellent technique to monitor changes in the APDS dimer and $\beta^{6.3}$-helical monomer composition in model membranes (Bañó et al., 1989), it seemed exciting to verify the possibility of obtaining detailed kinetic information on the conformational transitions taking place upon incubation of the vesicles, relating it to more conventional spectroscopic data. For this purpose, aliquots of a fresh sample of gramicidin-containing SUV, prepared by cosolubilization in THF, were incubated at room temperature and simultaneously analyzed at different times by HPLC and CD. The chromatographic results, illustrated in Figure 1A, show how HPLC permits the direct visualization of the slow transition undergone by gramicidin in the lipid environment, from an initial situation where the APDS dimers are predominant toward a final equilibrium state where the $\beta^{6.3}$-helical forms are clearly favored. In fact, when an aliquot of this sample was heated overnight at 68 °C, the corresponding chromatogram yielded >98% of monomers (see Figure 1A).

More interestingly, Figure 1B depicts the CD spectral changes associated with the transition visualized by HPLC. A gradual variation occurs from an ambiguous, typically defined as "nonchannel" state (zero time) toward a well-identified, characteristic $\beta^{6.3}$-helical conformation (channel configuration) (Urry et al., 1979), only completely reached after overnight heating at 68 °C (dashed curve). Note that the time-dependent changes in Figure 1B basically cover the range of differences in gramicidin conformational behavior observed by different authors in previous CD studies (Wallace et al.,

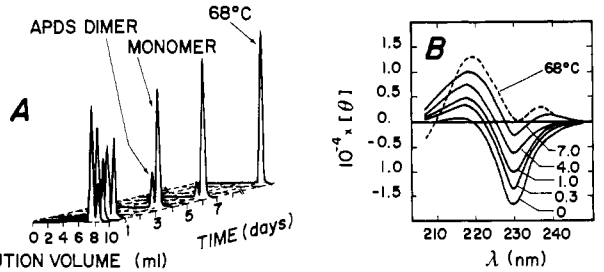

FIGURE 1: Time course of the slow conformational transition of gramicidin A reconstituted in phosphatidylcholine SUV, as simultaneously monitored by HPLC (A) and CD (B) at room temperature. The vesicles were obtained by cosolubilization of peptide and phospholipid in THF. Final lipid and gramicidin concentrations were 0.074 and 1.54 mg/mL, respectively, which corresponded to a mole ratio of about 50. (A) Chromatographic conditions: eluent, THF; flow rate, 1.0 mL/min; UV detection at 294 nm; injection volume, 2 μL. (B) The numbers in the figure indicate the time of incubation of the vesicles, in days. The elution profile and CD spectrum denoted by 68 °C correspond to an aliquot of the gramicidin-containing SUV sample which was heated overnight at this temperature.

1981; Sychev & Ivanov, 1982; LoGrasso et al., 1988; Killian et al., 1988). From the comparison of panels A and B of Figure 1, it can be suggested that any given CD curve could be essentially deconvoluted as the sum of two independent contributions assigned to two well-defined conformational states of the polypeptide (APDS dimers and $\beta^{6.3}$-helical monomers). It should be noted at this point that as previously described (Bañó et al., 1989) this chromatographic technique by itself does not allow a distinction between $\beta^{6.3}$-helical monomers and HH dimers (two juxtaposed monomers), as is also the case for CD (Killian et al., 1988). For this reason, we preferred to express all the results in this paper in terms of the mass fraction of APDS dimers.

Dependence of the Gramicidin Conformation in SUV on the Incubation Time in the Organic Solvent in the Presence of Phospholipid. Since there seems to be a "memory" of the inserted gramicidin conformation with respect to the situation in organic solvent before evaporation (Killian et al., 1988), we next tested alternatively this memory hypothesis by purposely altering the dimer:monomer ratio in the same organic solvent taking advantage of the fact that PC induces a time-dependent monomerization of the peptide (from APDS dimers) in relatively nonpolar solvents (Braco et al., 1986a,b). The strategy was as follows: a THF solution of PC and gramicidin was prepared and incubated at room temperature; 200-μL aliquots were withdrawn at different times (from 1 min to 15 h), the solvent was rapidly evaporated under a nitrogen stream, and the protocol was then followed as described under Materials and Methods, the resulting SUV being assayed by HPLC. The results in Figure 2A clearly corroborate the above hypothesis. The longer the incubation time of lipid and peptide in THF, the higher the extent of lipid-induced APDS dimer dissociation and, therefore, the lower the APDS dimer:$\beta^{6.3}$-helical monomer ratio in the corresponding resulting vesicles. In other words, it becomes again apparent that the actual gramicidin conformation in freshly prepared vesicles is determined by the dimer:monomer ratio in organic solution. Note that after 15-h incubation of lipid and peptide in THF, a solvent favoring per se the APDS dimeric species (Braco et al., 1986a,b), the resulting vesicles contain, however, <3% of these forms.

Interestingly, when these fresh liposome samples exhibiting very different dimer:monomer ratios (values for zero time in Figure 2B) were incubated at room temperature, a slow conformational transition was observed in all cases toward a

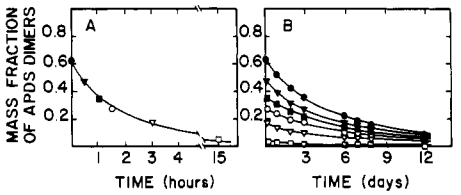

FIGURE 2: (A) Variation of the mass fraction of gramicidin APDS dimers determined by HPLC in fresh SUV as a function of the time of incubation of peptide and phospholipid in THF (cosolubilization step). Other conditions were as in Figure 1. (B) Time course of the gramicidin conformational transition taking place upon incubation at room temperature of the different SUV samples obtained in part A (symbols are maintained), expressed as the variation with time of the mass fraction of APDS dimers.

monomeric configuration, regardless of the percentage of APDS dimers in the starting, fresh SUV (Figure 2B).

Kinetics. Once the possibility was demonstrated of accurately quantifying the proportion of both interconverting conformational species in phospholipid vesicles, a more rigorous analysis of the data was needed to verify that they actually corresponded to a process of dissociation of APDS dimers into monomeric forms. Two kinetic models were tested: (I) an equilibrium model between APDS dimers (M_2) and $\beta^{6.3}$-helical monomers (M)

$$M_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2M \quad (1)$$

$$K_d = k_1/k_{-1} \quad (2)$$

and (II) an irreversible dissociation of APDS dimers into $\beta^{6.3}$-helical monomers

$$M_2 \xrightarrow{k_1} 2M \quad (3)$$

where k_1 and k_{-1} denote the rate constants for the direct and inverse processes, respectively. K_d refers to the equilibrium constant. The integrated equations for these kinetic models are easily obtained (Braco et al., 1986b):

$$\ln \frac{[M_2] - [M_2]_e}{([M_2]_0/[M_2]_e)([M_2]_0 - [M_2]_e) + ([M_2]_0 - [M_2])} = \ln \frac{[M_2]_e}{[M_2]_0} - \frac{[M_2]_0 + [M_2]_e}{[M_2]_0 - [M_2]_e} k_1 t \quad (4)$$

for model I, and

$$\ln [M_2] = \ln [M_2]_0 - k_1 t \quad (5)$$

for model II, where $[M_2]_0$, $[M_2]_e$, and $[M_2]$ refer to the APDS dimer concentration at zero time (freshly prepared liposomes), at equilibrium (after a long enough incubation period), and at any given time, t, respectively. Although the $\beta^{6.3}$-helical monomers are clearly the thermodynamically favored species, in order to test the equilibrium model (I), a finite value of $[M_2]_e$ must be used. Mass fraction values as low as 0.02, 0.01, and 0.005 were tested. A good fitting of the kinetic data in Figure 2B (four upper curves) was observed when either model was used, which demonstrates, on the one hand, the validity of the models assayed, and on the other, the accuracy in the chromatographic quantitation of the conformational species of gramicidin in the lipid environment. There was a remarkable consistency in the determination of k_1, regardless of the model used and even when different equilibrium concentrations were tested for APDS dimers in model I, the average value being $k_1 = 1.9 \times 10^{-6}$ s^{-1} for model I and $k_1 = 1.8 \times 10^{-6}$ s^{-1} for model II.

Dependence of the Initial Gramicidin Conformation in SUV on the Organic Solvent Total Volume before Drying. It has been shown above that in a relatively nonpolar solvent a phospholipid-induced time-dependent monomerization occurs

Table II: Dependence of the HPLC-Determined Mass Fraction of Gramicidin APDS Dimers in Fresh SUV on the Organic Solvent Total Volume[a]

solvent	volume (mL)	mass fraction of APDS dimers
THF	0.5	0.80
	2.0	0.73
	5.0	0.39
chloroform/methanol (2:1)	0.5	0.13
	2.0	0.12
	5.0	0.04
chloroform/methanol (1:1)	0.5	0.08
	2.0	0.07
	5.0	0.03

[a] For this experiment, rotary evaporation was used for solvent removal.

(see Figure 2A). Moreover, equilibrium in organic solvent is not always reached in a few minutes, but, on the contrary, it can take several hours or even days (Veatch & Blout, 1974; Braco et al., 1986a, 1988a). Therefore, we investigated the effect of the total volume of lipid+peptide organic solution to be rotary evaporated in order to determine whether this parameter would alter the dimer:monomer ratio in the resulting vesicles. In principle, we can assume that the longer the evaporation time, and, consequently, the higher the increase in concentration of lipid (and peptide) in the organic solution, the higher the extent of gramicidin monomerization (Braco et al., 1986b). If so, for a larger total volume of organic solution, a lower proportion of APDS dimers in the bilayer should be expected.

The results in Table II, obtained for three different solvents, seem to support the above assumption and show that this variable is also to be taken into account for a rigorous interpretation of reconstituted gramicidin conformational behavior. On the other hand, a direct comparison with recently published results from similar experiments using CD (Killian et al., 1988) may not be immediate because in the present case the samples were purposely not allowed to equilibrate before starting rotary evaporation.

Dependence of the Gramicidin Conformation in SUV on the Sonication Time. Sonication is another experimental variable extensively used in preparation protocols. It is agreed upon that it favors the formation of the channel state (LoGrasso et al., 1988), but in general, it has been employed to a very different extent by several groups in a sometimes rather arbitrary way (Shungu et al., 1986; Massari & Colonna, 1986; Takeuchi et al., 1990). Since a range of sonication times has been reported in the literature from a few minutes up to several hours, a chromatographic study was carried out covering this time interval to analyze the effect (if any) of ultrasonic radiation on the dimer:monomer ratio during conversion of MLV to SUV. Figure 3A depicts the results obtained expressed as the variation of the mass fraction of APDS dimers. Because there was no significant heating of the samples during exposure to ultrasound (see Materials and Methods), it can be concluded that sonication does alter the conformational equilibrium of inserted gramicidin and results in a partial dissociation of APDS dimers; i.e., it favors the formation of the $\beta^{6.3}$-helical configuration. Note that the effect per se can be important if exposure is long enough.

Dependence of the Gramicidin Conformation in SUV on the Phospholipid:Peptide Mole Ratio. It has been reported that gramicidin–gramicidin interactions in model membranes, and therefore the lipid:peptide mole ratio, can play an important role in channel formation and function (Wallace et al., 1981; Sychev & Ivanov, 1984; Wallace, 1986). In particular, it has been recently suggested that a nonchannel conformation can be stabilized by a higher ratio of gramicidin to lipid (Killian et al., 1988). Our chromatographic strategy was also applied to investigate the influence of this parameter, taking into account the wide range of mole ratios described in the literature for reconstitution of gramicidin. Figure 3B shows the variation of the mass fraction of peptide APDS dimers in freshly prepared SUV as a fuction of phospholipid:gramicidin mole ratio, R, in a range from 25 to 300. No measurement was carried out for $R < 25$ to ensure bilayer integrity. As can be observed, the lipid-to-peptide mole ratio does have a clear effect on the proportion of conformational species in fresh liposomes. In general, an increase in R favors the appearance of the $\beta^{6.3}$-helical monomeric (channel) configuration, this behavior being much more pronounced in the range between $R = 25$ and $R = 150$.

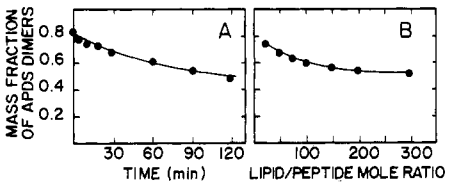

FIGURE 3: (A) Effect of sonication time on the mass fraction of APDS dimers in fresh SUV, as determined by HPLC. Other conditions were as in Figure 1. (B) Dependence of the mass fraction of APDS dimers in SUV on the PC:gramicidin mole ratio (varied by decreasing the peptide concentration). HPLC measurements were carried out after 1-h incubation of the vesicles at room temperature.

Dependence of the Gramicidin Conformation in SUV on the Incubation Temperature. It is now established that heat incubation is a factor affecting the conformational state of gramicidin in a phospholipid model membrane, which results in a shift toward a $\beta^{6.3}$-helical configuration. As it is the case for the variables analyzed above, a wide range of experimental conditions have been reported in the preparation protocols concerning both temperature and heat incubation time (Masotti et al., 1980; Wallace, 1984; Prasard et al., 1986; Shungu et al., 1986; Nicholson et al., 1987; Killian et al., 1988; LoGrasso et al., 1988). Nevertheless, the information obtained in most cases is only qualitative and interpreted in terms of spectral changes associated with heat-induced processes leading eventually to a channel configuration.

For HPLC measurements, experimental conditions were deliberately selected during liposome preparation so that the freshly prepared stock SUV contained a high percentage of APDS dimers (about 80% for a lipid:peptide mole ratio of 25), to make the extent of conformational changes more pronounced upon heat incubation. On the other hand, the use of egg yolk PC as a phospholipid ensured minimal heat exposure during hydration and sonication while keeping the sample above the transition temperature (see Materials and Methods). In this way, we can expect that the changes actually due to heat incubation can be properly analyzed without interference by other factors. Aliquots of the stock SUV suspension were then incubated at different temperatures, and the conversion kinetics were chromatographically monitored.

The results are shown in Figure 4A, expressed as the disappearance of APDS dimers as a function of incubation time at 4, 24, 30, 40, and 50 °C. As expected, a progressive dissociation of APDS dimers toward $\beta^{6.3}$-helical monomers occurs in all cases, regardless of incubation temperature, the conversion rate being, however, dramatically dependent on this variable. Thus, at 4 °C, the sample had to be incubated for as long as 2 weeks to reach hardly 15% of conversion. At this

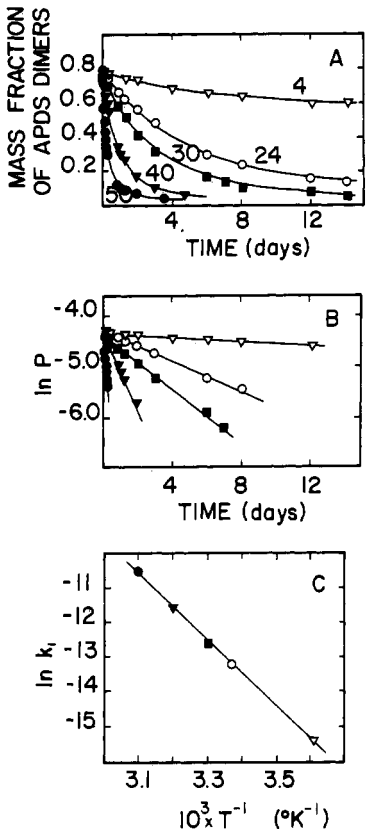

FIGURE 4: (A) Temperature dependence of the conformational transition of gramicidin in SUV, expressed as the variation with time of the mass fraction of APDS dimers. Aliquots of a stock suspension of vesicles were incubated at 4, 24, 30, 40, and 50 °C and monitored chromatographically at different times. The PC:gramicidin mole ratio was 25. (B) Fitting of the data in part A to eq 4 using an equilibrium mass fraction of APDS dimers of 0.01. Symbols have been maintained. (C) Arrhenius plot of the k_1 values obtained from the fittings in part B. Symbols have been maintained.

temperature, the APDS dimeric configuration could be considered as if "frozen" in the bilayer. Conversely, at 50 °C, an almost quantitative conversion has occurred after 1 day. In addition, after overnight heating of the sample at 68 °C, HPLC revealed >98% of monomers (see Figure 1A). It now seems obvious that an increase in temperature results in an acceleration of the conformational transition from APDS dimers to $\beta^{6.3}$-helical forms.

The results in Figure 4A were kinetically analyzed to verify whether in the whole range of temperatures assayed (phospholipid liquid-crystalline phase) the transitions observed actually corresponded to an APDS dimer–$\beta^{6.3}$-helical monomer equilibrium. Figure 4B shows the fitting of the data at different temperatures to eq 4, taking an equilibrium mass fraction of APDS dimers of 0.01. For simplicity, the whole fraction in the first member of eq 4 is referred to as P. The good linearity obtained in all cases supports that the conformational changes taking place in the vesicles upon heat incubation can be interpreted in terms of an equilibrium as simple as that proposed in model I.

More interestingly, the fittings in Figure 4B permit for the first time (and from chromatographic measurements) an accurate calculation of the rate constant, k_1, for the APDS dimer dissociation process as a function of the temperature at which the vesicles were incubated. Figure 4C shows the Arrhenius plot corresponding to these values. The good linearity obtained allowed the estimation of the activation energy for this process, the value being about 75 kJ/mol. Although energetic data on the channel properties of the APDS dimer have been reported from theoretical calculations (Sung & Jordan, 1988, 1989), they correspond to a dimerization energy rather than to an activation energy, and therefore a direct comparison with our results is not possible.

Table III: Relationship between HPLC-Determined Mass Fraction of Gramicidin APDS Dimers in LPC Micelles and Percentage of Incorporation upon Peptide External Addition from Different Organic Solvents

solvent	percentage of incorporation	mass fraction of APDS dimers
THF	35	0.86
chloroform	30	0.60
benzene/methanol (95:5)	47	0.53
ethanol	69	0.57
methanol	75	0.10
TFE	95	0.08
DMSO	>99	0.03

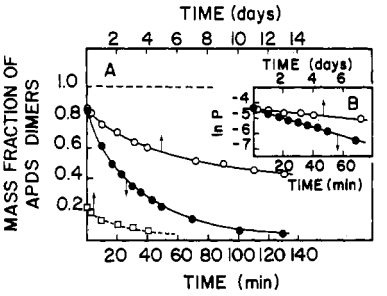

FIGURE 5: (A) Time course of the conformational transition of gramicidin incorporated from a THF solution into preformed LPC micelles. Ten microliters of a 7.4 mg/mL peptide THF solution was added to 1 mL of micelle suspension. Final LPC:gramicidin mole ratio was 142. The micelles were incubated at room temperature (○) and at 68 °C (●). The dashed curve corresponds to the room-temperature time course of gramicidin-containing micelles prepared by cosolubilization of LPC and peptide in chloroform solution; final LPC:gramicidin mole ratio, 50. (B) Fitting of the data in part A to eq 4 using an equilibrium mass fraction of APDS dimers of 0.01. Symbols have been maintained.

Conformational Behavior of Gramicidin in Micelles. The conformation of gramicidin was next investigated by HPLC in a different phospholipid environment such as LPC micelles. The chromatographic strategy was the same as for vesicle analysis. Given the variability of spectral patterns reported for gramicidin incorporated into phospholipid micelles (Urry et al., 1979; Masotti et al., 1980; Prasard et al., 1986; Killian & Urry, 1988; Bañó et al., 1989), we first checked the solvent dependence upon external addition of the peptide of the proportion of inserted APDS dimers and $\beta^{6.3}$-helical monomers. As expected, Table III shows that as solvent polarity decreases the mass fraction of incorporated APDS dimers increases, a behavior similar to that observed in SUV. This proves that also in this lipid environment the gramicidin conformational motif is significantly determined by the dimer:monomer ratio in the organic solvent. Interestingly, the percentage of peptide incorporation also resulted to be dependent on the solvent, so that a correlation can be established between the ratio of conformational species in the fresh LPC dispersion and the extent of gramicidin incorporation (see Table III).

Next, in order to characterize possible time-dependent conformational transitions of the peptide while it is in the lipid environment, we purposely prepared micelles by external addition of gramicidin from THF, which ensured an initial mass fraction of APDS dimers as high as 0.86. Figure 5A depicts the slow dissociation of these dimers at room temperature. Fitting of the data to eq 4 (Figure 5B) yielded a rate constant of 8.3×10^{-7} s^{-1} for this process, a value even lower than that

obtained in PC vesicles. As expected, when an aliquot of this sample was incubated at 68 °C, a dramatic acceleration of the transition was observed (Figure 5A) so that conversion was almost quantitative after a few hours. From the corresponding fitting in Figure 5B, a value of 4.6×10^{-4} s^{-1} was obtained for k_1 at this temperature.

On the other hand, when the sample was prepared by cosolubilization in chloroform, a low percentage of APDS dimers was observed in the fresh micelles (Figure 5A) as compared to the situation upon external addition from this solvent (see Table III), possibly due to some lysophosphatidylcholine-induced peptide monomerization in this nonpolar solvent. This evidences that the conformation of gramicidin is determined not only by the solvent used but also by the method of incorporation. Anyway, once the peptide was incorporated, the remaining APDS dimers also underwent in this case a slow transition toward monomeric forms, as can be seen in Figure 5A.

Discussion

The HPLC methodology used in this paper has proved to be a valuable technique to directly visualize conformational changes of gramicidin while it is in a lipid environment. It has been shown that it is now possible to chromatographically separate APDS dimers and $\beta^{6.3}$-helical monomers present at a given time in the model membrane and to quantitate the relative proportion of each one (for example, see Figure 1A), which offers the possibility for a better interpretation of the molecular basis for the reported spectroscopic changes of gramicidin. It now seems clear that a given CD spectroscopic pattern is actually the result of the individual contributions, at well-defined proportions, of dimeric and monomeric species in the bilayer. In fact, the more negative the ellipticity near 230 nm (nonchannel states), the higher the fraction of APDS dimers. On the contrary, a characteristic channel configuration correlates with a quantitative presence of monomeric forms. In this regard, since our kinetic results at room temperature demonstrate that the dissociation of intertwined dimers into monomers in the membrane is a very slow process (Figures 1A, 2B, and 5A), it is not surprising that in the time framework commonly used in spectroscopic experiments (from a few minutes to several hours) the bilayer can be regarded as an environment of minimal interconversion, as previously reported by Killian et al. (1988). Although related peptide conformational states other than APDS dimers and $\beta^{6.3}$-helical monomers (organized as HH dimers in the functionally active model membrane) cannot be completely discarded, it seems unlikely that they significantly contribute to the reported spectra, as is reinforced by studies of HPLC-aided simulation of CD spectra of gramicidin incorporated into vesicles (manuscript in preparation).

A plausible explanation can be now proposed accounting for many experimental observations and apparent discrepancies in the prior literature in a simple, straightforward manner, particularly regarding the laboratory-to-laboratory heterogeneity in the reported gramicidin CD spectral patterns in model membranes. We have learned here to which extent the variation of only one factor in a given preparation protocol affects the gramicidin dimer–monomer equilibrium in the resulting membrane. Thus, either an increase in solvent polarity, lipid:peptide mole ratio, or temperature as well as sonication or prolonged preincubation of the sample leads to a higher proportion of inserted β-helical monomers. On the contrary, an increase in peptide concentration in the organic solution favors the presence of APDS dimeric forms in the membrane. It is therefore not surprising that the confluence of effects of several more or less arbitrarily selected experimental parameters can lead to a marked variability in the proportion of both inserted peptide conformational species, and therefore very different spectra should be expected depending on the history of the specific sample preparation protocol used.

From the above considerations, it can now be easily understood that upon increasing gramicidin concentration in various solvents drastic changes occur in the CD characteristics of the inserted peptide, the ellipticity at 230 nm becoming more negative (Urry et al., 1975; Killian et al., 1988; Takeuchi et al., 1990). Also, the general observation that prolonged heat treatment is an effective means for generating the channel conformation in vesicles or micelles (Urry et al., 1979, 1985; Masotti et al., 1980; Shungu et al., 1986; Nicholson et al., 1987; LoGrasso et al., 1988; Killian et al., 1988; Takeuchi et al., 1990) can be explained on the basis of a marked acceleration of the conversion of APDS dimers into monomers (Figures 4A and 5A). It is also evident that when gramicidin is directly inserted as a monomer, e.g., from TFE or DMSO (Fossel et al., 1974; Hawkes et al., 1987), a $\beta^{6.3}$-helical conformation is immediately adopted and maintained in the model membrane without the need for further heat treatment (Masotti et al., 1980; LoGrasso et al., 1988; Killian et al., 1988). On the other hand, Sawyer et al. (1990) have recently suggested that the CD spectrum of inserted gramicidin at a short time after incorporation from a solvent favoring the dimeric state should be expected to resemble that of the dissolved peptide, since the folding the intertwined dimers into β-helices in the membrane is a slow process (Bañó et al., 1989). We have experimentally checked this point using THF as a solvent (results not shown) and found that this seems indeed to be the case, provided that experimental conditions are used that preserve APDS dimers in the fresh SUV. However, it must be noted that a high lipid:peptide ratio, a long preincubation time before drying, or extensive sonication (factors shifting the equilibrium toward the monomer) can yield a CD spectrum for inserted gramicidin (even in fresh SUV) markedly different from that obtained in the pure nonpolar solvent.

An additional advantage of HPLC for characterizing the conformational state of gramicidin while it is in the lipid environment is its versatility, because in addition to SUV and micelles, extensive bilayers (MLV) can be also analyzed by direct injection of a few microliters of sample. In fact, the behavior of gramicidin in an MLV sample was similar to that in SUV, the dissociation of APDS dimers being slightly slower in the extensive bilayers. In this case, the kinetic data also fitted the equilibrium model in eq 1 (results not shown).

In relation to gramicidin-containing micelles, in general, the effectiveness of incorporation at room temperature seems to increase with the proportion of monomeric forms in the organic solvent. It appears that upon external addition peptide insertion is somewhat hindered for a dimer with respect to a monomer. In fact, incorporation was negligible when gramicidin was added at room temperature as a powder (which is known from HPLC measurements to contain more than 98% of APDS dimeric forms). In this case, extensive sonication and heating of the sample were needed for the peptide to be inserted and reach the channel configuration, as has also been described by Urry et al. (1979) and Masotti et al. (1980).

In summary, in this preliminary report we have exploited a recently proposed facile, straightforward, reliable chromatographic methodology (Bañó et al., 1988) to get a deeper insight on the conformational behavior of gramicidin A in a model membrane system. The approach also offers the possibility to study in detail in the future the consequences of lipid

environment modulation by parameters such as type of phospholipid polar head, hydrocarbon chain length and unsaturation degree, and presence of cholesterol and fluidity perturbing agents such as anesthetics.

Finally, HPLC emerges as an exciting, powerful technique to be used in combination with other spectroscopic methods for the monitoring of other self-associating hydrophobic peptides incorporated in model membranes. In particular, due to the importance attributed to the tryptophan residues for proper functioning of gramicidin as a channel (Stark et al., 1986; Jones et al., 1986), this chromatographic approach will be of much interest for studying gramicidin analogues where tryptophan residues have been chemically modified (Prasard et al., 1983; Killian et al., 1987) or replaced by different amino acids, which have shown altered conductance behavior (Straessle et al., 1989; Heitz et al., 1989).

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Registry No. THF, 109-99-9; TFE, 116-14-3; gramicidin A, 11029-61-1; benzene, 71-43-2; methanol, 67-56-1; chloroform, 67-66-3; ethanol, 64-17-5.

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Average Structural and Motional Properties of a Diunsaturated Acyl Chain in a Lipid Bilayer: Effects of Two Cis-Unsaturated Double Bonds†

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ABSTRACT: Isolinoleic acid ($18:2^{\Delta 6,9}$) deuterated at 10 different positions was esterified to form 1-palmitoyl-2-isolinoleoyl-*sn*-glycero-3-phosphocholine (PiLPC), and the average structural and motional properties of the diunsaturated chain, in aqueous dispersions of PiLPC, were examined by ^2H NMR spectroscopy. For each sample, ^2H spectra were acquired over a temperature range of 1-40 °C and the quadrupolar splittings interpreted in terms of carbon-deuterium bond order parameters, S_{CD}. Furthermore, definition of the average orientation of the C8 methylene unit with respect to the bilayer normal [Baenziger, J. E., Smith, I. C. P., Hill, R. J., & Jarrell, H. C. (1988) *J. Am. Chem. Soc. 110*, 8229-8231] provided sufficient information to calculate both the average orientations and the molecular order parameters, S_{mol} (which reflects the amplitudes of motion), for the C6-C7 and the C9-C10 double bonds. The results indicate that both the motional freedom (reflected in the order profile) and the average structure (reflected in the orientation of carbon segments with respect to the bilayer normal) are strongly affected by the presence of two cis-unsaturated double bonds. The data were interpreted in terms of two possible models whereby, in each case, the chain adopts a conformation consistent with the low-energy conformation of 1,4-pentadiene [Applegate, K. R., & Glomset, J. A. (1986) *J. Lipid Res. 27*, 658-680] but undergoes a two-site jump between the conformations. The jump motion arises mainly from rotations about the C7-C8 and the C8-C9 single bonds that disorder the C8 and the C9-C10 segments (S_{mol} = 0.15 and 0.08, respectively) but leave the C6-C7 double bond relatively immobile (S_{mol} = 0.55; all at 40 °C). It is suggested that acyl chains containing three or more double bonds could not undergo a similar jump motion and therefore would be highly ordered and not "fluid" as is generally thought.

Polyunsaturated fatty acyl chains are common components of most mammalian cell membranes and are concentrated at high levels at postsynaptic neurons, in the rod outer segment, and in other excitable cells (Nielson et al., 1970; Stone et al., 1979; Crawford et al., 1977). Considerable evidence also indicates that highly unsaturated fatty acids, such as docosahexaenoic acid (DHA,[1] $22:6^{\Delta 4,7,10,13,16,19}$), play an essential role in the biological function of these tissues (Lamptey & Walker, 1976; Wheeler et al., 1975; Neuringer et al., 1984; Wiedmann et al., 1988). Therefore, defining the physicochemical properties of polyunsaturated lipids in bilayers is an important step to gaining a complete understanding of membrane structure and function.

It has been proposed that the role of polyunsaturated lipids is to increase and perhaps even modulate the "fluidity" of membranes. This hypothesis arose from calorimetry experiments (DSC) which showed that saturated lecithin bilayers exist in the relatively rigid gel phase, at physiological temperatures, whereas bilayers containing monounsaturated acyl chains exist in the liquid crystalline state (Chapman et al., 1966). This observation has been extrapolated to suggest that there is a correlation between the number of double bonds and the temperature of the gel-to-liquid crystal phase transition and a direct relationship between the degree of unsaturation and the "fluidity" of a lipid bilayer. In contrast, recent DSC and NMR studies have shown that bilayers composed of highly unsaturated lipids have phase transition temperatures that are similar to, or even higher than, those composed of their less unsaturated counterparts (Coolbear et al., 1983; Deese et al., 1981; Drat & Deese, 1986). Furthermore, only subtle differences in the order and dynamics of lipid probes in bilayers of varying degrees of unsaturation have been observed (Stubbs et al., 1981; Straume & Litman, 1987; Deinum et al., 1988; Yeagle et al., 1987). However, the probe studies are only sensitive to those motions that occur over a limited range of

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[1] Abbreviations: DHA, *cis,cis,cis,cis,cis,cis*-4,7,10,13,16,19-docosahexaenoic acid; DSC, differential scanning calorimetry; FID, free induction decay; iLPPC, 1-isolinoleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; PC, *sn*-glycero-3-phosphocholine; PiLPC, 1-palmitoyl-2-isolinoleoyl-*sn*-glycero-3-phosphocholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; T_c, gel-to-liquid crystal transition temperature.

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