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Structural Polymorphism of Gramicidin A Channels: Ion Conductivity and Spectral Studies

SERGEI V. SYCHEV, STANISLAV V. SUKHANOV, LEONID I. BARSUKOV and VADIM T. IVANOV

Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

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Abstract: The relation between the various spatial structures of the gramicidin A channels and their ionic conductance has been studied. For this aim, various conformations of the peptide were pre-formed in liposomal bilayer and after subsequent fusion of liposomes with planar lipid bilayer the measured channel conductance was correlated with gramicidin structures established in liposomes. To form the single-stranded $\pi 6.3\pi 6.3$ helix the peptide and lipid were co-dissolved in TFE prior to liposome preparation. THF and other solvents were used to form parallel ($\uparrow\uparrow$ $\pi\pi$) and antiparallel ($\uparrow\downarrow$ $\pi\pi$) double helices. Conformation of gramicidin in liposomes made by various phosphatidylcholines was monitored by CD spectroscopy, and computer analysis of the spectra obtained was performed. After fusion of gramicidin containing liposomes with planar bilayer membranes from asolectin, the histograms of single-channel conductance were obtained. The histograms had one or three distinct peaks depending on the liposome preparation. Assignment of the structure of the channel to conductance levels was made by correlation of CD data with conductance histograms. The channel-forming analogue, des(Trp-Leu)$_2$-gramicidin A, has been studied by the same protocol. The channel conductances of gramicidin A and the shortened analogue increase in the following order: $\uparrow\downarrow$ $\pi\pi$ < $\uparrow\uparrow$ $\pi\pi$ < $\pi 6.3\pi 6.3$. Single-channels formed by double helices have higher dispersity of conductance than the $\pi 6.3\pi 6.3$ helical channel. Lifetimes of the double helical and the $\pi 6.3\pi 6.3$ helical channels are very close to each other. The data obtained were compared with theoretically predicted properties of double helices [1].

Keywords: gramicidin A; channel-forming peptides; peptide conformational analysis; circular dichroism

INTRODUCTION

Synthetic and native polypeptides mimic many properties of ion channels existing in biological membranes [2–4]. The progress achieved in the study of cation selectivity and the interactions in the ion–water–channel system to a large extent has been made due to research of linear gramicidines A, B, C, produced by *Bacillus brevis* at the sporulation stage [5–8]. Gramicidins are pentadecapeptides with alternating L and D amino acid residues. These peptide antibiotics can adopt various helical conformations typical for poly-LD-peptides. Therefore gramicidins represent a good model for correlating the channel properties with spatial structures.

Val-gramicidin A (GA), HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$ [9], forms in solution four structural species which are in slow conformational equlibrium [10]. The NMR studies of Bystrov and Arseniev and coworkers [11,12] have shown that in solution GA forms antiparallel (left-handed) and parallel (left- and right-handed) double helices ($\uparrow\downarrow$ $\pi\pi 5.6$ and $\uparrow\uparrow$ $\pi\pi 5.6$ helices) proposed earlier by Veatch *et al.* [10] (Figure 1). Another dimer structure, left handed $\pi 6.3\pi 6.3$ helix (N-terminal-to-N-terminal, figures indicate the number of residue

Abbreviations: CTH, current transition histograms; DPPC, dipalmitoylphosphatidylcholine; GA, gramicidin A; NRMSD, normalized root mean square deviation; SSPC, PC from sea star *Asteria amurensis*; TFE, trifluorethanol; THF, tetrahydrofuran; SD, standard deviation.

Address for correspondence: Prof. Vadim T. Ivanov, Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry, U1. Miklukho-Maklaya 16/10, 117871 GSP-7 Moscow V-437, Russia. Fax: (095) 310-70-07 (095) 325-71-03; e-mail: ssv@ibch.siobc.msk.su.

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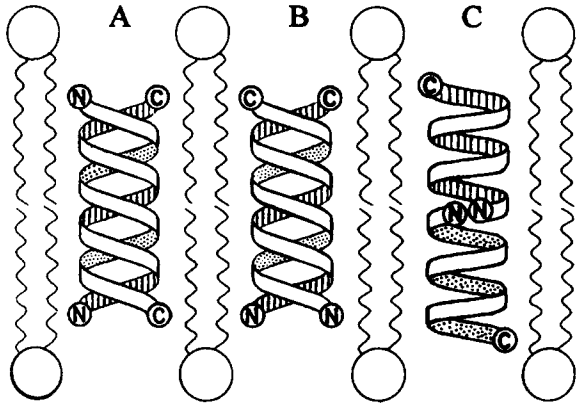

Figure 1 Schematic presentation of the left-handed antiparallel (A) and parallel (B) double helices and right-handed N-to-N terminal helical dimer (C).

per turn) has been proposed by Urry [13] and Urry *et al.* [14] for GA both in solution and in the membrane channel. On the basis of NMR and CD data [15,16] the structure of the transmembrane channel is now generally accepted to be the right-handed $\pi 6.3\pi 6.3$ helix. At the same time 'non-standard' channels have been observed by Busath and Szabo [17–19] and Busath *et al.* [20], with lower Na^+ and Cs^+ and higher H^+ conductances than the 'standard' channel. It has been shown in these studies that low-conductance channels are dimers formed by Val-GA and they have a lifetime similar to that of the 'standard' channels. However, the structure of low-conductance channels remained uncertain.

Variation of organic solvents in which GA was dissolved prior to incorporation into the lipid bilayer did not result in the formation of channels with structures other than $\pi 6.3\pi 6.3$ dimer [21]. A certain expectation of obtaining double helical channel was connected with preparation [22] and study [23,24] of GA analogues wherein the N–C or C–C terminals were linked by covalent bridges. It was found that each bis-derivative forms two types of the channels with different lifetimes. The short-living channel was assigned to double helical structure [24] formed, however, by interdimer associates rather than within the respective monomeric dimer [23,24]. This circumstance did not allow the straightforward interpretation of the experimental data. As a result, formation of GA channels by double helices remained a possible, but never proven, phenomenon. Therefore, a direct comparison of the results of theoretical analysis (including those obtained by the method of molecular dynamics [6,25]) with the experimentally observed parameters of the double helical channels, as well as with the properties of the $\pi 6.3\pi 6.3$ channel, could not be carried out.

Whereas in liposomes prepared from saturated PC the $\pi 6.3\pi 6.3$ helix predominates [26], in unsaturated PC thermodynamically preferred structures are the left-handed $\uparrow\uparrow$ $\pi\pi 5.6$ and $\uparrow\downarrow$ $\pi\pi 5.6$ helices [27]. Various non-equilibrium conformational states of GA in liposomes can be obtained by changing the solvent in which peptide and lipid are codissolved before drying. Bano *et al.* [28] have shown that the $\pi 6.3\pi 6.3$ dimer is formed when TFE is used as a solvent, while in THF or benzene the $\pi\pi 5.6$ double helices predominate.

To determine the structure of GA in liposomes Bano *et al.* [28] proposed quantitative analysis of HPLC data. However this approach allows the detection of only the overall $\uparrow\uparrow$ $\pi\pi 5.6 + \uparrow\downarrow$ $\pi\pi 5.6$ content of helices. More detailed information was provided by computer analysis of CD spectra (and FTIR spectroscopy) [27], whereby the assignments of CD and FTIR spectra were based on the NMR data [11,15].

The aim of the present work is to correlate the ionic conductances of GA channels in lipid bilayers to their polymorphous structures. The experimental approach is based on the formation in liposomes of gramicidin channels with predetermined structures and their subsequent transfer into planar bilayer by fusion of liposomes with the lipid bilayer. THF and a few other solvents were used to form the double helices in the liposomes while TFE was used for $\pi 6.3\pi 6.3$ helix formation. Liposomes were prepared from the lipids containing unsaturated fatty acid residues (asolectin, soybean PC and SSPC) in order to shift the equilibrium towards the $\pi\pi 5.6$ helices. The conformation of GA in liposomes was monitored by CD spectroscopy with computer analysis of the data obtained. After fusion of GA-containing liposomes with the planar lipid bilayer, the single channel currents were recorded and analysed for different kinds of GA channels. In other words we used liposomes as a transport vehicle for the delivery of GA channels with predetermined spatial structures into the planar bilayer. The combined analysis of CD data and channel parameters allowed channel structures to be related to their transport function.

MATERIALS AND METHODS

Materials

Val-GA was obtained from GD (Boehringer) by countercurrent distribution [29]. The amino acid analysis on Mark II (Durrum) detected *ca.* 6% of Ile-

GA in the sample. DPPC was from Serva, soybean PC and asolectin (IIS) were from Sigma, SSPC was obtained from Dr. E. Ya. Kostetsky (Far East University, Vladivostok). In the latter preparation the fraction of unsaturated fatty acids with >2 double bonds is *ca.* 50%, similarly to the soybean PC [30]. Lipids were stored at −20°C under nitrogen, their purity was checked by TLC. TFE was from Fluka and THF was from Merk. Water was deionized on a Milli-Q water system (Millipore Corp.) and degased under vacuum. A chromium mixture was used to clean the glassware.

Preparation of Liposomes from TFE and Ethanol Solutions

The procedure was described by Killian *et al.* [26]. Stock solutions of the appropriate amount of GA (0.1–0.5 mg) and lipids were prepared in methanol (0.8 ml) and chloroform (0.8 ml), respectively. The solutions were mixed and solvents were removed in a rotary evaporator. The dry mixture of peptide and lipid was dissolved in TFE or ethanol (1.6 ml) to obtain GA concentration $\sim 10^{-4}$ M. After incubation for 2 h at 20°C the solvent was removed by rotary evaporation (at 45°C) and samples were dried for 2 h under $\sim 10^{-3}$ torr. The dry lipid–peptide film was hydrated in 10 mM NaCl in H_2O (pH 6.5) to 5–7.5 $\times 10^{-4}$ M GA concentration at 1:25–200 peptide: lipid molar ratios and to 1×10^{-4} M GA concentration at 1:600–1000 ratios. The samples were allowed to swell for 30 min at 20°C and then were sonicated at 20°C (or at 45°C for DPPC) by Braunsonic 1510 sonicator for 2–3 min until they became optically clear.

Preparation of Liposomes from THF, Benzene, Ethylacetate and Isopropanol Solutions

Appropriate amounts of GA and a lipid as dry powders were dissolved separately in the solvent of choice and the solutions were combined. The GA concentration was $\sim 10^{-3}$ M (i.e. higher than in TFE and ethanol in order to increase the fraction of the dimer). The solvent was rapidly evaporated under a nitrogen stream and then under high ($\sim 10^{-3}$ torr) vacuum [28]. The rest of the preparation procedure was as described above. For other details see [27].

Measurement of CD Spectra

Spectra were recorded at 20°C on the Jasco 500 C dichrograph in demountable cells (Hellma) with 10^{-3} cm and 10^{-2} cm optical path length. The spectra reported are the average of two or three scans. The spectrum of the appropriate suspension of liposomes without GA was used for the baseline.

CD Data Analysis

As shown by CD and FTIR spectroscopy [27] GA forms three structures in liposomes, namely the right-handed $\pi 6.3\pi 6.3$ helix (designated as U) and left-handed $\uparrow\uparrow$ $\pi\pi 5.6$ and $\uparrow\downarrow$ $\pi\pi 5.6$ helices. The measured spectrum can be expressed as a linear combination of the spectra of these basic structures:

$$[\Theta]_\lambda = f_u[\Theta]_{\lambda u} + f_{\uparrow\uparrow}[\Theta]_{\lambda\uparrow\uparrow} + f_{\uparrow\downarrow}[\Theta]_{\lambda\uparrow\downarrow}$$

where f_u, $f_{\uparrow\uparrow}$, $f_{\uparrow\downarrow}$ are their molar fractions determined by the least-squares method (the $f_i > 0$ constraint was not applied). The spectra of $\uparrow\uparrow$ $\pi\pi 5.6$ and $\uparrow\downarrow$ $\pi\pi 5.6$ helices (Figure 2) were taken from [10] (structures established in [11] and [12] and the GA spectrum in liposomes made of DPPC was taken as a spectrum of $\pi 6.3\pi 6.3$ helix [27]. CD spectra were calculated in the 204–248 nm region at 2 nm intervals. The quality of each computed fit was evaluated by a normalized root mean square deviation as

$$\mathrm{NRMSD} = \left[\frac{\sum_N (\theta_{\mathrm{expt}} - \theta_{\mathrm{calc}})^2}{\sum_N \theta_{\mathrm{expt}}{}^2}\right]$$

where θ_{expt} and θ_{calc} are experimental and calculated residue ellipticities, and N is the number of data

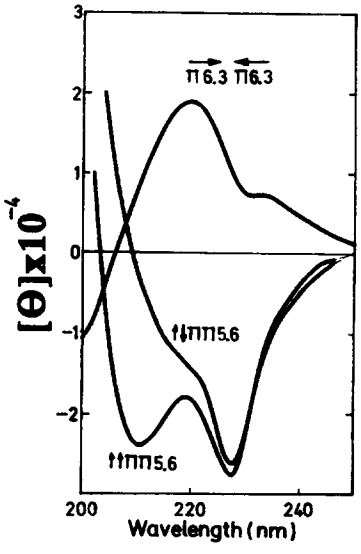

Figure 2 Reference CD spectra used for computer analysis of CD data (see text).

points used. Molar fractions of the structures f_i were determined both with constraint $\sum_i f_i = 1$ and without it. In the latter case the results were normalized to 100% in order to remove variations due to concentration errors [31]. Such an approach as a rule results in a drop of NRMSD (in comparison with $\sum_i f_i = 1$ version) and these results are shown in Tables 1–3 and in Figures 3–5. Although in the case of $\sum_i f_i = 1$ somewhat different f_i values were obtained (similar to Mao and Wallace [31], the order of f_u, $f_{\uparrow\uparrow}$, $f_{\uparrow\downarrow}$ preference remains the same as without the constraint (with the exception of soybean PC liposomes prepared from ethanol, see below). Calculations performed with adding of the fourth basic spectrum of right-handed $\uparrow\uparrow$ $\pi\pi$5.6 helix (species 4 [10]) showed the absence of this structure in the equilibrium ($f_i = 0$) as well as in the lipids used by us earlier [27]. Additional correction of the spectra of GA analogues with a variable number of tryptophans was made according to [32].

Gel Filtration

After CD measurements the sample was diluted by a double volume of the same solution (10 mM NaCl) and 15–20 μl of it was applied to Sephadex G-50 (medium) column 1 × 45 cm (Whatman). The 2238 Uvicord SII (LKB) detector was used at $\lambda = 280$ nm. Gel was used only once; for the next run the column was prepared afresh. A typical elution profile of the liposomes is shown in Figure 3. The collected liposome fraction (~0.5 ml) was diluted to the GA concentration 10^{-9}–10^{-11} M before adding to the pre-formed planar lipid bilayer.

Single-channel Recording

Planar lipid bilayers were formed from asolectine (type IIS, Sigma) in *n*-decane (40 mg/ml) across a hole (diameter 1 mm) in a Teflon partition separating two 2 ml chambers. The experiments were done with solutions of 100 mM NaCl (or KCl) at 19–21 °C. Aliquots (1–3 μl) of the liposome suspension with GA concentration 10^{-9}–10^{-11} M were added to both aqueous phases. Recordings of the current traces were started at once after the appearance of the first spikes, usually in 10–30 min after addition of liposomes. The single-channel measurements were done at 70 mV potential which was applied to the membrane by Ag–AgCl electrodes. Current transitions were amplified by a Keithly-427 amplifier and recorded on a 7005 Brul and Kjer F–M recorder for the subsequent computer analysis. The current traces were observed simultaneously on the 5525 Tektronix oscilloscope. The records of single-channel currents obtained were filtered at the low-pass filter cut of frequency 100 Hz (8-pole Bessel) and stored on an F–M recorder. Recorded data were digitized at 10 ms intervals. Records were eliminated from the final data set if there were overheads of spikes produced by several simultaneous open transitions.

Digitized records were analysed using a PClamp 5.5 software package (Axon Instruments, USA). The channels with a current amplitude that was greater than 20% of the maximum amplitude of the current transitions were analysed. The portion of the transitions falling below this threshold was negligible. Determination of current transition amplitudes was based on current transition histograms (CTHs). Approximation of the CTH obtained was performed by a many-component Gaussian distribution by the PClamp 5.5 statistical module using linear regression analysis. The same software was used for calculating the average channel durations of the open time distributions.

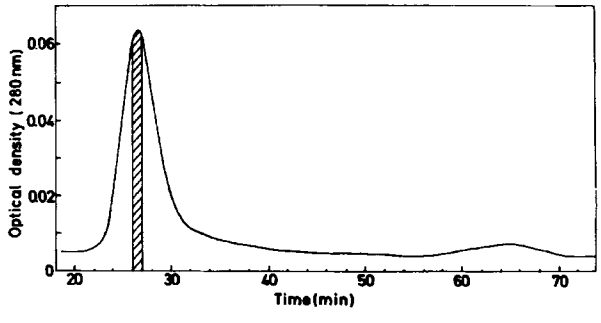

Figure 3 Elution profile of sonicated GA/SSPC dispersion (prepared from TFE) at 1:25 peptide:lipid molar ratio.

RESULTS

Conformational Equilibrium of GA in the Membranes from CD and Single-channel Data

Asolectin. At first the fusion of GA-containing liposomes from asolectin was performed with the planar bilayer of the same lipid. The observed and calculated CD spectra of GA in liposomes are shown in Figure 4. The results of corresponding quantitative analysis of the spectra, i.e. relative fractions of $\uparrow\downarrow$ $\pi\pi$5.6, $\uparrow\uparrow$ $\pi\pi$5.6 and π6.3π6.3 helices, are listed in Table 1. Usage of TFE results in the formation mainly of the π6.3π6.3 helix (~75%) in asolectin liposomes, which corre-

sponds to the data of Killian *et al.* [26] and Sychev *et al.* [27] obtained for other lipids.

Usage of THF at the same peptide:lipid molar ratio (1:150) results in predominant formation of $\pi\pi 5.6$ helices. The initial molar fraction of double helices (~65%) as well as its drop with time up to ~55% are in agreement with the data of Bano *et al.* [28] obtained by HPLC. The content of double helices observed when using benzene (~65%) is somewhat lower than that obtained by HPLC for liposomes prepared from egg yolk PC. A still lower fraction of $\pi\pi 5.6$ helices is observed in the cases of ethylacetate and isopropanol which have not been used before (Table 1). It is worth noting that the content of $\uparrow\downarrow$ $\pi\pi 5.6$ helix is about twice as high as that of $\uparrow\uparrow$ $\pi\pi 5.6$ helix. The portion of double helices in asolectin (from THF) drops to ~50% at the reduction of the peptide:lipid ratio to 1:600, in accord with [28]. At the 1:600 molar ratio the CD spectrum can be reliably measured only up to 210 nm and the NRMSD in this case increases; nevertheless the fraction of $\uparrow\downarrow$ $\pi\pi 5.6$ helix is higher than that of $\uparrow\uparrow$ $\pi\pi 5.6$ helix, similar to the 1:150 ratio.

Figure 5 shows typical single-channel traces after fusion of GA-containing liposomes prepared from various lipids with the planar bilayer made of asolectine. The corresponding CTHs in the case of asolectin liposomes are shown in Figure 6. When using THF three levels of conductance can be seen on the CTH in accord with the number of the GA main structures in liposomes. It is tempting to assume that each of these three forms has its own level of conductance. When GA dissolved in methanol is added (to both aqueous compartments) the high-conducting channel 3 is observed and the peaks 1 and 2 on the CTH are practically absent (Figure 7). The structure of this channel is well known as a right-handed $\pi 6.3\pi 6.3$ helix [15,16,33]. Conductance levels 1 and 2 are also manifested very weakly when preparing liposomes from TFE (Figure 6D). This implies that the causative factor of conductances 1 and 2 is the formation of double helices in liposomes where their portion falls to 25% when using TFE (Table 1). The assignment of peaks 1 and 2 to $\uparrow\downarrow$ $\pi\pi$ and $\uparrow\uparrow$ $\pi\pi$ helices respectively (see Table 1) is based on the correlation of the relative integral intensities of peaks 1–3 and the fractions of the corresponding structures according to the CD data. Thus when using THF, a higher content of the $\uparrow\downarrow$ $\pi\pi 5.6$ helix in comparison with $\uparrow\uparrow$ $\pi\pi 5.6$ corresponds to a higher intensity of peak 1 relatively to peak 2. Other data favouring this assignment will be given below. The total intensity of peaks 1 and 2 assigned to double helices is ~55% and changes slightly with time (Figure 6 (A)–(C)).

SSPC. As seen from Figure 8 and Table 2 THF in this lipid also favours double helices, their content reaching ~75% at 1:150 peptide: lipid ratio. Similar values

Table 1 Conformational States of GA in Liposomes from Asolectin (Estimated from the CD Data) and Relative Integral Intensities of the Peaks 1–3 in CTH after Fusion of Liposomes with Planar Bilayer from Asolectin

Method	Solvent	Peptide: lipid (M:M)	Time (days)	GA structure in liposomes		
				$\uparrow\downarrow$ $\pi\pi$	$\uparrow\uparrow$ $\pi\pi$	$\pi 6.3\pi 6.3$
				Peak number in the CTH		
				1[a]	2[a]	3
CD	TFE	1:150	0–1	0.00	0.25	0.75
CTH	TFE	1:150	0	0.04	0.11	0.85
CD	THF	1:150	0	0.56	0.10	0.34
			1	0.48	0.08	0.44
		1:600[b]	0	0.31	0.15	0.54
CTH	THF	1:600	0	0.28	0.16	0.56
CD	Benzene	1:150	0	0.49	0.16	0.35
			1	0.49	0.04	0.47
	Ethylacetate	1:150	0	0.52	0.05	0.43
			1	0.48	0.00	0.52
	Isopropanol	1:150	0	0.46	0.08	0.46
			1	0.38	0.09	0.53

[a]Tentative assignment.
[b]NRMSD (~20%) is higher than for the rest of the spectra.

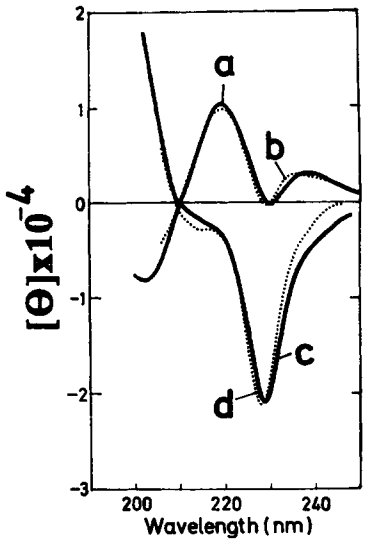

Figure 4 CD spectra of GA in asolectin liposomes prepared from TFE (a) and THF (c); (b) and (d) are corresponding calculated curves. NRMSD values are 12% and 17% respectively. Peptide:lipid molar ratio is 1:150.

were obtained by Bano *et al.* [28] in HPLC experiments. At the 1:600 ratio (THF or benzene) the fraction of $\pi\pi 5.6$ helices falls according to the CD data (qualitative estimation because of difficulties in recording spectra at $\lambda < 220$ nm) and correspondingly low relative intensity of peaks $1+2$ ($\sim 25\%$) is observed in the CHT. An increase in the peptide:lipid ratio of 1:25 is followed by some decrease in the double helical content. However, with this lipid the single-channel experiments are more reproducible at a 1:25 ratio (Figure 9) than at the ratio $1: > 150$. The relative intensity of the peaks $1+2$ assigned to $\uparrow\downarrow \pi\pi$ and $\uparrow\uparrow \pi\pi$ helices is $\sim 50\%$. As for asolectin, that ratio does not change with time (Figure 9 (A)–(C)). The change in the intensities of peaks 1 and 2 in the CTH with time (Figure 9 (A)–(C)) is of interest, because a similar time dependence of the content of $\uparrow\downarrow \pi\pi 5.6$ and $\uparrow\uparrow \pi\pi 5.6$ helices is observed according to the CD data ($0.35/0.20 \rightarrow 0.23/0.33$, Table 2).

With TFE the $\pi 6.3\pi 6.3$ helix predominates in the liposomes ($\sim 80\%$, Table 2), in accordance with the data of single-channel experiments (Figure 9 (D) and

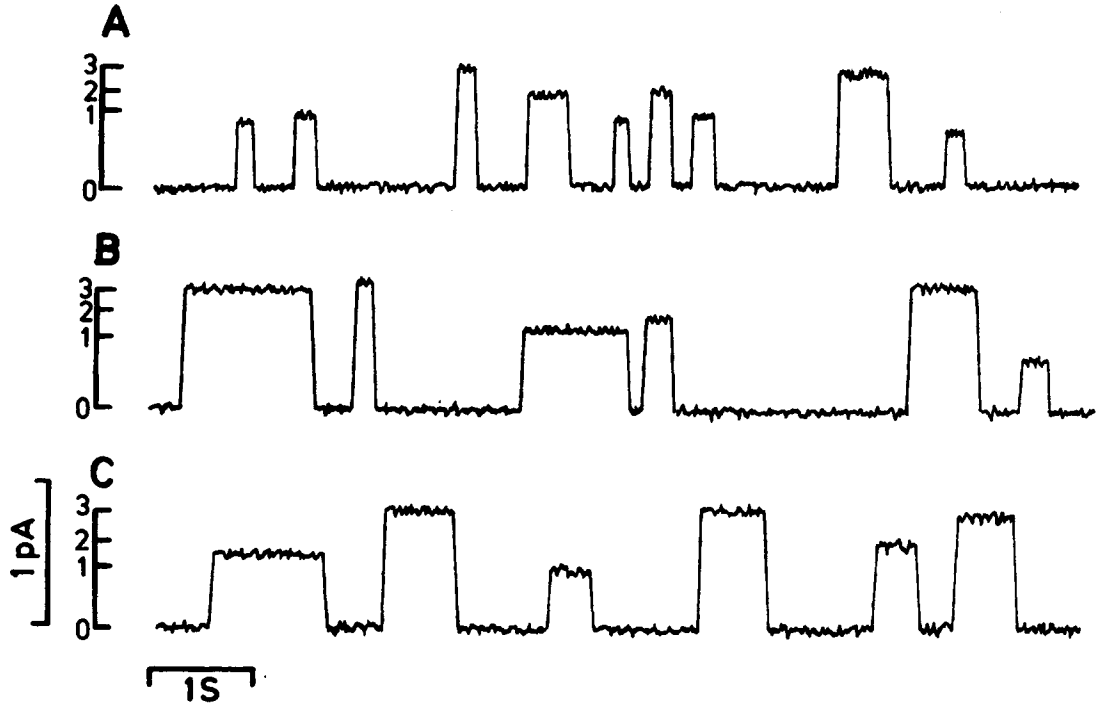

Figure 5 Single-channel current traces in asolectin/decane planar bilayer after its fusion with GA-containing liposomes from asolectin (A), SSPC (B) and soybean PC (C). The conductances denoted at the vertical scale (on the left) are the values corresponding to the maxima of the peaks on the current transition histograms (Figures 6, 9, 13). In all records, channels are seen with conductances less than for the 'standard' level 3. These low-conductance channels have about the same lifetime as 'standard' ones.

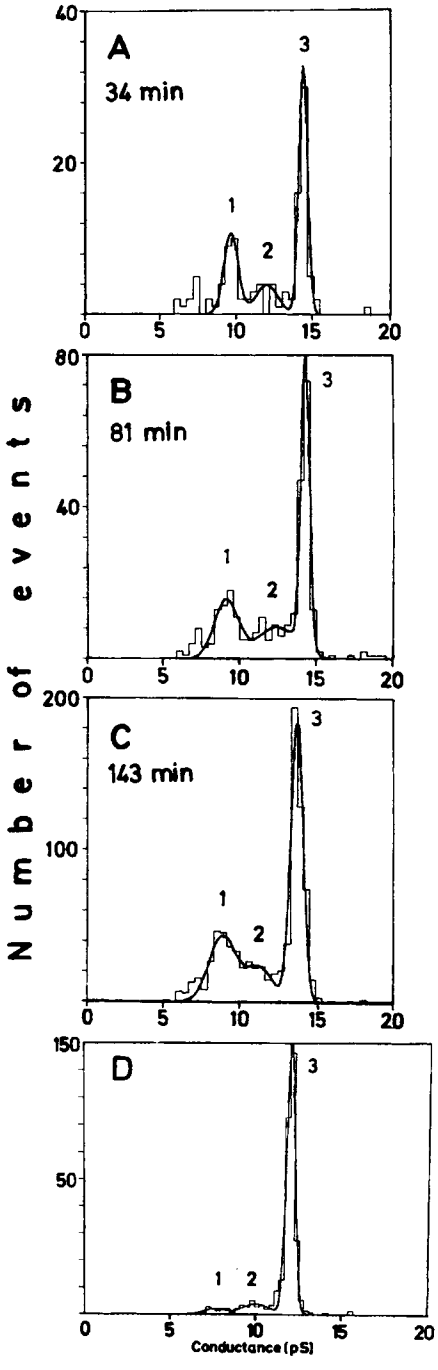

Figure 6 Current transition histograms obtained after fusion of GA-containing liposomes made of asolectin with planar bilayer. Liposomes were prepared from THF (A–C) and TFE (D). The time of channel recording after fusion event is indicated. Relative integral intensities of peaks 1–3 are: 29:16:55 (A); 27:25:48 (B); 28:16:56 (C); and 4:11:85 (D). The total number of transitions are 858 (C) and 395 (D). The conductances corresponding to the maxima of the peaks are presented in Table 4.

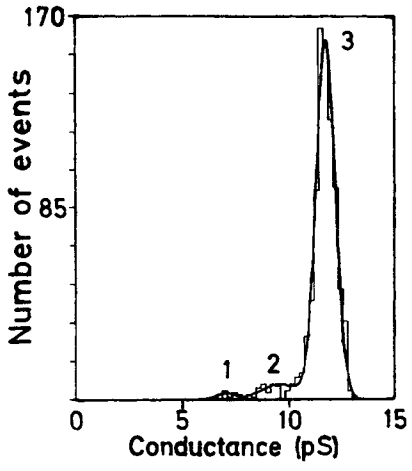

Figure 7 Current transition histogram obtained after addition of GA solution in methanol (2.5×10^{-4} M) to aqueous phase (on both sides of the membrane). Relative integral intensities are 1.5:7.5:91. Conductance corresponding to the main peak is 11.8 pS. The total number of transitions was 544.

Table 2). The result can be considered as a control experiment to that shown in the Figure 9(A)–(C).

Des(Trp-Leu)$_2$-GA. To facilitate the assignment of peaks 1 and 2 in CTH to particular channel structures we have studied the shortened membrane active analogue, des(Trp-Leu)$_2$-GA, for which conformational equilibrium in dioxane and THF is shifted to the left-handed $\uparrow\downarrow$ $\pi\pi$5.6 helix (up to 90% [32]). For comparison, GA in dioxane has the ratio $\uparrow\downarrow$ $\pi\pi$5.6/ $\uparrow\uparrow$ $\pi\pi$5.6 = 1 [34]. According to the CD data (Figure 10), the $\uparrow\downarrow$ $\pi\pi$5.6/ $\uparrow\uparrow$ $\pi\pi$5.6 ratio of the analogue falls to 0.56/0.16 = 3.5 (Table 2) after incorporation into SSPC liposomes. This is still higher than the ratio for GA 0.35/0.20 = 1.75. Finally, after fusion of the liposomes with planar bilayer the intensity of peak 1 ($\uparrow\downarrow$ $\pi\pi$ helix) in the case of the analogue is higher than for GA (cf. Figures 11 and 9 (B) and (C)).

Soybean PC. Usage of THF to form double helices is based on the so-called 'molecular memory' of the polypeptide [26], i.e. having being transferred from THF to the lipid bilayer, it still 'remembers' the starting structure adopted in that solvent. For asolectine and SSPC, the relative content of the π6.3π6.3 helix increases with time (Tables 1 and 2), implying that the $\pi\pi$ and π6.3π6.3 helices are not in equilibrium. Another way to obtain the double helices is by the selection of the proper lipid favouring those structures. In this case double helices repre-

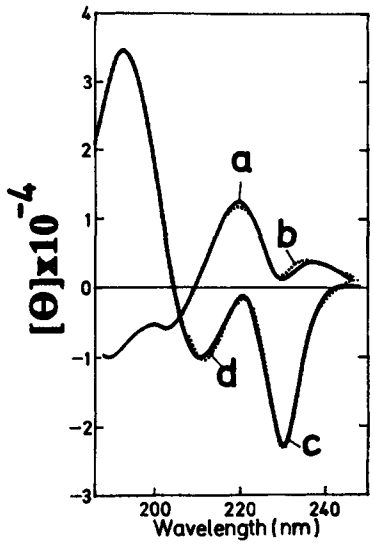

Figure 8 CD spectra of GA in SSPC liposomes prepared from TFE (a) and THF (c); (b) and (d) are corresponding calculated curves. NRMSD values are 5% and 8% respectively. The peptide:lipid molar ratio 1:25. Curve (c) was obtained at the next day after liposome preparation.

sent thermodynamically preferred structures. In order to reach the equilibrium state it is better to use ethanol as a solvent for a GA/lipid mixture and to incubate the prepared liposomes additionally at 68 °C for 4–5 h [27]. The study of GA by the protocol described for liposomes made of SSPC and asolectine has shown that the relative content of double helices in the equilibrium state is ~40% and ~30% respectively, while in soybean PC it is more than 60% (Figure 12, Table 3). Therefore soybean PC was used for a single-channel experiment. The $\uparrow\uparrow\pi\pi$ helix predominates over the $\uparrow\downarrow$ $\pi\pi$ structures in liposomes made of this lipid and this dominance is maintained after fusion of liposomes with planar bilayer (Figure 13 (A), Table 3). The high value of SD for the component 2 (Figure 13) makes the analysis of CTH difficult. Decomposition of the profile into three components was made in this case by analogy with the data for other lipids.

With TFE and liposomes made of soybean PC, the $\pi 6.3\pi 6.3$ helix is the main form (~75%, Table 3) which agrees with the data of the single-channel experiment (Figure 13 (B)). The result can be considered as a control experiment to that shown in Figure 13 (A).

Single-channel Parameters

Conductance. As seen from Table 4 the three levels of GA conductance both in asolectin and SSPC membranes relate to each other as 0.64:0.82:1. In KCl solution low-conductance levels in CTH are of low intensity, so that only their mean value conductance can be determined. Such low intensity is in accord with the lower content of double helices in the presence of K^+ shown by CD for GA in liposomes [27].

Table 2 Conformational States of GA and des(Trp-Leu)$_2$-GA in Liposomes from SSPC and DPPC (Estimated from the CD Data) and Relative Integral Intensities of Peaks 1–3 in CTH after Fusion of Liposomes with Planar Bilayer from Asolectin

Lipid, peptide	Method	Solvent	Peptide: lipid (M:M)	Time (days)	GA structure in liposomes: $\uparrow\downarrow\pi\pi$ Peak number in the CTH 1[a]	$\uparrow\uparrow\pi\pi$ 2[a]	$\pi 6.3\pi 6.3$ 3
SSPC, GA	CD	THF	1:150	0	0.52	0.25	0.23
				1	0.46	0.18	0.36
			1:25	0	0.35	0.20	0.45
				1	0.23	0.33	0.44
	CTH	THF	1:25	0	0.28	0.22	0.50
	CD	TFE	1:25	0	0.00	0.20	0.80
	CTH	TFE	1:25	0	0.09	0.15	0.76
SSPC	CD	THF	1:25	0	0.56	0.16	0.28
Des(Trp-Leu)$_2$-GA	CTH	THF	1:25	0	0.35	0.26	0.39
DPPC, GA	CD	THF	1:25	0	0.35	0.14	0.51
	CTH	THF	1:25	0	0.22		0.78

[a]Tentative assignment.

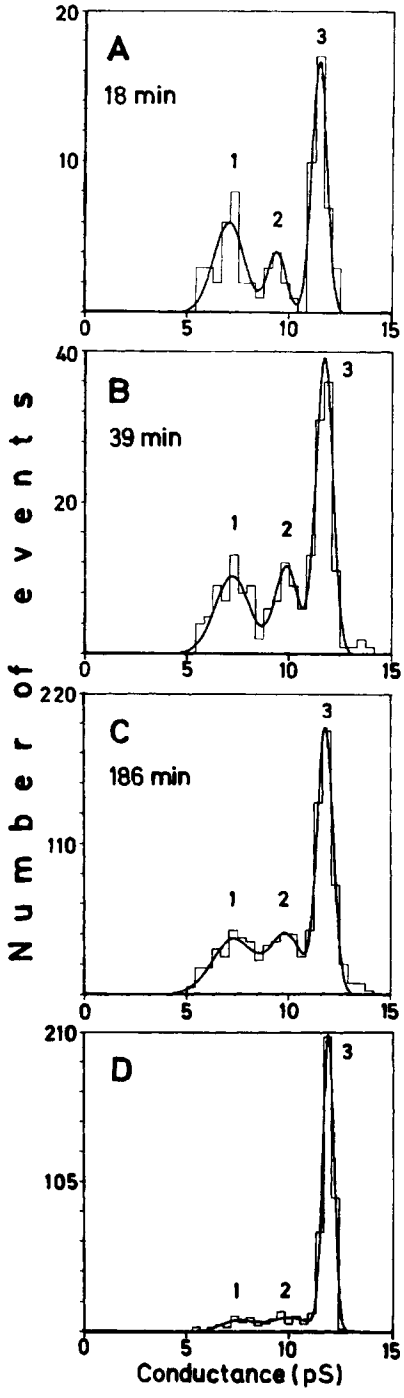

Figure 9 Current transition histograms obtained after fusion of GA-containing liposomes made of SSPC with the planar bilayer. Liposomes were prepared from THF (A)– (C) and TFE (D). The time of channel recording after the fusion event is indicated. Relative integral intensities of the peaks 1–3 are 36:15:49 (A), 28:21:51 (B), 28:22:50 (C) and 9:15:76 (D). The total number of transitions are 524 (C) and 488 (D). The conductances corresponding to the maxima of the peaks are shown in Table 4.

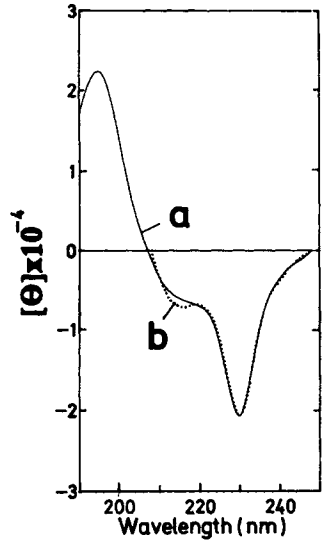

Figure 10 CD spectrum of des(Trp-Leu)$_2$-GA in SSPC liposomes prepared from THF (a) and the corresponding calculated curve (b). NRMSD = 7%. The peptide:lipid molar ratio is 1:25.

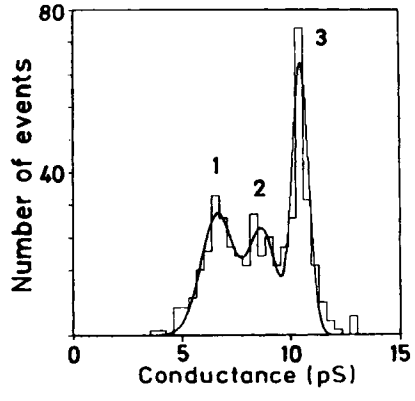

Figure 11 Current transition histograms obtained after fusion of des(Trp-Leu)$_2$-GA-containing liposomes made of SSPC with planar bilayer. Liposomes were prepared from THF. The total number of transitions is 408. Relative integral intensities and the conductances corresponding to the maxima of the peaks 1–3 are presented in Tables 2 and 4.

But the ratio of Na^+/K^+ conductances for the low-conducting channels is about the same as for the π6.3π6.3 channel (Table 4), i.e. channels formed both by double helices and the π6.3π6.3 helix apparently do not show Na^+/K^+ selectivity.

The level 3 conductance of the 11-membered analogue (6.6 pS, Table 4) is somewhat lower than

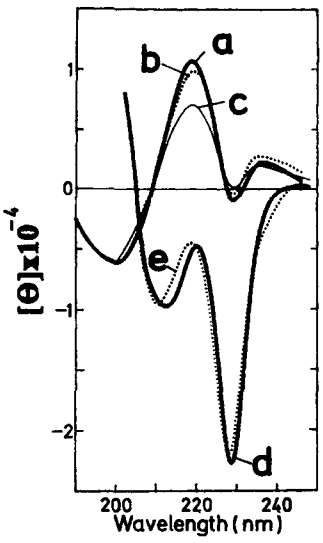

Figure 12 CD spectra of GA in soybean PC liposomes prepared from TFE (a) and ethanol (d); (b) and (e) corresponding calculated curves with NRMSD values 19% and 24% respectively; (c) spectrum of GA in asolectin liposomes prepared from ethanol (corresponding calculated curve with NRMSD value 14% is not shown). The liposomes prepared from ethanol were incubated for 5 h at 68°C.

the corresponding value for GA (7.2 pS) in the same membrane. This result is in accord with the data of Kolb and Bamberg [35], who found that the single-channel conductance decreases with increasing fatty acid chain length of the lipid, i.e. the membrane thickness. The same is true for the low-conductance double helical channels, since the conductances of all three levels for the analogue relative to GA are 0.90 ± 0.01.

Lifetime of the Channels

In Figure 14 lifetimes for channels are plotted against conductance. One can see that the average lifetime of low-conductance channels (~ 1 s) is not different from the lifetime of the $\pi 6.3\pi 6.3$ channel. In general this is in agreement with the data of Busath and Szabo [18] obtained for the planar bilayer made of diphytanoyl-PC. Average lifetime of the channels formed by fusion of GA containing liposomes prepared from soybean PC with the planar bilayer is three to four times lower than for asolectin and SSPC liposomes.

Standard Deviation. As noted above, peaks 1+2 amounting to 50–55% of the total intensity of CTH do not change with time when using asolectin and SSPC for liposome preparation. At the same time in the case of SSPC (and to the lesser extent, of asolectin) components 1 and 2 in CTH obtained by channel recording for the first 35–40 min after fusion are sharper than in the CTH obtained by channel recording during ~ 3 h (Figures 6 and 9). This is due to the increase in SD of peaks 1 and 2 with time (Figure 15). On the other hand, the SD of component 3 is lower than that of components 1 and 2 and it does not change with time. In the case of soybean PC liposomes, all three channels have a conductance SD higher than the rest of studied lipids (Table 4).

The results obtained in the present study can be summarized as follows:

Table 3 Conformational States of GA in Liposomes[a] from Soybean PC and Asolectin (Estimated from the CD Data) and Relative Integral Intensities of Peaks 1–3 in CTH after Fusion of Liposomes from Soybean PC with Planar Bilayer

Lipid	Method	Solvent	GA structure in liposomes		
			$\uparrow\downarrow\pi\pi$	$\uparrow\uparrow\pi\pi$	$\pi 6.3\pi 6.3$
			Peak number in the CTH		
			1[b]	2[b]	3
Soybean	CD	Ethanol[c]	0.28	0.34	0.38
PC	CTH	Ethanol	0.91	0.43	0.38
	CD	TFE	0.12	0.14	0.74
	CTH	TFE	0.18	0.18	0.64
SSPC	CD	Ethanol	0.20	0.21	0.59
Asolectin	CD	Ethanol	0.11	0.17	0.72

[a]The liposomes prepared from ethanol were incubated at 68°C for 5 h.
[b]Tentative assignment.
[c]Calculation with the constraint $\sum_i f_i = 1$ results in the relationship: 0.34:0.42:0.2, i.e. in such cases the $\uparrow\uparrow\pi\pi 5.6$ helix becomes the most abundant.

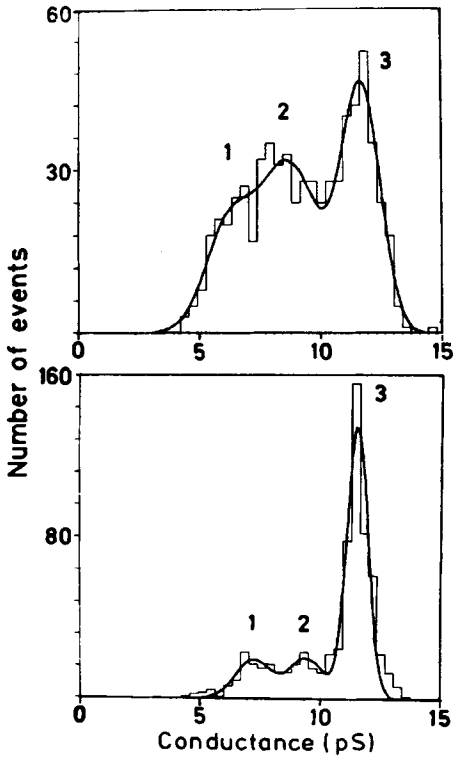

Figure 13 Current transition histograms obtained after fusion of GA-containing liposomes from soybean PC with planar bilayer. Liposomes were prepared from ethanol and incubated for 5 h at 68°C (A) and from TFE (B). The total number of transitions are 653 (A) and 1309 (B). Relative integral intensities and the conductances corresponding to maxima of the peaks 1–3 are shown in Tables 3 and 4.

(1) According to the CD data three structures are formed in the membrane: left-handed ↑↓ $\pi\pi$5.6 and ↑↑ $\pi\pi$5.6 and right-handed π6.3π6.3 helices. The peaks 1–3 in CTH are assigned to distinct channel structures on the basis of the data obtained for GA in three different lipids as well as the data obtained for des(Trp-Leu)$_2$-GA.

(2) The ratio of ↑↓ $\pi\pi$5.6 and ↑↑ $\pi\pi$5.6 structures depends on the composition of liposomes and generally correlates with the ratio of the intensities of peaks 1 and 2 in CTH.

(3) The channel conductances 1–3 depend weakly on the liposome composition.

(4) The lifetime of channels 1 and 2 assigned to the double helices is very close to the lifetime of the π6.3π6.3 channel.

(5) The SD of channel conductances 1 and 2 is higher than that of π6.3π6.3 channel conductance and slowly increases with time.

DISCUSSION

In the present study, GA-containing liposomes were used to deliver channels onto the planar bilayer. Two ways of GA insertion into bilayer can be generally envisaged: (1) via transfer through the aqueous phase and (2) by direct liposome–bilayer interaction. The former cannot serve as the main way since the aqueous phase favours the GA monomeric state (Alimpiev *et al.*, unpublished), which obviously does not 'remember' the double helical structures adopted by GA in organic solvents. Owing to this fact, the technique used earlier and based on adding GA in different organic solvents to the water (up to 10^{-8}–10^{-10} M concentration) [21] results in the formation of only one type of the channel structure.

There are a number of ways by which the GA-loaded liposome can interact with the bilayer, such as fusion, semifusion, adsorption or collision. In this paper we do not specify these mechanisms and we use the term 'fusion' in a broad context, implying incorporation of GA into bilayer without going into the water phase.

In another set of experiments by Sawyer *et al.* [21] the peptide and the lipid were joined in different solvents, the overall GA concentration being 2×10^{-9} M and the peptide:lipid molar ratio being $1{:}10^7$. The solution was equilibrated for 1 h, the solvent was removed and the dry mixture was dissolved in *n*-decane at the GA concentration 2×10^{-9} M, where peptide is present as a monomer. The planar bilayer was formed from the latter solution. The presence of lipid in a nonpolar solvent increases the decay rate of double helix by four orders of magnitude due to the interaction of polypeptide with the PC polar heads [36,37]. Therefore, even if some monomer transforms to double helix in the course of evaporation it should decay again to a monomer in the decane solution. Therefore it is not surprising that in the described experiments only 'standard' channels were observed [21].

As we have shown [27] the higher fluidity of unsaturated membranes is responsible for the conformational shift towards the left-handed $\pi\pi$5.6 helix, which predominates in solution. Accordingly the lipids containing unsaturated fatty acid residues were used in the present study to form double helical channels. It is of interest that Busath *et al.* [20] observed that the rise of temperature from 25°C to 37.5°C results in an increase of low-conducting channels. Such behaviour can also be explained by the increase of membrane fluidity with temperature.

Table 4 Parameters of GA and des(Trp-Leu)$_2$-GA Single Channels formed by Fusion of Peptide-containing Liposomes[a] from Different Lipids with Planar Bilayer from Asolectin

Peptide, lipid	Electrolyte	T(°C)		Peak number in the CTH 1 $\uparrow\downarrow \pi\pi$[b]	2 $\uparrow\uparrow \pi\pi$[b]	3 $\pi 6.3\pi 6.3$
GA, Asolectin	NaCl	21	A[c]	8.80	11.16	13.61
			SD	0.86	0.90	0.40
		19	A	8.11	10.12	12.42
			SD	0.69	0.66	0.44
	KCl	19	A	20.5		25.1
			Na^+/K^+	0.5		0.49
GA, SSPC	NaCl	19	A	7.24	9.81	11.69
			SD	1.04	0.77	0.39
	KCl		A	19.3		22.92
			SD			0.70
			Na^+/K^+	0.4		0.51
Des(Trp-Leu)$_2$-GA, SSPC	NaCl	19	A	6.61	8.67	10.45
			SD	0.76	0.65	0.38
GA, DPPC	NaCl	19	A	8.4		12.0
			SD			0.39
GA, soybean PC	NaCl	19	A	6.2	8.7	11.69
			SD	0.96	1.27	0.79

[a]Liposomes were prepared from THF.
[b]Tentative structures of the channels.
[c]A = single-channel conductance (pS).

As follows from the Results, computer analysis of CD spectra provides useful information on the conformational equilibrium of GA in the membrane. In principle one may assume that the observed channels 1–3 have the same polypeptide backbone structure and differ only by the orientation of the tryptophan side chains. However, the change of CD spectra is unequivocally correlated with the changes of FTIR spectra in the amide I and II bands range [27]. Such a correlation negates the assumption made.

As the reference spectra of $\uparrow\downarrow \pi\pi 5.6$ and $\uparrow\uparrow \pi\pi 5.6$ helices we have used the spectra of these structures obtained for solutions. The orientation of indole rings of tryptophans may be somewhat different in solution and in membrane which may contribute to the error of calculation when using the spectra of GA in solution. However, the CD curves observed for the liposomes with high content of $\uparrow\downarrow \pi\pi 5.6$ helix (Figures 4(c) and 10(a)) and $\uparrow\uparrow \pi\pi 5.6$ helix (Figures 8(c) and 12(d)) are close to the corresponding spectra for solution (Figure 2). In addition, the results of our computer analysis for liposomes prepared from THF, benzene and TFE are in agreement with the data of Bano *et al.* [28] obtained by HPLC.

Similarity of the spectra of $\uparrow\downarrow \pi\pi 5.6$ and $\uparrow\uparrow \pi\pi 5.6$ helices in the 215–250 nm range is another potential source of error as noted earlier [27]. In general the formation of both $\uparrow\downarrow \pi\pi 5.6$ and $\uparrow\uparrow \pi\pi 5.6$ helices is proven by our CD data, although the assignment of the peaks 1 and 2 in CTH to $\uparrow\downarrow \pi\pi$ and $\uparrow\uparrow \pi\pi$ helices (and not vice versa) should be considered as tentative. At the same time this assignment is in accord with the results of theoretical analysis of energetic profiles of $\uparrow\downarrow \pi\pi$ and $11\pi\pi$ channels made by Sung and Jordan [1,38]. According to this study the channel conductance should increase in the sequence: $\uparrow\downarrow \pi\pi < \uparrow\uparrow \pi\pi < \pi 6.3\pi 6.3$.

There is no reason to believe that the error in the computer analysis of CD curves arise as a consequence of not taking into account other structures, in particular the monomer. Actually, since the dimerization constant of GA in bilayers is as high as $\sim 3 \times 10^{14}$ cm^2 M^{-1} [39] and the surface area/pospholipid molecule is 0.6–0.7 nm^2, the monomer has been shown [21] to be almost absent at the peptide/lipid molar ratios used in our experiments.

When fusing liposomes prepared from SSPC and especially DPPC with a planar bilayer made of asolectin, the content of double helices in the bilayer diminishes compared with the liposomes (Table 2). This means that under fusion there occurs a fast shift of the equilibrium ratio of double helices and $\pi 6.3\pi 6.3$ helix. Fast conformational rearrangement of the channel in planar

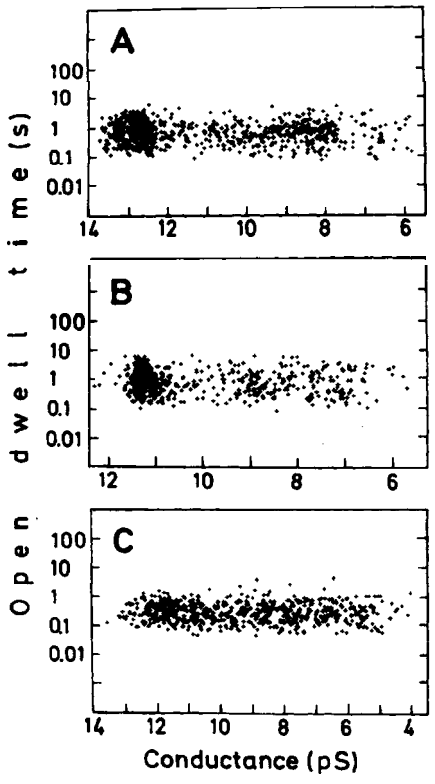

Figure 14 Correlation between the open channel lifetimes and their conductances. Channels were formed by fusion of GA-containing liposomes (from THF) with planar bilayer. Liposomes were prepared from asolectin (A), SSPC (B) and soybean PC (C). The average lifetimes are 0.97 s (A), 1.39 s (B) and 0.33 s (C). The total number of transitions are 755, 540 and 625 respectively. The corresponding current histograms are shown in Figures 6, 9 and 13.

bilayer is in particular due to the presence of decane (Sychev and Barsukov, unpublished). One can assume that other conformational rearrangements in particular $\pi\pi5.6 \rightarrow \pi\pi7.2$ also proceed faster in a planar bilayer than in liposomes. If so, such rapid transition cannot be excluded after incorporation of the $\pi\pi5.6$ helix into the planar bilayer and its interaction with Na^+ ions at the membrane potential applied (see also [40]). Then the low-conductance states observed (or one of them) may correspond to the $\pi\pi7.2$ helix. At the same time there is no *a priori* reason to expect the formation of such helices in the membrane since they were not found in liposomes in the presence of Na^+ ions [27]. In any case this ambiguity does not abolish our conclusion that the helical structures of channels 1–3 differ in the type of the peptide backbone conformation rather than in minor

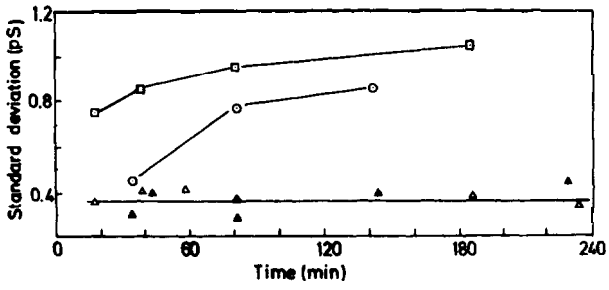

Figure 15 The time course of SD for the peaks 1–3 in the current transition histograms: (□) peak 1, (△) peak 3, liposomes made of SSPC; (○) peak 1, (▲) peak 3, liposomes made of asolectin.

structural features, e.g. at the channel entrance. Our suggestion is based particularly on the control experiments where the 'standard' channels were formed by the fusion of the $\pi6.3\pi6.3$ helix containing liposomes with the planar bilayer (see Results).

Comparative study of the structure of water and the hydration of ions in the $\pi\pi7.2$ and $\pi\pi5.6$ helices by the Monte-Carlo method resulted in the conclusion that the $\pi\pi5.6$ helix cannot be filled with water and therefore cannot act as a channel [41]. However, these calculations were done with atomic coordinates obtained by theoretical analysis [42], while according to X-ray data of Langs [43] and Langs *et al.* [44] the inner diameter of the $\pi\pi5.6$ helix (4.6–5.5 Å) is much larger than proposed from theoretical analysis and NMR data for solutions (~3 Å [11]). The latter figures are large enough to accommodate water molecules and Na^+ ions. Considering the $\pi\pi5.6$ helix as a putative channel structure we primarily refer to the Na^+ channel studied in the present work. On the other hand a $\uparrow\downarrow$ $\pi\pi7.2$ helix forms upon interaction of GA with Cs^+ in solution [45] as well as in crystals [46].

The average lifetime of low-conductance channels is similar to that of the $\pi6.3\pi6.3$ channel. This result does not accord with the assumption that double helical channels should have a longer lifetime because of the high number of intermonomeric H-bonds [18,47]. We interpret the result on the basis of a sliding mechanism of channel opening (for details see [23,27,48]). We suppose that the main factors determining the lifetime of the channels are the surface tension of the membrane and the mismatch between the channel length and the thickness of the lipid bilayer. In the double helix channel model we suggest that the switching on of the channel is connected with local thinning of the membrane as

was supposed [48,49] for the $\pi 6.3\pi 6.3$ channel as well.

The average lifetime of the channel formed by a considerably, ~8 Å, shortened analogue, 1.31 s, does not differ from that of GA channel (1.33 s) in a planar bilayer made of SSPC/decane. This result would seem rather unexpected except for the data on Kolb and Bamberg [35] and Hladky and Haydon [49] which showed that in thick membranes (>40 Å) formed with decane the mean lifetime of GA channels decreases only slightly with increasing the chain length of the lipid; with thinner membranes formed in the presence of hexadecane, this dependence becomes stronger. Thus, to shorten the lifetime tenfold the thickness of the 'decane' membrane has to be increased from 41.8 Å (palmitolein, 16:1 chain) to 58.4 Å (erucin, 22:2 chain), i.e. by ~17 Å. Since the $\pi\pi 5.6$ helix of GA (~31 Å [43]) is only ~4–5 Å longer than the $\pi\pi 7.2$ and $\pi 6.3\pi 6.3$ helices (~26–27 Å, [15,45,46]) the similar lifetimes of low-conductance and $\pi 6.3\pi 6.3$ channels do not exclude formation of both $\pi\pi 7.2$ and $\pi\pi 5.6$ helices in the bilayer.

Bamberg *et al.* [50] have shown that the SD of the channel conductance in the CTH depends on the membrane lipid composition. As we see now this applies to the $\pi 6.3\pi 6.3$ channel. Increase of SD of channels 1–3 observed in the present study with soybean PC in comparison with the values obtained with other lipids implies that this effect may be associated with the double helical channels (especially if one assumes that in this case GA incorporates into the planar bilayer with its boundary lipid). In the case of asolectin and SSPC the SD of low-conductance channels is more than two times higher than the SD for the $\pi 6.3\pi 6.3$ channel. Flexibility of side chains, in particular of Trp in double helix, might contribute to such an increase as suggested by Urry *et al.* [51] for the enhanced dispersity of des(Val^7-Val^8)-GA channel conductance relative to that of GA. The second reason for the exceptionally low dispersity of the $\pi 6.3\pi 6.3$ channel conductance might be formation of the channel by junction of two helical monomers, i.e. by a simple mechanism, which is not used in other polypeptide and protein channels. On the other hand, the double helical channels penetrate the membrane and have a more complicated mechanism of opening. It is worth noting in this connection that the SD of the double helical channel approaches that of the Na channels (e.g. from rabbit T-tubular membranes [52]).

In conclusion, the data presented here provides considerable, though indirect, evidence supporting the assumption that low-conductance channels are formed by double helices. An alternative explanation of low-conductance channels was proposed in [18] and [19]. The appearance of these channels was assigned to a conformational transition near the channel mouth; however, no structural studies were performed to prove that assumption. Direct information on the structure of low-conductance channels would require performing spectroscopic and single-channel measurements on the same system. Such work is currently in progress.

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REFERENCES

1. S. -S. Sung and P. C. Jordan (1989). The channel properties of gramicidin dimers. *J. Theor. Biol. 140*, 369–380.
2. G. Molle, J. Y. Dugast, H. Duclohier, P. Daumas, F. Heitz and G. Spach (1988). Ionophore properties of a synthetic alpha-helical transmembrane fragment of the mitochondrial H^+ ATP synthetase of *Saccharomyces cerevisiae*. *Biophys. J. 53*, 193–203.
3. G. Spach, H. Duclohier, G. Molle and J-M. Valleton (1989). Structure and supramolecular architecture of membrane-forming peptides. *Biochimie 71*, 11–21.
4. H. Duclohier, G. Molle and G. Spach (1989). Antimicrobial peptide megainin I from *Xenopus* skin forms anion-permeable channels in planar lipid bilayers. *Biophys. J. 56*, 1017–1021.
5. D. B. Sawyer, L. P. Williams, W. L. Whaley, R. E. Koeppe II and O. S. Andersen (1990). Gramicidins A, B and C form structurally equivalent ion channels. *Biophys. J. 58*, 1207–1212.
6. P. C. Jordan (1990). Ion-water and ion-polypeptide correlations in a gramicidin-like channel. *Biophys. J. 58*, 1133–1156.
7. B. Roux and M. Karplus (1991). Ion transport in a model gramicidin channel. *Biophys. J. 59*, 961–981.
8. M. Akeson and D. Deamer (1991). Proton conductance by the gramicidin water wire. Model for proton conductance in F_1F_0 ATPases? *Biophys. J. 60*, 101–109.
9. R. Sarges and B. Witkop (1965). The structure of valine- and isoleucine-gramicidin A. *J. Am. Chem. Soc. 87*, 2011–2020.
10. W. R. Veatch, E. T. Fossel and E. R. Blout (1974). The conformation of gramicidin A. *Biochemistry 13*, 5249–5256.
11. A. S. Arseniev, V. F. Bystrov, V. T. Ivanov and Yu. A.

Ovchinnikov (1984). NMR solution conformation of gramicidin A double helix. *FEBS Lett. 165*, 51–56.

12. V. F. Bystrov and A. S. Arseniev (1988). Diversity of the gramicidin A spatial structure: Two-dimensional 1H NMR study in solution. *Tetrahedron 44*, 925–940.
13. D. W. Urry (1971). The gramicidin A transmembrane channel: Proposed π(L,D) helix. *Proc. Natl Acad. Sci. USA 68*, 672–676.
14. D. W. Urry, M. C. Goodall, J. D. Glickson and D. F. Mayers (1971). The gramicidin A transmembrane channel: characteristics of head-to-head dimerized π(L,D) helices. *Proc. Natl Acad. Sci. USA 68*, 1907–1911.
15. A. S. Arseniev, I. L. Barsukov, V. F. Bystrov, A. L. Lomize and Yu. A. Ovchinnikov (1985). H-NMR study of gramicidin A transmembrane ion channel. *FEBS Lett. 186*, 168–174.
16. R. E. Koeppe 2nd, L. L. Providence, D. V. Greathouse, F. Heitz, Y. Trudelle, N. Purdie and O. S. Anderson (1992). On the helix sense of gramicidin A single channels. *Proteins 12*, 49–62.
17. D. Busath and G. Szabo (1981). Gramicidin forms multi-state rectifying channels. *Nature 294*, 371–373.
18. D. Busath and G. Szabo (1988). Low conductance gramicidin A channels are head-to-head dimers of β6.3-helices. *Biophys. J. 53*, 689–695.
19. D. Busath and G. Szabo (1988). Permeation characteristics of gramicidin conformers. *Biophys. J. 53*, 697–707.
20. D. Busath, O. S. Andersen and R. E. Koeppe II (1987). On the conductance heterogeneity in membrane channels formed by gramicidin A. *Biophys. J. 51*, 79–88.
21. D. B. Sawyer, R. E. Koeppe II and O. S. Andersen (1990). Gramicidin single-channel properties show no solvent-history dependence. *Biophys. J. 57*, 515–523.
22. L. A. Fonina, A. M. Demina, S. V. Sychev, V. T. Ivanov and Ya. Hlavacek (1984). Synthesis of gramicidin A dimeric analogues. *Bioorgan. Khimia 10*, 1073–1079.
23. S. V. Sychev, L. A. Fonina and V. T. Ivanov (1984). Conformational states of gramicidin A bis-derivatives. *Bioorgan. Khimia 10*, 1080–1088.
24. A. I. Irkhin and S. M. Bezrukov (1984). Properties of ionic channels formed by gramicidin A dimeric analogs in lipid bilayer membranes. *Biol. Membrany 1*, 739–748.
25. S. Kh. Aityan and Yu. A. Chizmadzhev (1986). Simulation of molecular dynamics of water movement in ion channels. *Gen. Physiol. Biophys. 5*, 213–229.
26. J. A. Killian, K. U. Prasad, D. Hains and D. W. Urry (1988). The membrane as an environment of minimal interconversion. A CD study on the solvent dependence of the conformational behavior of gramicidin in diacylphosphatidylcholine model membranes. *Biochemistry 27*, 4848–4855.
27. S. V. Sychev, L. I. Barsukov and V. T. Ivanov (1993). The double $\pi\pi$5.6 helix of gramicidin A predominates in unsaturated lipid membranes. *Eur. Biophys. J. 22*, 279–288.
28. M. C. Bano, L. Braco and A. Abad (1991). Conformational transitions of gramicidin A in phospholipid model membranes. A HPLC assessment. *Biochemistry 30*, 886–894.
29. E. Gross and B. Witkop (1965). Gramicidin IX. Preparation of gramicidin A,B, and C. *Biochemistry 4*, 2495–2501.
30. T. Galliard in: *Form and Function of Phospholipids*, G. B. Ansell, J. N. Hawthorne and R. M. C. Dawson, Eds., pp. 253–288, Elsevier, Amsterdam 1973.
31. D. Mao and B. A. Wallace (1984). Different light scattering and absorption flattening optical effects are minimal in the CD spectra of small unilamellar vesicles. *Biochemistry 23*, 2667–2673.
32. S. V. Sychev, N. A. Nevskaya, St. Jordanov, E. N. Shepel, A. I. Miroshnikov and V. T. Ivanov (1980). The solution conformation of gramicidin A and its analogs. *Bioorgan. Chem. 9*, 121–151.
33. E. Bamberg, H. J. Apell and H. Alpes (1977). Structure of the gramicidin A channel: Discrimination between the πL,D and β helix by electrical measurements with lipid bilayer membranes. *Proc. Natl Acad. Sci. USA 74*, 2402–2406.
34. W. R. Veatch and E. R. Blout (1974). The aggregation of gramicidin A in solution. *Biochemistry 13*, 5257–5264.
35. H.-A. Kolb and E. Bamberg (1977). Influence of membrane thickness and ion concentration on the properties of the gramicidin A channel. *Biochem. Biophys. Acta 464*, 127–141.
36. L. Braco, C. Abad, A. Campos and J. E. Figueruelo (1986). Time-dependent monomerization of gramicidin A, enhanced by phosphatidylcholine in non-polar solvents. *J. Chromatogr. 353*, 181–192.
37. L. Braco, M. C. Bano, F. Chillaron and C. Abad (1986). Conformation species of gramicidin A in non-polar solvent. A kinetic and thermodynamic treatment in the absence and presence of phosphatidylcholine as studied by HPLC. *Biophys. Chem. 25*, 297–305.
38. S. -S. Sung and P. C. Jordan (1988). Theoretical study of the antiparallel double-stranded helical dimer of gramicidin as an ion channel. *Biophys. J. 54*, 519–526.
39. W. R. Veatch, R. Mathies, M. Eisenberg and L. Stryer (1975). Simultaneous fluorescence and conductance studies of planar bilayer membranes containing a highly active and fluorescent analog of gramicidin A. *J. Mol. Biol. 99*, 75–92.
40. H. Rottenberg and R. E. Koeppe II (1989). Stimulation of cation transport in mitochondria by gramicidin and truncated derivatives. *Biochemistry 28*, 4361–4367.
41. V. E. Khutorsky (1984). A molecular model for permeability of the gramicidin A transmembrane channel. *Biol. Membrany 1*, 244–253.
42. B. Lotz, F. Colonna-Cesari, F. Heitz and G. Spach (1976). A family of double helices of alternating poly (γ-benzyl-DL-glutamate), a stereochemical model for gramicidin A. *J. Mol. Biol. 106*, 915–942.

43. D. A. Langs (1988). Three-dimensional structure at 0.86 Å of the uncomplexed form of the transmembrane ion channel peptide gramicidin A. *Science 241*, 188–191.
44. D. A. Langs, G. D. Smith, C. Courseille and G. Precigoux (1991). Monoclinic uncomplexed double-straned, antiparallel, left-handed β5.6-helix (1.β5.6) structure of gramicidin A: Alternate patterns of helical association and deformation. *Proc. Natl Acad. Sci. USA 88*, 5345–5349.
45. A. S. Arseniev, I. L. Barsukov and V. F. Bystrov (1985). NMR solution structure of gramicidin A complex with caesium cations. *FEBS Lett. 180*, 33–39.
46. B. A. Wallace (1991). Crystallographic studies of a transmembrane ion channel, gramicidin A. *Prog. Biophys. Molec. Biol. 57*, 59–69.
47. J. T. Durkin, L. L. Providence, R. E. Koeppe 2nd and O. S. Andersen (1992). Formation of non-beta 6.3-helical gramicidin channels between sequence-substituted gramicidin analogs. *Biophys. J. 62*, 145–157.
48. D. W. Urry, M. M. Long, M. Jacobs and R. D. Harris (1975). Conformation and molecular mechanisms of carriers and channels. *Ann. N. Y. Acad. Sci. 264*, 203–220.
49. S. B. Hladky and D. A. Haydon (1972). Ion transfer across lipid membranes in membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochim. Biophys. Acta 274*, 294–312.
50. E. Bamberg, K. Noda, E. Gross and P. Lauger (1975). Single-channel parameters of gramicidin A, B and C. *Biochim. Biophys. Acta 419*, 222–228.
51. D. W. Urry, S. Alonso-Romanowski, C. M. Venkatachalam, R. D. Harris and K. U. Prasad (1984). Dispersity of des-LVal^7-DVal^8 gramicidin A single channel conductances argues for different side chain orientations as basis. *Biochem. Biophys. Res. Commun. 118*, 885–893.
52. R. E. Furman, J. C. Tanaka, P. Mueller and R. L. Barchi (1986). Voltage-dependent activation in purified reconstituted sodium channel from rabbit T-tubular membranes. *Proc. Natl Acad. Sci. USA 83*, 488–492.