Gramicidins A, B, and C form structurally equivalent ion channels

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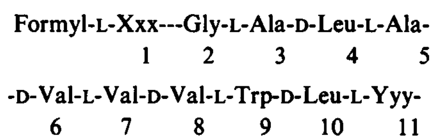
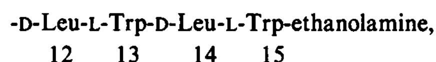
ABSTRACT The membrane structure of the naturally occurring gramicidins A, B, and C was investigated using circular dichroism (CD) spectroscopy and single-channel recording techniques. All three gramicidins form channels with fairly similar properties (Bamberg, E., K. Noda, E. Gross, and P. Läuger. 1976. *Biochim. Biophys. Acta.* 419:223–228.). When incorporated into lysophosphatidylcholine micelles, however, the CD spectrum of gramicidin B is different from that of gramicidin A or C (cf. Prasad, K. U., T. L. Trapane, D. Busath, G. Szabo, and D. W. Urry. 1983. *Int. J. Pept. Protein Res.* 22:341–347.). The structural identity of the channels formed by gramicidin B has, therefore, been uncertain. We find that when gramicidins A and B are incorporated into dipalmitoylphosphatidylcholine vesicles, their CD spectra are fairly similar, suggesting that the two channel structures could be similar. In planar bilayers, gramicidins A, B, and C all form hybrid channels with each other. The properties of the hybrid channels are intermediate to those of the symmetric channels, and the appearance rates of the hybrid channels (relative to the symmetric channels) corresponds to what would be predicted if all three gramicidin molecules were to form structurally equivalent channels. These results allow us to interpret the different behavior of channels formed by the three gramicidins solely on the basis of the amino acid substitution at position 11.

INTRODUCTION

Ion channels are an excellent choice of molecular species with which to investigate the relation between protein structure and function, because the effects of amino acid substitutions on protein function can be determined using single-channel recording techniques, i.e., at the single molecule level. Among ion channels, the linear gramicidins have served as reliable and durable prototypes for such studies because they perform similarly to physiological integral membrane protein channels using only 1% of the molecular mass, which simplifies the molecular interpretation of the results. Gramicidin channels are, thus, the "Rambo" of ion channels (e.g., Canby, 1985).

During sporulation, *Bacillus brevis* synthesizes a mixture of linear gramicidins, having these amino acid sequences (Gross and Witkop, 1965):





where Xxx denotes Val or Ile, and Yyy denotes Trp, Phe, or Tyr in gramicidins A, B, and C, respectively. (In addition to these "standard" gramicidins, commercial samples contain smaller amounts of acylated forms that have fatty acids esterified to the ethanolamine hydroxyl group [Koeppe et al., 1985, 1988; Peart-Williams et al., 1988].) The three different gramicidins form well-defined single-channels with different single-channel conductances and average durations (Bamberg et al., 1976; Becker et al., 1989). Ideally, one would like to relate these functional differences to the different aromatic residues at position 11 in the amino acid sequence. But to make such correlations, one must first determine whether the channels formed by the three gramicidins are structurally equivalent. It is disturbing in this context that circular dichroism (CD) spectra of gramicidins A and B in lysophosphatidylcholine micelles differ significantly (Prasad et al., 1983), suggesting that the (membrane) structure of gramicidin B channels could be different from that of gramicidin A channels.

We have examined this question further by comparing the CD spectra for the naturally occurring gramicidins in lysophosphatidylcholine micelles and in phospholipid vesicles. In addition, we addressed the structural question in single-channel studies, where we tested for the ability of

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Biophys. J. © Biophysical Society
Volume 58 November 1990 1207–1212
0006-3495/90/11/1207/06 $2.00

gramicidin B to form conducting heterodimers (i.e., hybrid channels) with gramicidins A and C in a functional assay of structural equivalence (cf. Durkin et al., 1990).

MATERIALS AND METHODS

L-α-Lysophosphatidylcholine (Lyso-PC) from egg yolk, containing primarily palmitic and stearic acids at position 1, and L-α-dipalmitoylphosphatidylcholine (DPPC) were from Sigma Chemical Co. (St. Louis, MO); they were used as supplied. Diphytanoylphosphatidylcholine (DPhPC) from Avanti Polar Lipids Inc. (Birmingham, AL) was further cleaned by ion-exchange chromatography (Andersen, 1983). *n*-Decane from Wiley Organics (Columbus, OH) was used without further purification.

Methanol was "HPLC" grade from various suppliers (Fisher Scientific Co., Pittsburgh, PA; Baker Instruments Corp., Allentown, PA; and Mallinckrodt Inc. Science Products Div., St. Louis, MO). Ethanol (absolute) was from US Industrial Chemicals (Tuscola, IL). Water was deionized and glass distilled (REK), or deionized Millipore/Continental Water Systems, Milli-Q water (Bedford, MA) (OSA). NaCl was Suprapur grade from E. Merck (through MCB, Cincinnati, OH), it was roasted at 500°C for 24 h and stored over NaOH in evacuated desiccators.

The gramicidins were purchased as mixtures of the naturally occurring gramicidins A, B, and C from Sigma Chemical Co. or United States Biochemical Corp. (Cleveland, OH). The preparation from United States Biochemical Corp. had the highest content of gramicidin B and was used for purifying gramicidin B. The individual A, B, and C components were separated by high-performance liquid chromatography (HPLC) on a 0.78 × 240 cm column of Chromosorb LC-5 (Johns-Manville, Denver, CO), using a multistep 70 h water-methanol gradient, as previously described (Koeppe et al., 1981). The gramicidins were further purified on Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ). Gramicidin concentrations were determined by vacuum drying to constant weight and verified by UV spectroscopy.

Circular dichroism spectroscopy

Gramicidins A, B, and C were incorporated into lyso-PC micelles using the sonication-plus-heat-treatment method (Urry et al., 1979). Solutions containing 6 mM gramicidin (monomer) and 60 mM lyso-PC were placed into polycarbonate tubes and sonicated at 5°C for three 2-min intervals with vortexing between intervals, using a Branson W-185 cell disruptor (power level 5) fitted with a model 431-A cup horn accessory (Branson Sonic Power Co., Danbury, CT). The heated samples of gramicidins A, B, and C were then heated for 23 h with constant stirring at 70°C and stored in a Dewar flask above 50°C. The "unheated samples" were heated for only 45 min at 70°C and stored at room temperature; such brief heating was sufficient to solubilize most of the gramicidin A in the micelles, but not to achieve the channel CD spectrum (Masotti et al., 1980).

Gramicidins A and B were incorporated into DPPC vesicles by a procedure adapted from Barrow and Lentz (1980). Solutions were prepared by dissolving 1 μmol of gramicidin A or B and 30 μmol of DPPC in 2 ml of chloroform/methanol (50/50), evaporating the solvent, resuspending the gramicidin-lipid mixture in 1 ml of N_2-flushed water at 40°C over a period of 1 h, and finally sonicating the suspension at power level 5 (see above) for 2.5 h at 45°C. The solutions were then centrifuged at 16,000 *g* at room temperature for 30 min.

The lyso-PC/gramicidin and DPPC/gramicidin stock solutions were transported to the laboratory of Dr. Neil Purdy (Oklahoma State University). Samples for CD spectroscopy were prepared by diluting the stock solutions with distilled water just before analysis (see figure legends). The spectra were obtained using a J-500A spectrometer (Jasco Inc., Easton, MD).

Single-channel recordings

Planar lipid bilayers were formed from diphytanoylphosphatidylcholine (DPhPC) in *n*-decane (2.5% wt/vol). The general experimental procedures were as described in Andersen (1983) and Sawyer et al. (1989). The experiments were done with symmetrical unbuffered solutions of 1.0 M NaCl at 25 ± 2°C. Doubly HPLC-purified gramicidin A, B, or C was dissolved in methanol, diluted 1:100 in ethanol (to ~10 nM), and added to the electrolyte surrounding the membrane during vigorous stirring, to a nominal concentration of gramicidin in the electrolyte solution of ~10 pM. The stirring was continued for more than 1 min before the measurements began.

Single-channel measurements with [Val¹]gramicidin A, B, and C were done at 200 mV applied potential using the bilayer punch technique (Andersen, 1983). Single-channel current transitions were detected and their amplitudes were determined as described above.

To assess whether the three different peptides form structurally equivalent channels, we examined whether heterodimers, or hybrid channels, could form between the chemically dissimilar gramicidin monomers (Durkin et al., 1990). In these experiments, equipotent amounts of two different gramicidin molecules were added to both aqueous phases, and the membrane was broken and reformed to ensure that both peptides were adsorbed in equal amounts at both membrane/solution interfaces. The hybrid channels were identified by their appearance in current transition amplitude histograms. In experiments with a single species of gramicidin, the histograms consisted of a single major peak that included ~95% of the well-defined transitions in that experiment. In the hybrid channel experiments, there were three (or four) peaks in the histograms: two corresponding to the two symmetrical channel types, and one (or two) corresponding to the hybrid channels.

Channel durations were determined by matching channel appearances with disappearances and measuring the interval between these. Only events with amplitudes that fell within the desired distribution in the amplitude histogram for that experiment were used in the analysis. Where channels occurred singly, this was straightforward. When a new channel appeared, while one (or more) channels were conducting, channel appearances and disappearances that fell within the desired distribution were matched using a randomized assignment, which is valid as long as all appearances (and disappearances) of the same amplitude represent the same type of molecular event. Channel durations were plotted as normalized survivor plots (e.g., Andersen and Muller, 1982). To estimate the average duration (τ) for each distribution, a single exponential decay, $N(t)/N(0) = \exp(-t/\tau)$, was fitted to the results using a maximum likelihood estimator (Hall and Sellinger, 1981), where $N(t)$ denotes the number of channels with a duration $\geq t$, and $N(0)$ is the estimated total number of channels in the population. ($N(0)$ differs from the number of events actually observed because the on-line channel detection algorithm does not define unambiguously events with durations <40 ms. Such events may, thus, be lost in the subsequent analysis.)

RESULTS

The CD spectra of gramicidin A solubilized in lyso-PC by sonication and mild heating ("unheated" gramicidin A), and of gramicidins A, B, and C incorporated into lyso-PC

micelles by sonication and extended heating, are illustrated in Fig. 1. As has been reported previously, the CD spectrum of gramicidin A in lyso-PC undergoes a dramatic change upon extended heating at 70°C (Masotti et al., 1980). This change has been attributed to a conformational change from a lyso-PC-associated solvent form to the final "channel" state. This change is illustrated for gramicidin A in Fig. 1. Heat-incorporated gramicidins A and C have similar CD spectra, but that of gramicidin B is more similar to the "unheated" gramicidin A spectrum (c.f. Prasad et al., 1983). It thus appears that the structure of gramicidin B in lyso-PC differs from that of gramicidins A and C.

The CD spectra of gramicidins A and B incorporated into DPPC vesicles are illustrated in Fig. 2. The spectrum for gramicidin A is similar, but not identical, to that obtained in the lyso-PC samples. More significantly, the spectra for gramicidins A and B are more similar in DPPC than in lyso-PC. The spectrum for gramicidin B in DPPC more nearly resembles that of "heated" gramicidin A in lyso-PC (or in DPPC) than the spectrum for "heated" gramicidin B in lyso-PC. Thus, based on the DPPC results one would surmise that the channels formed by gramidicin B could be structurally equivalent to those formed by gramicidins A and C; but they could, for example, have a different helix pitch.[1]

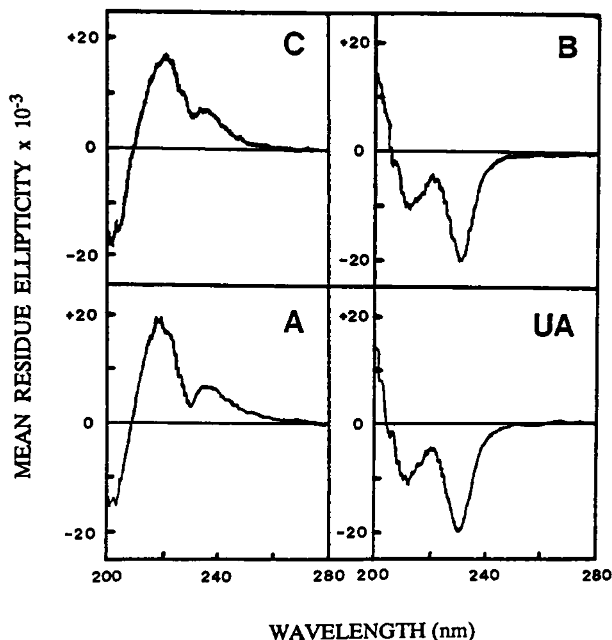

FIGURE 1 Circular dichroism spectra of 0.06 mM gramicidin A, B, or C (monomer) dispersed in 0.6 mM aqueous lyso-PC. All samples except "UA" ("unheated A") sample were heated for 23 h at 70°C after 6 min of sonication (see Methods). *A* and *UA* refer to heated and unheated gramicidin A, respectively, *C* to heated gramicidin C, and B to heated gramicidin B.

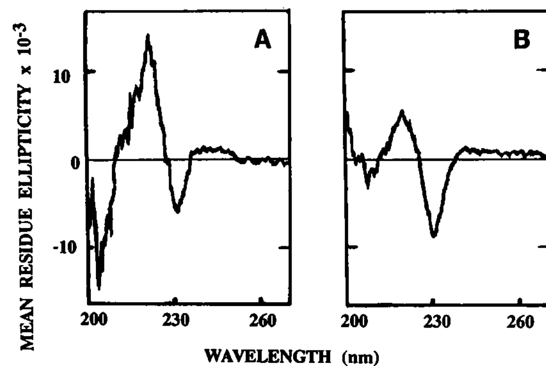

FIGURE 2 Circular dichroism spectra of 0.045 mM gramicidin A or gramicidin B dispersed in 1.4 mM aqueous DPPC. The samples were sonicated for 2.5 h at 45°C (see Methods). *A* refers to gramicidin A, and *B* to gramicidin B.

Single-channel experiments

The single-channel characteristics of gramicidins A, B, and C are shown in Fig. 3. All three peptides form well-defined channels whose amplitudes fall within a narrow distribution in an amplitude histogram. The amplitude of channels formed by gramicidin B is smaller than that of gramicidin C, which is smaller than that of

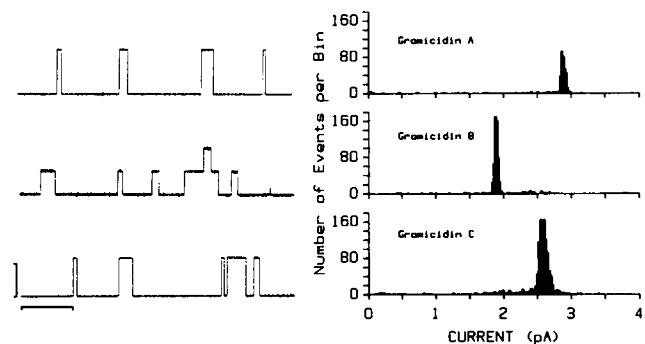

FIGURE 3 Single-channel behavior induced by [Val[1]]gramicidins A, B, and C at 200-mV applied potential in 1 M NaCl. (*A*) Single-channel current traces. (*B*) The corresponding current transition amplitude histograms. Statistics for the experiments are summarized in Table 1. The vertical calibration bar denotes 2 pA, the horizontal bar, 5 s. The current traces were filtered at 50 Hz.

[1]The CD spectrum of the gramicidins is dominated by the absorption of the Trp residues (Urry et al., 1975), such that there is no rigorous method to relate changes in the CD spectrum to structural changes.

gramicidin A. In addition, the durations of the three channels differ, with gramicidin B channels having the longest average duration, followed by gramicidin A, then gramicidin C.

When any two of these gramicidin analogues were incorporated into the bilayer at the same time, at least three distinct channel types were observed: two channel types with the characteristics of the respective homodimeric channels, and at least one channel type with characteristics intermediate to those of the symmetrical channels and representing heterodimers formed by two different monomers (Fig. 4). For the case where hybrid channels formed between gramicidins B and A (or B and C), two different hybrid-channel amplitudes were detected corresponding to the two possible orientations of the asymmetrical heterodimers with respect to the transmembrane potential.

If a given monomer cannot distinguish between two different analogues when pairing with another monomer to form a channel (i.e., when the two monomers are structurally equivalent), and if the gramicidin monomers of each species are evenly distributed in the two monolayers of the membrane, then the frequency of the pure channels f_x and f_y will be related to the frequency of hybrid channels f_h by $f_h/2(f_x f_y)^{1/2} \geq 1$ (Veatch and Stryer, 1977; Mazet et al., 1984; Durkin et al., 1990). The observed ratio is 1.13 for A–B hybrids, 1.28 for B–C hybrids, and 0.97 for A–C hybrids. Moreover, we can use this ratio to calculate the difference in activation energy for heterodimer vs. homodimer channel formation, $\Delta\Delta G^{\ddagger}$, because (Durkin et al., 1990)

$$\Delta\Delta G^{\ddagger} = -R \cdot T \cdot \ln(f_h/2(f_x f_y)^{1/2}). \quad (1)$$

In addition, we can calculate the standard free energy difference for heterodimers vs. homodimers, $\Delta\Delta G°$, using the average durations (τ) of each of the channel types as

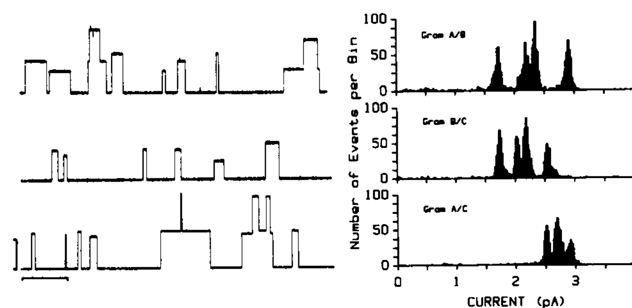

FIGURE 4 Formation of heterodimeric channels by gramicidins A, B, and C at 200-mV applied potential in 1 M NaCl. (*A*) Single-channel current traces. (*B*) The corresponding current transition amplitude histograms. Statistics for the experiments are summarized in Table 1. The vertical calibration bar denotes 2 pA, the horizontal bar, 5 s. The current traces were filtered at 50 Hz.

well (Dürkin et al., 1990),

$$\Delta\Delta G° = -RT\ln\{[f_h/2(f_x f_y)^{1/2}] \cdot [\tau_h/(\tau_x \tau_y)^{1/2}]\}. \quad (2)$$

The channel durations and calculated free energy differences are presented in Table 1. Both activation and standard free energy differences are in all cases close to 0, which indicates that for these three gramicidin analogues there is no energetic cost to hybrid channel formation, and that channels formed by these three analogues thus have equivalent conformations (Durkin et al., 1990).

DISCUSSION

The main conclusion of this paper is that in bilayer systems the channel structures of gramicidins A, B, and C are equivalent. This conclusion is supported by the similar CD spectra of the gramicidins in lipid vesicles and, in particular, by the ability of the three gramicidins to form

TABLE 1 Single channel parameters and energetic calculations for gramicidins A, B, and C and hybrid channels

Analogue	I(pA) ± SD	Frequency	$F^{\ddagger}$	τ*	$N(0)$*	$\Delta\Delta G^0$	$\Delta\Delta G$
				s			*kJ/mol*
A	2.89 ± 0.05	0.25	—	0.99	180	—	—
B	1.71 ± 0.06	0.22	—	2.1	150	—	—
Hybrid	2.26 ± 0.1	0.53	1.13	1.3	450	−0.04	−0.29
B	1.83 ± 0.06	0.25	—	2.3	180	—	—
C	2.67 ± 0.03	0.19	—	0.87	160	—	—
Hybrid	2.22 ± 0.04	0.56	1.28	1.2	360	−0.21	−0.59
A	2.90 ± 0.05	0.29	—	1.1	100	—	—
C	2.51 ± 0.04	0.22	—	0.95	90	—	—
Hybrid	2.70 ± 0.05	0.49	0.97	0.96	210	0.25	0.08

*τ and $N(0)$ are estimates based upon maximum likelihood estimator fits of the survivor plots of the channel durations.
‡F is the fraction $f_h/(2(f_x f_y)^{1/2}$.

hybrid channels among each other. The differences in the properties (conductance and average duration) of the channels formed by these gramicidins do not result from any major conformational change induced by the amino acid substitutions, but result from differences in the physicochemical properties of the aromatic residues at position 11.

The different CD spectra of the gramicidins in lyso-PC dispersions are a curious phenomenon and may have some bearing on the properties of gramicidin channels. Lyso-PC/gramicidin mixtures adopt a variety of micellar and vesicular structures (Killian et al., 1983). The change in conformation of gramicidin A upon heating implies that gramicidin A is trapped in its solvent conformation during its initial interactions with lyso-PC, and can only reach the energetically preferred conformation with the input of energy that enables it to overcome a large activation energy barrier. This observation is consistent with CD and NMR studies of the solvent history dependence of the membrane conformations of gramicidin, where gramicidin tends to retain its solvent conformation when added to membranes and will only upon heating relax toward the preferred "channel" conformation (Killian et al., 1988; LoGrasso et al., 1988).[2]

The inability of gramicidin B to relax toward the "channel" conformation in lyso-PC even upon prolonged heating implies that either the barrier for such a conformational change is much higher for gramicidin B than for A and C, or that such a structure is not the energetically most favorable conformation for gramicidin B in the lyso-PC environment.

These differential effects of the lyso-PC environment on gramicidin conformational changes could be evidence for specific gramicidin/lipid interactions. In planar bilayers, lyso-PC and other detergents alter the conformational equilibrium of conducting gramicidin dimers in a more subtle manner, inducing the formation of conductance variants (Sawyer et al., 1989).

From the hybrid channel experiments, the channel conformations of the three gramicidins are very similar. Considering the different number of tryptophans in gramicidins A and B, their CD spectra in DPPC vesicles are probably also indicative of their structures being very similar in that environment. Thus, to the extent that the CD spectrum of gramicidin A in DPPC vesicles report on the same structure as the conducting channel in planar bilayers, it is likely that the channel conformation of gramicidin B, as recorded in planar bilayers, is like that in DPPC vesicles. This indicates that DPPC vesicles are a better system for structural studies of gramicidins, and perhaps other membrane proteins, than are detergent micelles, when one is interested in drawing conclusions about the active conformation of the peptide/protein.

[2]But this depends on the lipid/peptide ratio, because gramicidin added to bilayers forms single channels without heating, and gramicidin channels display no solvent history dependence (Sawyer et al., 1990).

We wish to thank Drs. N. Han and N. Purdy for permission to use the circular dichroism spectrometer, and for assistance with the measurements.

This work supported by the United States Public Health Service through National Institutes of Health grants GM 21342 and GM 34968. D. B. Sawyer was supported by the M.D./Ph.D. student fellowship program at Cornell University Medical College.

Received for publication 21 May 1990 and in final form 27 July 1990.

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