Gramicidin Channels in Phospholipid Bilayers with Unsaturated Acyl Chains

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ABSTRACT In organic solvents gramicidin A (gA) occurs as a mixture of slowly interconverting double-stranded dimers. Membrane-spanning gA channels, in contrast, are almost exclusively single-stranded $\beta^{6.3}$-helical dimers. Based on spectroscopic evidence, it has previously been concluded that the conformational preference of gA in phospholipid bilayers varies as a function of the degree of unsaturation of the acyl chains. Double-stranded $\pi\pi^{5.6}$-helical dimers predominate (over single-stranded $\beta^{6.3}$-helical dimers) in lipid bilayer membranes with polyunsaturated acyl chains. We therefore examined the characteristics of channels formed by gA in 1-palmitoyl-2-oleoylphosphatidylcholine/*n*-decane, 1,2-dioleoylphosphatidylcholine/*n*-decane, and 1,2-dilinoleoylphosphatidylcholine/*n*-decane bilayers. We did not observe long-lived channels that could be conducting double-stranded $\pi\pi^{5.6}$-helical dimers in any of these different membrane environments. We conclude that the single-stranded $\beta^{6.3}$-helical dimer is the only conducting species in these bilayers. Somewhat surprisingly, the average channel duration and channel-forming potency of gA are increased in dilinoleoylphosphatidylcholine/*n*-decane bilayers compared to 1-palmitoyl-2-oleoylphosphatidylcholine/*n*-decane and dioleoylphosphatidylcholine/*n*-decane bilayers. To test for specific interactions between the aromatic side chains of gA and the acyl chains of the bilayer, we examined the properties of channels formed by gramicidin analogues in which the four tryptophan residues were replaced with naphthylalanine (gN), tyrosine (gT), and phenylalanine (gM). The results show that all of these analogue channels experience the same relative stabilization when going from dioleoylphosphatidylcholine to dilinoleoylphosphatidylcholine bilayers.

INTRODUCTION

The linear gramicidins, as exemplified by gramicidin A (gA), are channel-forming pentadecapeptide antibiotics that are conformationally polymorphic in organic solvents (Veatch et al., 1974; Bystrov and Arseniev, 1988) and yet assume a single, well-defined structure in lipid bilayer membranes (Andersen et al., 1986; Arseniev et al., 1986; Nicholson and Cross, 1989; Sawyer et al., 1989; Koeppe et al., 1992; see Koeppe and Andersen, 1996, for a recent review). In organic solvents the gramicidins exist as a mixture of monomers with no defined structure (Roux et al., 1990) and slowly interconverting parallel and antiparallel intertwined double-stranded (DS) dimers (Veatch et al., 1974; Bystrov and Arseniev, 1988; Abdul-Manan and Hinton, 1994). In lipid bilayers (or bilayer-like environments) with saturated acyl chains, gA occurs as either monomers or as membrane-spanning channels that are dimers, which are created by the transmembrane association (O'Connell et al., 1990) of two right-handed (RH), single-stranded (SS) β-helical monomers with 6.3 residues per turn (Arseniev et al., 1986; Baño et al., 1988; Nicholson and Cross, 1989; Koeppe et al., 1992) that form a head-to-head (formyl-NH-terminal-to-formyl-NH-terminal) dimer (Bamberg and Läuger, 1973; Cifu et al., 1992). Fig. 1 shows the primary sequence of gA (Fig. 1 *A*), schematic representations of DS and SS dimers (Fig. 1 *B*), as well as schematic representations of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1,2-dioleoylphosphatidylcholine (DOPC), and 1,2-dilinoleoylphosphatidylcholine (DLoPC) (Fig. 1 *C*).

The variety of structural themes in organic solvents and lipid bilayers makes gramicidin channels well suited for examining questions pertaining to the folding and function of membrane proteins (Andersen and Koeppe, 1992; Koeppe and Andersen, 1996) and for understanding membrane-protein interactions (Andersen et al., 1992; Killian, 1992).

Cox et al. (1992) and Sychev et al. (1993) reported that the structure of membrane-spanning gA dimers varies as a function of the degree of unsaturation of the acyl chains of the bilayer-forming phospholipids in which the channels are imbedded. Cox et al. (1992) found that the kinetics of the conformational transitions between DS and SS conformers are faster in bilayers with polyunsaturated acyl chains. Sychev's group found that the DS/SS equilibrium is shifted toward the DS form in bilayers formed by polyunsaturated phospholipids. In addition, Greathouse et al. (1994) found that the conformational preference of gA in micelles formed by short-chain phosphatidylcholines varies as a function of the acyl chain length: SS $\beta^{6.3}$-helices dominate at acyl chain lengths above eight; DS conformers predominate when the acyl chain length is less than eight. Similarly, at very long chain lengths (e.g., in di-$C_{22:1}$-PC bilayers), one observes conducting DS gA channels, but these conformers are not observed in the amino acid sequence-extended analogue [l-Ala^{0a}-d-Ala^{0b}]gA (Nielsen et al., 1997). These results

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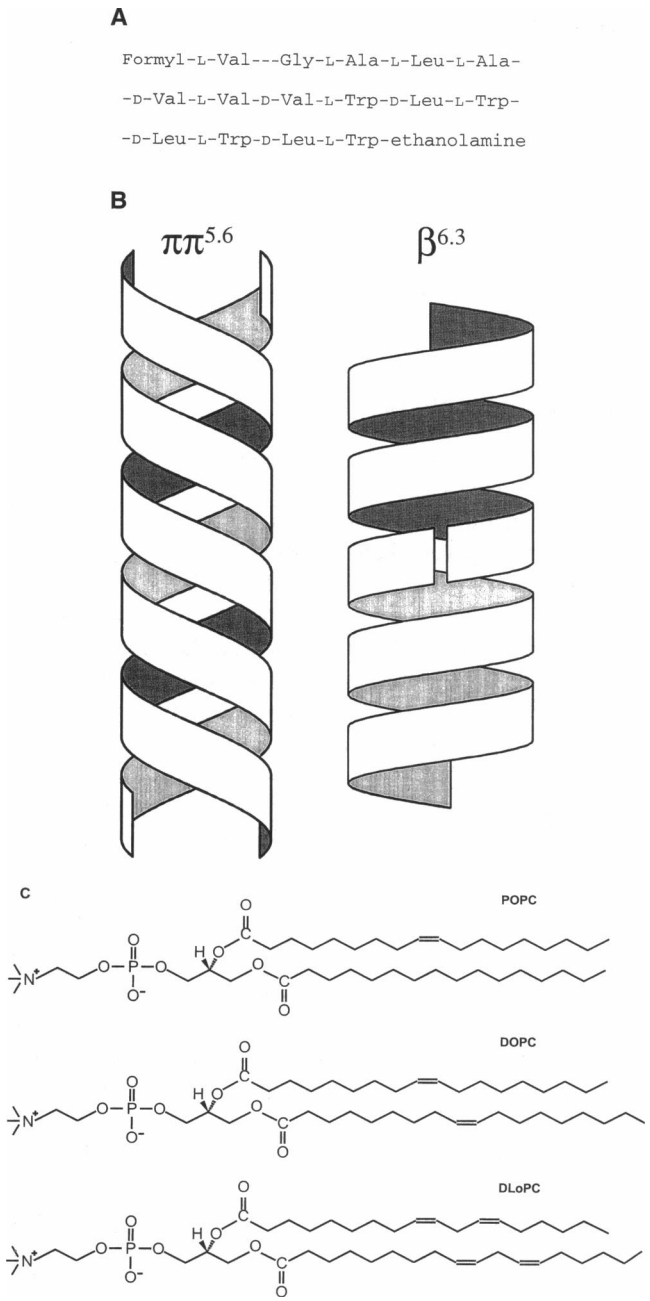

FIGURE 1 (*A*) Primary sequence of gA (Sarges and Witkop, 1965). (*B*) Schematic representation of a gramicidin double-stranded $\pi\pi^{5.6}$-helical dimer (*left*) and a single-stranded $\beta^{6.3}$-helical dimer (*right*). Comparing the sequence and backbone structures, it is apparent that the tryptophan residues will be spaced uniformly along the double-stranded helix, whereas they will be localized at the ends of the single-stranded dimer. (*C*) Schematic representations of POPC, DOPC, and DLoPC.

show that the conformational preference of gA is determined by an interplay between the primary sequence and the lipid environment. The question remains whether the effect(s) of the membrane is specific (resulting from specific side-chain/lipid interactions) or is a reflection of more global membrane properties.

Both Cox et al. (1992) and Sychev et al. (1993) used a combination of infrared absorbance and circular dichroism (CD) spectroscopy to examine the structural preference of gA in lipid vesicles formed by phosphatidylcholines with unsaturated acyl chains. They concluded that gA dimers in bilayers formed by either POPC/*n*-decane or DOPC/*n*-decane are almost exclusively SS, RH $\beta^{6.3}$-helical dimers, whereas the predominant dimer species in DLoPC/*n*-decane bilayers are left-handed parallel and antiparallel DS dimers with 5.6 residues per turn (DS $\pi\pi^{5.6}$-helical dimers). The (equilibrium) result was independent of the solvent from which gA was added, but it was not established whether the $\pi\pi^{5.6}$-helical DS dimers in fact span the membrane.

Sychev et al. (1993) also found that the distribution between SS and DS dimers varied as a function of the aqueous electrolyte composition. In the absence of salt, ~20% of the gA occurred as $\beta^{6.3}$-helical dimers and ~80% occurred as $\pi\pi^{5.6}$-helical dimers. In the presence of Na^+ the fraction of gA in the $\pi\pi^{5.6}$-helical dimer form was increased, whereas Cs^+ and K^+ increased the fraction of gA in the $\beta^{6.3}$-helical form. Na^+ therefore binds to $\pi\pi^{5.6}$-helical dimers, which stabilizes them, whereas Cs^+ and K^+ bind to $\beta^{6.3}$-helices and thereby stabilize this conformer. This observation raises the possibility that one might be able to examine the functional characteristics of DS gramicidin channels—assuming that the $\pi\pi^{5.6}$-helical DS dimers are membrane-spanning.

We therefore examined channel formation by gA in POPC, DOPC, and DLoPC (*n*-decane containing) bilayers. The results show that gA forms only a single type of conducting channel in all three lipid environments (irrespective of the degree of unsaturation). There is no evidence for functional (conducting) gA DS helical channels in any of the membrane environments we examined. Surprisingly, the channel-forming potency and the average duration are higher in DLoPC/*n*-decane than in DOPC/*n*-decane and POPC/*n*-decane bilayers. To test whether this stabilization was dependent on the tryptophans, we examined the properties of channels formed by gramicidin analogues in which the four tryptophan residues were replaced with naphthylalanine (gN), tyrosine (gT), and phenylalanine (gM) (Fig. 2).

The results show that all of these analogue channels experience the same relative stabilization when going from DOPC/*n*-decane to DLoPC/*n*-decane bilayers. Some of this material has appeared in a preliminary form (Girshman et al., 1996).

MATERIALS AND METHODS

DOPC, POPC, and DLoPC (>99% pure) were from Avanti Polar Lipids (Alabaster, AL) and were used as supplied. *n*-Decane was from Wiley Organics (Columbus, OH) and was used without further purification. GA was purified from the naturally occurring mixture of gramicidin A, B, and C as described previously (Koeppe and Weiss, 1981). gT was a gift from Y. Trudelle and F. Heitz (Trudelle and Heitz, 1987); gN was a gift from Y. Lazaro and F. Heitz (Ranjalahy-Rasoloarijao et al., 1989); gM was synthesized as described by Providence et al. (1995). The gA and gT were dissolved in absolute ethanol (U.S. Industrial Chemicals, Tuscola, IL). gN

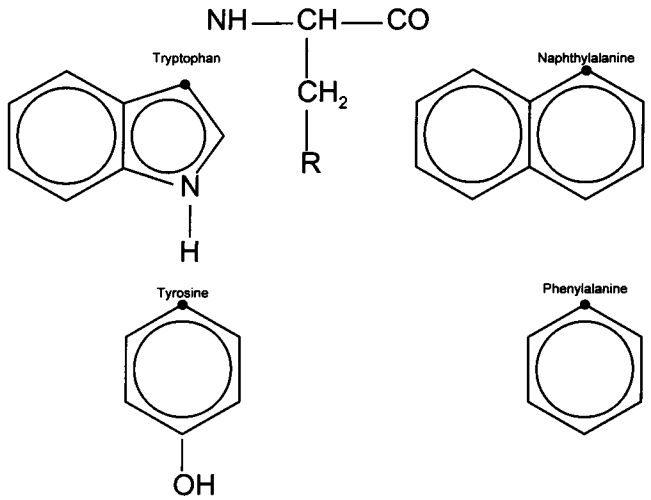

FIGURE 2 Chemical modifications at positions 9, 11, 13, and 15. The peptide backbone and the β-methylene group are illustrated at the top. The aromatic rings are attached to the β-methylene at the filled circle (●).

and gM were dissolved in ethanol, but we did not observe reproducible current transitions. Subsequently, gN and gM were dissolved in dimethylsulfoxide (DMSO) (American Burdick and Jackson, Muskegon, MI), which is known to promote the monomeric form of gramicidin (Glickson et al., 1972; Hawkes et al., 1987), which then goes directly into the channel form when incorporated into a membrane environment (Masotti et al., 1980; Tournois et al., 1987). NaCl was analytical grade from EM Science (Cherry Hill, NJ) and was roasted at 550°C for 24 h and stored over $CaSO_4$ in an evacuated desiccator. CsCl was ultrapure grade from United States Biochemicals (Cleveland, OH). Water was deionized Milli-Q water (Millipore Corp., Bedford, MA).

Planar bilayers were formed from *n*-decane solutions (2.5% wt/vol) of the different lipids across a hole (~1.6 mm diameter) in a Teflon partition separating two aqueous solutions of either unbuffered 1.0 M NaCl or 1.0 M CsCl, which were prepared the day of the experiment. Single-channel experiments were done at 25 ± 1°C with a Dagan 3900 (Minneapolis, MN) patch clamp, using the bilayer punch technique, with pipette tip diameters of ~30 μm (Andersen, 1983). Single-channel current transitions were detected with a PC/AT-compatible computer employing the algorithm described by Andersen (1983). Single-channel current transition amplitude histograms and duration distributions were determined as previously described (Andersen, 1983; Sawyer et al., 1989; Durkin et al., 1990), using programs written in AxoBasic (Axon Instruments, Foster City, CA). The distribution of single-channel current transitions can be characterized by the mini-channel frequency (f_m), the percentage of transitions that fall outside the major peak in the histogram (cf. Busath et al., 1987):

$$f_m = \frac{100 \cdot t_m}{t_m + t_s} \quad (1)$$

where t_m and t_s denote the number of transitions that fall outside and within the major peak in the histogram. A mini-channel frequency >10% is indicative of a significant heterogeneity among the single-channel events (Busath and Szabo, 1981; Busath et al., 1987). The duration distributions were determined only for the channel events in the major peak of the current transition amplitude histogram. The duration histogram was transformed into survivor plots, and the average duration (τ) was determined by fitting a single exponential decay ($N(t) = N(0)\cdot\exp\{-t/\tau\}$, where $N(t)$ denotes the number of channels with a duration longer than time t) to each distribution (Sawyer et al., 1989; Durkin et al., 1990).

gA usually was added to both sides of the bilayer, which is necessary for the formation of SS gramicidin channels (O'Connell et al., 1990). In some experiments, where we wanted to determine whether DS channels could form, gA was added asymmetrically (to only one side of a preformed bilayer). These experiments also were done with the bilayer punch.

Membrane capacitance measurements were done by applying a sawtooth potential across the large membrane (area ~2.5 mm^2). The bilayer area was determined by using a microscope with a calibrated reticule.

The relative channel-forming potency for gA (Russell et al., 1986) was determined as the relative appearance rate, the ratio of the gramicidin concentrations that had to be added to the aqueous phase to give a particular channel appearance rate (~1/s). The results are given relative to the appearance rate in POPC/*n*-decane bilayers.

RESULTS

Experiments with gA

The basic characteristics of gA channels in POPC/*n*-decane, DOPC/*n*-decane, and DLoPC/*n*-decane bilayers are shown in Fig. 3 and summarized in Table 1.

In all three bilayers there is a single dominant conducting channel species. There is no evidence for a well-defined secondary channel type with a very long-lived duration, as would be expected for channels that are DS dimers, which are stabilized by 28 intermolecular hydrogen bonds (Langs, 1988; Durkin et al., 1992), in contrast to the six intermolecular hydrogen bonds stabilizing SS $\beta^{6.3}$-helical dimers (Arseniev et al., 1986). The current traces for channels in POPC/*n*-decane and DOPC/*n*-decane bilayers (Fig. 3 *A*) show channels with uniform transitions. The corresponding amplitude histograms (Fig. 3 *B*) have a single major peak that encompasses ~90% of the transitions. In contrast, the current trace and amplitude histogram for channels in DLoPC/*n*-decane bilayers show a relatively high mini-channel frequency (conductance variants that fall outside of the major peak). We believe this is an experimental artifact because DLoPC/*n*-decane bilayers are unstable (average duration of only ~10 min), which made the experiments difficult because more bilayers had to be formed. We generally observe high mini-channel frequencies under such conditions.

The duration distributions for the major channel type (Fig. 3 *C*) can be described by a single exponential distribution, again indicative of a uniform population of channels. (The results obtained in DLoPC/*n*-decane bilayers are not as well fitted by a single exponential distribution. We do not attach significance to this, again because of the poor stability of these bilayers.) The average durations and relative appearance rates in POPC/*n*-decane and DOPC/*n*-decane bilayers are comparable. Surprisingly, the relative appearance rate is ~10-fold higher in DLoPC/*n*-decane bilayers, and τ is ~10-fold longer than in POPC/*n*-decane and DOPC/*n*-decane bilayers (Table 1). In contrast to the large increase in τ, the single-channel conductance (g) increases by only ~25% (Fig. 3, Table 1).

The results in Fig. 3 strongly suggest that gA forms only SS $\beta^{6.3}$-helical channels in all three bilayers. To further substantiate this conclusion, experiments were done to compare the channel activities seen after symmetrical and asymmetrical gA addition (Fig. 4).

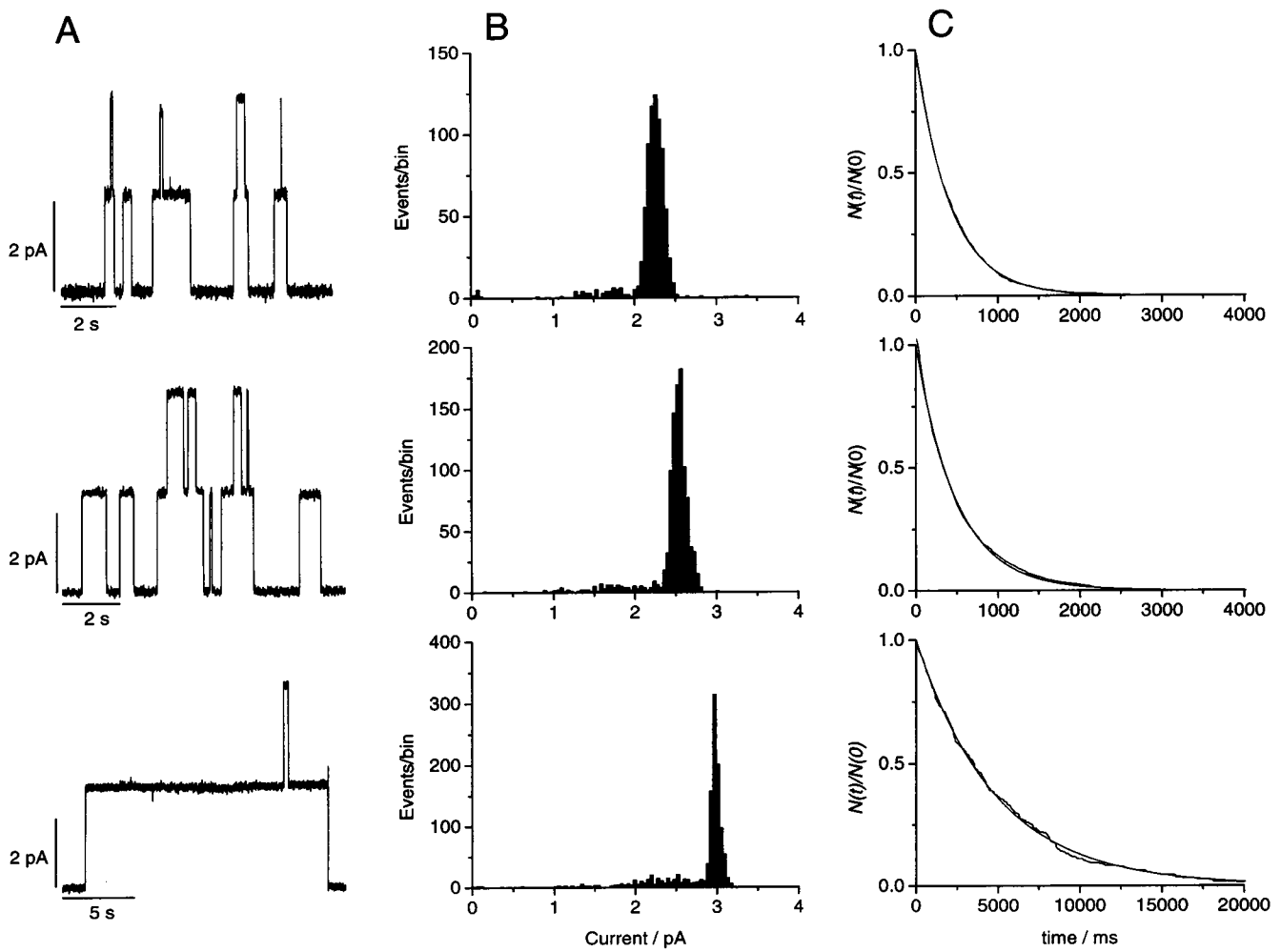

FIGURE 3 (*A*) Single-channel current traces of gA in POPC/*n*-decane (*top*), DOPC/*n*-decane (*middle*), and DLoPC/*n*-decane (*bottom*) bilayers. The corresponding amplitude histograms (*B*) and normalized duration distributions are displayed as survivor plots (*C*). The smooth curves in the duration plots denote the fits of a single exponential distribution to the results. The results are summarized in Table 1. Conditions: 1.0 M NaCl, 200 mV, 100 Hz, 25 ± 1°C.

When gA is added to both sides of a bilayer (as is usually the case), membrane-spanning SS $\beta^{6.3}$-helical dimers will form. In addition, if there were conducting DS dimers, one would see channel activity associated with such conformers. When gA is added asymmetrically (to one side only), SS channels should not form readily (O'Connell et al., 1990), whereas DS channels do form readily (Durkin et al., 1992).

TABLE 1 Summary of results for gA channels

Lipid	Capacitance (μF/cm^2)	Relative channel-forming potency*	g (pS)	f_m	τ (ms)
POPC	0.40 ± 0.05	1	11.3 ± 0.5	9.8	420 ± 40
DOPC	0.41 ± 0.07	1	13.0 ± 0.5	9.6	500 ± 100
DLoPC	0.47 ± 0.06	10	14.9 ± 0.4	24	5200 ± 300

Mean ± standard deviation. Conditions: 1.0 M NaCl, ±200 mV, 25 ± 1°C.

*1/(the amount of gA necessary to give a channel appearance rate of ~1/s), normalized by the value for POPC.

Fig. 4 *A* shows a current trace obtained in an experiment where gA was added symmetrically to a preformed DLoPC/*n*-decane bilayer (nominal aqueous gA concentration ~2 pM). There was high channel activity (5–10 conducting channels) immediately after the membrane potential was applied (at ~60 s after addition); a similar result was obtained in one other experiment. Fig. 4 *B* shows a current trace obtained in an experiment where gA was added asymmetrically to a preformed DLoPC/*n*-decane bilayer (nominal aqueous gA concentration ~4 pM). No channel activity was observed for more than 10 min (the figure shows the first 5 min); a similar result was obtained in one other experiment. When the large membrane was broken and reformed, which allowed gA to be present on both sides of the membrane, there was immediate channel activity when a new small membrane was isolated with the punch (results not shown). Fig. 4 *C* shows a current trace at very high amplification (filtered at 1 Hz), which shows no convincing evidence for long-lived low-conductance events. (There

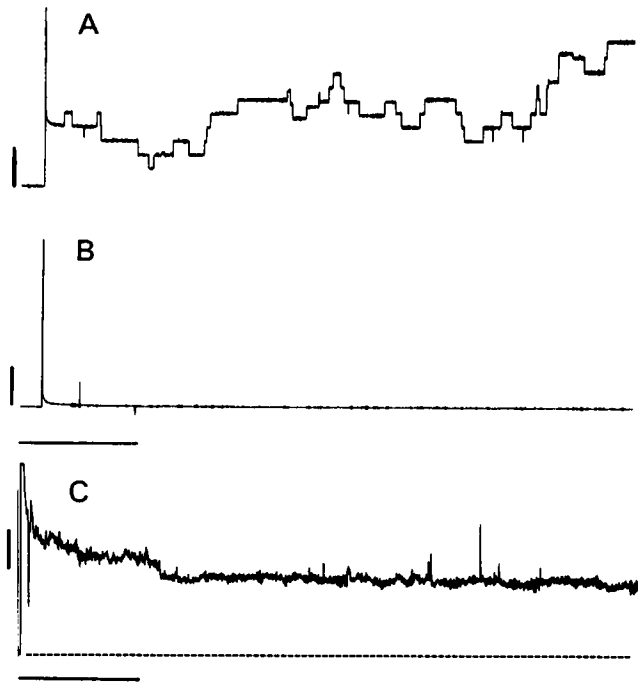

FIGURE 4 (*A*) Single-channel current trace obtained after symmetrical addition of gA to both sides of a preformed DLoPC/*n*-decane bilayer. There is immediate channel activity. (*B*) Single-channel current trace obtained after asymmetrical addition of gA. There is no channel activity for more than 10 min (figure shows the first 5 min). (*C*) Single-channel current trace shown at a 100-fold increased amplification, to demonstrate the absence of well-defined long-lived current transitions. Calibration bars for *A* and *B*: 10 pA (*vertically*); 5 s (*horizontally*). Calibration bars for (*C*): 0.1 pA (*vertically*); 60 s (*horizontally*); 1 Hz. Conditions: 1.0 M NaCl, 200 mV, 100 Hz, 25 ± 1°C.

may be three current transitions with an amplitude of ~0.03 pA; similar transitions can be seen in the absence of gA. We do not attach any significance to them.) The conductance of the pipette plus bilayer was ~1 pS, which is the background we usually obtain; it is unlikely that we missed very long-lived events (that did not disappear during the 10-min observation period).

The increased channel-forming potency, taken together with the longer average channel duration, shows that SS, RH $\beta^{6.3}$-helical dimers form more readily (and are more stable) in DLoPC/*n*-decane bilayers than in POPC/*n*-decane and DOPC/*n*-decane bilayers, in apparent conflict with the spectroscopic results of Sychev et al. (1993). The changes in channel-forming potency and average duration could, however, result from changes in membrane thickness (Kolb and Bamberg, 1977). The specific capacitance of DLoPC/*n*-decane bilayers is ~20% higher than that of POPC/*n*-decane and DOPC/*n*-decane bilayers (Table 1). The change in specific capacitance cannot be quantitatively related to a change in membrane hydrophobic thickness, because the dielectric constant of the hydrocarbon core of DLoPC/*n*-decane bilayers will be higher than that of POPC/*n*-decane and DOPC/*n*-decane bilayers because of the increased density of double bonds. Nevertheless, the results show that the thickness of the hydrophobic core of DLoPC/*n*-decane bilayers is less than that of POPC/*n*-decane or DOPC/*n*-decane bilayers. The dielectric constant of hydrocarbons increases by ~0.2/double bond (Weast, 1972). The dielectric constant of a DLoPC/*n*-decane bilayer would be at most 10% larger than that of a DOPC/*n*-decane bilayer, which could account for only half the capacitance increase. (The actual increase in dielectric constant will be less because of the *n*-decane in the bilayer's hydrophobic core.)

Experiments with Trp→aromatic substituted gramicidins

Given the spectroscopic results of Cox et al. (1992) and Sychev et al. (1993), it is surprising that conducting SS $\beta^{6.3}$ gA channels are stabilized in DLoPC/*n*-decane, as compared to DOPC/*n*-decane bilayers. Assuming that DS dimers in DLoPC bilayers are membrane-spanning, the predominance of DS dimers in these bilayers could arise because their hydrophobic core is more polar (due to the increased number of double bonds), which will reduce the energetic penalty of burying the dipolar indole groups in the membranes' interior. The lower energetic cost associated with SS dimer (channel) formation also could arise from this mechanism. To address this question, additional experiments were carried out to determine whether the enhanced stability of gA in DLoPC/*n*-decane bilayers is due solely to the decrease in membrane hydrophobic thickness or rather to some more favorable interactions between the tryptophan indole moieties and the more polar membrane interior (the conjugated double bonds in DLoPC). We examined the functional characteristics of three different gA analogues in which the four tryptophan residues were replaced by naphthylalanine (gN), tyrosine (gT), or phenylalanine (gM) (see Fig. 2). These analogues were chosen because of the different shapes of their aromatic side chains (gA and gN versus gT and gM) and different abilities to form hydrogen bonds (gA and gT versus gN and gM). These experiments were done using Cs^+ as the permeant ion, to facilitate the observation of gM and gN channels. The results show that all of the analogue channels experience the same relative stabilization when going from DOPC/*n*-decane to DLoPC/*n*-decane bilayers (about fivefold) (Table 2). The relative channel-forming potencies of gA in DOPC/*n*-decane and DLoPC/*n*-decane bilayers differ by an order of magnitude (results not shown), as was the case with Na^+ as the permeant ion. These results provide no evidence for specific interactions between the aromatic residues of gA and the acyl chains of DLoPC. We ascribe the increased stability and channel-forming potency of the gramicidin channels in the more unsaturated lipid environment to changes in the membrane's bulk properties (membrane thickness).

One should note, however, that specific interactions cannot be ruled out when examining a broader spectrum of lipids. The relative ranking of the average durations among

TABLE 2 Summary of results for Trp-substituted gA analogue channels

	DOPC		DLoPC		DPhPC*		GMO*	
Analog	g (pS)	τ (ms)	g (pS)	τ (ms)	g (pS)	τ (ms)	g (pS)	τ (ms)
gA	35.9 ± .9	600 ± 140	46.8 ± 2.7	3100 ± 150	48.5 ± 1.0	570	83.5 ± 0.5	540
gN	5.8 ± .5	1100 ± 180	9.5 ± 1.1	4100 ± 1500	6.5 ± 0.5	1000	15.0 ± 1.0	60
gT	29.3 ± 1.5	110 ± 15	37.9 ± 1.9	520 ± 140	32.0 ± 0.5	80	62.5 ± 0.5	130
gM	7.4 ± .3	670 ± 100	12.0 ± .5	3400 ± 760	7.7 ± .4	420	15.0 ± 1.0#	50#

Mean ± standard deviation. Conditions: 1.0 M CsCl, ±200 mV, 25 ± 1°C.
*The DPhPC/*n*-decane and GMO/*n*-decane results are from Fonseca et al. (1992).
#These results are for gM^-, the enantiomer of gM.

the Trp→aromatic substituted gramicidin analogues in phospholipid and GMO bilayers is very different (Table 2).

DISCUSSION

The dominant gA channel type in POPC/*n*-decane, DOPC/*n*-decane, and DLoPC/*n*-decane bilayers is the garden-variety SS $\beta^{6.3}$-helical dimer. We see no evidence of conducting DS helical dimers in any of the three membrane environments. In addition, gA forms channels more readily in DLoPC/*n*-decane bilayers than in POPC/*n*-decane and DOPC/*n*-decane bilayers, and the average channel duration is longer in DLoPC/*n*-decane bilayers than in POPC/*n*-decane and DOPC/*n*-decane bilayers. Finally, experiments with other gramicidin analogues (gN, gT, gM) suggest that the changes in average duration result from changes in membrane material properties (membrane thickness) and not from tryptophan stabilization in the more polar membrane interior of DLoPC/*n*-decane bilayers. It should be noted, however, that only ~1% of the gA in DOPC/*n*-decane bilayers are conducting SS channels (Durkin et al., 1990). This could produce disparate trends in spectroscopic and functional results.

gA predominantly forms single-stranded $\beta^{6.3}$-helical channels in DLoPC/*n*-decane bilayers

Only a single-channel type is observed in any of the bilayer environments investigated (Fig. 3). Specifically, there is no evidence for a well-defined minor population of channels in DLoPC/*n*-decane bilayers, which could denote the presence of both DS and SS channels. The increased channel duration in DLoPC/*n*-decane bilayers could suggest that gA in this membrane environment forms DS channels. Several observations argue that is not the case, however. First, we note that the increased duration is likely to be a direct result of the reduced thickness of DLoPC/*n*-decane bilayers (Table 1). Second, the results of experiments with the asymmetrical addition of gA to preformed DLoPC/*n*-decane bilayers (Fig. 4) tend to exclude this possibility. Conducting channels formed only when gA was present on both sides of the membrane, which demonstrates that the conducting channels form by the transmembrane dimerization of $\beta^{6.3}$-helical monomers (O'Connell et al., 1990). We did not observe very long-lived conductance events with either symmetrical or asymmetrical addition, and we conclude that such channels do not form. Conducting, membrane-spanning DS dimers should be able to form with symmetrical or asymmetrical addition; the absence of channel activity with asymmetrical gA addition thus provides a strong argument that the channels we observe are not DS dimers. Third, DS dimers form in hydrocarbon-containing bilayers (Durkin et al., 1992); the use of *n*-decane-containing bilayers should not inhibit the formation of DS $\pi\pi^{5,6}$-helical dimers. Fourth, the increased channel duration was observed not only when the permeant ion was Na^+, but also when it was Cs^+ (Table 2), which converts gA into the $\beta^{6.3}$-helical form (Sychev et al., 1993). We conclude that the channels we observe are the standard SS $\beta^{6.3}$-helical dimers.

In addition to the larger channel duration in DLoPC/*n*-decane bilayers, the single-channel conductance is also increased (Fig. 3, Tables 1 and 2). This is most likely a consequence of the increased dielectric constant of DLoPC/*n*-decane bilayers, which will reduce the electrostatic energy for ion movement through the pore (e.g., Jordan, 1981). The hydrophobic core of 1-chlorodecane-containing bilayers, for example, has a higher dielectric constant than *n*-decane-containing bilayers (Dilger et al., 1979), and in diphytanoylphosphatidylcholine bilayers the single-channel conductance is 15 pS when the hydrocarbon is *n*-decane (1.0 M NaCl, 200 mV; e.g., Durkin et al., 1990) as compared to 17 pS when the hydrocarbon is 1-chlorodecane (1.0 M NaCl, 200 mV; O. S. Andersen, unpublished results).

The increased gA single-channel conductance in DLoPC/*n*-decane bilayers (as compared to POPC/*n*-decane and DOPC/*n*-decane bilayers) has additional importance. In DPhPC/*n*-decane bilayers there is observed, in addition to the standard gA channels, a rare type of very long-lived events (durations of >100 s) with an appearance rate less than 10^{-4} of the standard channel appearance rate (Koeppe and Andersen, 1996). These rare events have a single-channel conductance that is about one-third of the standard channel conductance (J. T. Durkin and O. S. Andersen, unpublished experiments). These long-lived, low-conductance events are likely to be conducting DS dimers. Similarly, the DS heterodimeric channels that form between des-Val-[Tyr^{11}]gA and the enantiomeric gA analogue [d-$Phe^{9,11,13,15}$]gA^- (also called gM^-; Heitz et al., 1982) have a conductance that is less than that of either parent channel (Durkin et al., 1992). The increased single-channel

conductance in DLoPC/*n*-decane bilayers thus provides additional support for our conclusion that the channels are SS $\beta^{6.3}$-helical dimers.

Membrane-incorporated DS $\pi\pi^{5.6}$-helical dimers are nonconducting

The absence of conducting DS channels (in DLoPC/*n*-decane bilayers) could result for two reasons: because there are no membrane-spanning DS dimers; or because the conductance of DS $\pi\pi^{5.6}$-helical dimers is immeasurably low.

To address the first point, we note that the equilibrium distribution between SS and DS dimers should be independent of the gA concentration in the membrane, because the membrane concentration of either channel type varies as a function of $[gA]^2$. For SS dimers, the monomer $\leftrightarrow$ dimer equilibrium is

$$2M \leftrightarrow D_{SS}; \quad K_{SS} = \frac{[D_{SS}]}{[M]^2} \tag{2}$$

For DS dimers, the monomer $\leftrightarrow$ dimer equilibrium is

$$2M \leftrightarrow D_{DS}; \quad K_{DS} = \frac{[D_{DS}]}{[M]^2} \tag{3}$$

Consequently,

$$\frac{[D_{SS}]}{[D_{DS}]} = \frac{K_{SS}}{K_{DS}}, \quad \text{independent of } [M] \tag{4}$$

That is, DS $\pi\pi^{5.6}$-helical dimers should form under our experimental conditions. Deviations from Eq. 4 will occur only if there are lateral interactions among the various gA conformers, i.e., if the system does not conform to ideal solution theory. This qualification is important; the insertion of a membrane-spanning gA channel into a bilayer will perturb the immediately adjacent bilayer because the length of the channel's hydrophobic exterior (~2.2 nm for SS $\beta^{6.3}$-helical channels (Elliott et al., 1983) and ~2.6 nm for DS $\pi\pi^{5.6}$-helical channels (Langs, 1988)) is usually less than the average thickness of the bilayer's hydrophobic core (the hydrophobic thickness of solvent-free DOPC bilayers is ~2.7 nm; Lewis and Engelman, 1983). (Langs (1988) reports that the length of the DS $\pi\pi^{5.6}$ dimer is ~3.1 nm, which is the length from ethanolamide to ethanolamide; we subtracted 0.5 nm to get the length of the channel's hydrophobic exterior surface.) Generally, a membrane distortion can lead to lipid-mediated protein-protein interactions (e.g., Sackmann et al., 1984). Specifically, the energetic cost associated with the membrane deformation will contribute to the standard free energy of dimerization (Huang, 1986; Helfrich and Jakobsson, 1990; Lundbæk and Andersen, 1994), which therefore could vary as a function of the gA/lipid molar ratio.

Single-channel measurements are done at gA/lipid ratios of ~$1/10^7$, where only ~1% of the gA forms membrane-spanning channels (cf. Durkin et al., 1990), which should be compared to the gA/lipid ratios of ~1/100 to 1/10 that are used for spectroscopic measurements. When the gA/lipid ratio is increased, the average distance between membrane-spanning channels will decrease. Eventually, the perturbed membrane regions will begin to overlap and the standard free energy of dimerization (and the dimerization constant) will vary as a function of channel density. Direct evidence for the build-up of a mechanical strain in the membrane is evidenced by the bilayer$\rightarrow H_{II}$ transition that is induced by gA in DOPC bilayers at gA/DOPC ratios of ~1/20 (Killian et al., 1985). This transition depends on the presence of hydrogen bond-donating indole groups (Killian et al., 1987) that are able to form hydrogen bonds with polar groups at the membrane/solution interface (O'Connell et al., 1990; Becker et al., 1991; Durkin et al., 1992; Providence et al., 1995). The bilayer$\rightarrow H_{II}$ transition results from the build-up of mechanical strain due to a mismatch (Killian et al., 1989) between the SS $\beta^{6.3}$-helical gA dimer's hydrophobic exterior surface and the DOPC bilayer. The spectroscopic studies of Cox et al. (1992) and Sychev et al. (1993) were done at a gA/lipid molar ratio of ~1/25, which means that the equilibrium constants for SS and DS membrane-spanning dimers could be affected by the build-up of mechanical strain in the host bilayer. The mechanical strain would be attenuated if gA formed the longer DS $\pi\pi^{5.6}$-helical dimers, rather than the shorter SS $\beta^{6.3}$-helical dimers. (Consistent with this argument, the ability of gA to form the H_{II} phases in DOPC vesicles depends on the solvent the gA was dissolved in before addition (Tournois et al., 1987): when gA is dissolved in ethanol, where it predominantly occurs as DS conformers, the H_{II} phase does not form; when gA is dissolved in trifluoroethanol or DMSO, where it is monomeric and readily forms SS $\beta^{6.3}$-helical dimers, the H_{II} phase forms.) A build-up of mechanical strain cannot account, however, for the present results, as the hydrophobic thickness of DLoPC/*n*-decane bilayers is less than that of DOPC/*n*-decane bilayers.

Why is the conductance of the DS $\pi\pi^{5.6}$-helical dimers immeasurably low? One possibility is that the DS $\pi\pi^{5.6}$-helical dimers do not span the membrane. The spectroscopic results obtained so far do not distinguish between membrane-associated and membrane-spanning dimers. Assuming that the DS $\pi\pi^{5.6}$-helical dimers do span the membrane, the pore diameter is less for DS $\pi\pi^{5.6}$-helical dimers than for SS $\beta^{6.3}$-helical channels. Based on x-ray crystallographic analysis (Langs, 1988), the diameter of the "pore" lumen of a DS antiparallel $\pi\pi^{5.6}$-helical dimer varies between ~0.11 and ~0.27 nm. (These values refer to the luminal diameter. Langs (1988) reports diameters that vary between 0.39 and 0.55 nm, but these values must be adjusted for the average width of the peptide backbone atoms, which will decrease the actual luminal diameter by ~0.28 nm.) Cross and co-workers found average luminal diameters of ~0.25 nm for both parallel and antiparallel DS $\pi\pi^{5.6}$ dimers (Pascal and Cross, 1992; Zhang et al., 1992). Given these dimensions, DS $\pi\pi^{5.6}$-helical dimers may be too narrow to accommodate H_2O molecules (diameter ~0.3

nm; Eisenberg and Kauzmann, 1969) throughout the length of the pore, but H_2O could bind in the larger cavities seen in the crystal structure. Na^+ has a diameter of ~0.19 nm (e.g., Hille, 1992) and should be able to bind to DS $\pi\pi^{5,6}$-helical dimers, which also is suggested by the observation that the (DS $\pi\pi^{5,6}$-helical dimer)/(SS $\beta^{6,3}$-helical) equilibrium is favored in the presence of Na^+ (Sychev et al., 1993). Na^+, however, does not permeate the pore at a rate sufficient to allow for the electrophysiological observation of DS $\pi\pi^{5,6}$-helical dimers. In the DLoPC experiments we would be able to detect current transitions at ~0.03 pA (Fig. 4 *C*). Reproducible current transitions of that magnitude were not observed; the net Na^+ flux through DS $\pi\pi^{5,6}$-helical dimers is therefore less than 2×10^5 ions/s (1.0 M NaCl, 200 mV). This (relative) impermeability to Na^+ could arise because Na^+ cannot pass the narrow regions of the pore: because of the geometrical constraints seen in the crystal structure; because the absence of H_2O results in an energy barrier that is prohibitive to Na^+ permeation; or because H_2O molecules, situated in the largest cavities of the DS $\pi\pi^{5,6}$-helical dimer (Langs, 1988), cannot permeate through the channel and therefore "plug" the lumen and preclude Na^+ translocation. (Similar constrictions also are seen in the recent crystal structure of a gramicidin/K^+CN^- complex (Doyle and Wallace, 1997).)

SS $\beta^{6,3}$-helical channels in DLoPC/*n*-decane bilayers

The most conspicuous changes in the gA single-channel characteristics that occur as we go from POPC/*n*-decane and DOPC/*n*-decane to DLoPC/*n*-decane bilayers are the higher channel-forming potency (higher rate constant for dimerization) of gA and the longer average duration of gA channels in DLoPC/*n*-decane, as compared to POPC/*n*-decane and DOPC/*n*-decane bilayers (Tables 1 and Table 2).

The average duration of gA channels increases as the membrane thickness is decreased (Hladky and Haydon, 1972; Kolb and Bamberg, 1977; Elliott et al., 1983; N. Mobashery and O. S. Andersen, unpublished observations). The specific capacitance of DLoPC/*n*-decane bilayers is ~20% higher than that of either POPC/*n*-decane or DOPC/*n*-decane bilayers. Part of the increase in specific capacitance is due to the higher unsaturation of the acyl chains in DLoPC/*n*-decane bilayers, which will increase the dielectric constant of the hydrocarbon core. The remaining capacitance increase results from a decreased thickness of the bilayer's hydrophobic core. Kolb and Bamberg (1977) found that a 20% decrease in membrane hydrophobic thickness caused a twofold increase in gA channel duration (monoglyceride/*n*-decane bilayers). In phosphatidylcholine/*n*-decane bilayers, the average duration increases ~10-fold with each successive elimination of two methylene groups per lipid acyl chain (N. Mobashery and O. S. Andersen, unpublished observations). The changes in channel-forming potency and duration that we observe can therefore be explained simply by changes in bilayer thickness.

Specifically, we note that the similar changes in behavior of the gramicidin analogue channels when we go from DOPC/*n*-decane to DLoPC/*n*-decane mean that we can eliminate the hypothesis that the stabilization of gA channels in DLoPC/*n*-decane bilayers is due to specific interactions between the tryptophan indole side chains and the more polar membrane interior of the DLoPC/*n*-decane bilayers. The aromatic rings may interact directly with the π electrons in the conjugated double bonds in the acyl chains. We do not believe that to be the case, however, as it is difficult to understand how the four side chains, with their very different geometries, could interact so similarly with the π electrons in the double bonds. In fact, the experiments with the gA analogues suggest that the membrane's bulk mechanical properties (membrane thickness) rather than chemical specificity or shape of the gramicidin molecules in our system are responsible for the observed effects.

An unresolved question is why gA does not form DS $\pi\pi^{6,4}$-helical dimers (Wallace and Ravikumar, 1988), which, based on their dimensions, are likely to be conducting. One possible explanation is that the shorter hydrophobic length of the DS $\pi\pi^{6,4}$-helical dimer prohibits it from forming membrane-spanning channels. We note, however, that conducting DS-helical dimers (presumably $\pi\pi^{6,4}$ helices) are formed by some gA analogues with special amino acid sequences (Koeppe et al., 1991; Durkin et al., 1992; Koeppe and Andersen, 1996).

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