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On the Helix Sense of Gramicidin A Single Channels

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ABSTRACT **In order to resolve whether gramicidin A channels are formed by right- or left-handed β-helices, we synthesized an optically reversed (or mirror image) analogue of gramicidin A, called gramicidin A^-, to test whether it forms channels that have the same handedness as channels formed by gramicidin M^- (F. Heitz et al., Biophys. J. 40:87–89, 1982). In gramicidin M^- the four tryptophan residues have been replaced with phenylalanine, and the circular dichroism (CD) spectrum therefore reflects almost exclusively contributions from the polypeptide backbone. The CD spectrum of gramicidin M^- in dimyristoylphosphatidylcholine vesicles is consistent with a left-handed helical backbone folding motif (F. Heitz et al., Biophys. Chem. 24:149–160, 1986), and the CD spectra of gramicidins A and A^- are essentially mirror images of each other. Based on hybrid channel experiments, gramicidin A^- and M^- channels are structurally equivalent, while gramicidin A and A^- channels are nonequivalent, being of opposite helix sense. Gramicidin A^- channels are therefore left-handed, and natural gramicidin A channels in phospholipid bilayers are right-handed $\beta^{6.3}$-helical dimers.**

Key words: β-helix, circular dichroism, tryptophan, phenylalanine

INTRODUCTION

Gramicidin A (gA^+) is a pentadecapeptide (Table I) that dimerizes to form cation selective channels in biological and artificial membranes.[1,2] The antiparallel single-stranded $\beta^{6.3}$- helical structure proposed by Urry[3] for these channels has received general support (reviewed in 4), but the absolute helix sense has remained an enigma. In this article, we make use of sequence-substituted and optically reversed analogues of gA^+ to provide support for a right-handed helix sense for gA^+ channels under single-channel conditions in phospholipid bilayers.

The $\beta^{6.3}$-helical motif requires an alternating sequence of L- and D-amino acids. In such a structure, the repeating unit is an L,D-dipeptide, and all the side chains project to the same side of the peptide backbone. For a poly-(D,L)-alanine sequence the only difference between left-handed (LH) and right-handed (RH) helices would be the disposition of the end groups. For the gramicidins there are, in addition, small differences in the environments of the individual L- and D-side chains. Both LH and RH $\beta^{6.3}$-helical models can be constructed from the same sequence, and the conformational energies of the LH and RH models for gramicidin A channels have been predicted to be similar.[5,6]

The difference between LH and RH helices is in the orientation of the first carbonyl group (toward the N- or the C-terminal[3,7] and in whether $Leu^{10,12,14}$ or $Trp^{11,13,15}$ contribute the carbonyl groups at the channel entrance. The handedness should thus be determined by a combination of three factors: the detailed amino acid sequence, the ends of the molecule, and the environment. For example, specific interactions with optically active lipids, with the membrane–water interface itself, or at the monomer–monomer junction could influence the channels' helix sense. A priori, it is difficult to know whether the amino acid sequence, the end effects, or the environment should be of primary importance.

Originally a LH helix sense was favored,[3] but more recent evidence has favored an RH motif.[8,9] Circular dichroism (CD) spectra of phospholipid-packaged gA^+ show a positive ellipticity near 220 nm,[10,11] which would seem to indicate an LH motif,[10,12] but that interpretation is problematic because of overlap of the indole absorption bands with those of the peptide bond. Arseniev et al.[8] found by two-dimensional nuclear magnetic resonance

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Abbreviations: gA^+, gramicidin A; gA^-, optically reversed (mirror compound) gramicidin A; gM^-, optically reversed [Phe^9, Phe^{11}, Phe^{13}, Phe^{15}]gramicidin; gP^+, C-methylamine-des(ethanolamine) gramicidin A; $gK\text{–}A^+$, ethanolamine-O-acyl gramicidin A; F_3Val, 4,4,4-trifluorovaline; DMPC, dimyristoylphosphatidylcholine; DPhPC, diphytanoylphosphatidylcholine; SDS, sodium dodecyl sulfate; CD, circular dichroism; NMR, nuclear magnetic resonance.

TABLE I. Amino Acid Sequences of Gramicidin Analogues Used in This Study*

Name	Sequence	Ref	Sense
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-NEtOH	58	RH
gA^-	HCO-D-Val-Gly-D-Ala-L-Leu-D-Ala-L-Val-D-Val-L-Val-D-Trp-L-Leu-D-Trp-L-Leu-D-Trp-L-Leu-D-Trp-NEtOH	This work	LH
gM^-	HCO-D-Val-Gly-D-Ala-L-Leu-D-Ala-L-Val-D-Val-L-Val-D-Phe-L-Leu-D-Phe-L-Leu-D-Phe-L-Leu-D-Phe-NEtOH	26	LH
$[F_3Val^1]gA^+$	HCO-L-F_3V-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-NEtOH	16	RH
$gK\text{-}A^+$	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-OFA	23	RH
gP^+	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-$NHCH_3$	28	RH
endo-D-Ala^{Oa}-gA^+	HCO-D-Ala-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-NEtOH	33	RH

*Abbreviations: NEtOH, ethanolamine; O-FA, O-fatty acyl ethanolamine, where the fatty acid is either octadecanoic, hexadecanoic, pentadecanoic, or 12-methyltetradecanoic acid.

(NMR) that gA^+ in sodium dodecyl sulfate (SDS) micelles in water–trifluoroethanol (90:10) is folded into RH $\beta^{6.3}$-helices. The question remained, however, whether the conformation in micelles is equivalent to that in membranes,[13] even though the CD spectra of gramicidin in the two environments were similar.[8] Solid-state NMR spectra of ^{13}C-labeled gramicidins were consistent with LH helices or a mixture of LH and RH forms.[14] Solid-state NMR spectra of ^{15}N-labeled gramicidins, however, revealed peptide plane orientations that define an RH helix in stacked dimyristoylphosphatidylcholine (DMPC) membranes at gramicidin/lipid molar ratios in the range of 1/15 to 1/10.[9]

In order to clarify the relative importance of sequence and environment for the handedness of gramicidin-like helices, we have sought to examine the helix sense of several natural and analogue gramicidins under single-channel conditions. For that purpose, we have made use of an optically reversed analogue of gA^+ that lacks the four indole chromophores, gM^- (Table I). The CD spectrum of gM^- in DMPC vesicles (gramicidin–lipid ratio of 1/30) is consistent with an LH-helical motif,[15] a result that serves as our point of reference for determining the helix sense of gA^+ channels. To test for the possibility of a sequence or environment dependence of helix sense we synthesized gA^- (Table I).

The experimental approach makes use of the fact that gramicidin channels are symmetrical dimers and that asymmetrical heterodimers (hybrid channels) readily form between chemically dissimilar gramicidin monomers.[16,17] Heterodimer formation, however, requires that the "opposing" monomers have the same handedness. This requirement is illustrated schematically in Figure 1: the first two panels show viable channel formation between opposing pairs of RH and LH helices, respectively; the third panel shows the incompatibility of an LH monomer opposite an RH monomer; and the fourth panel shows that two helices of the same handedness but different end structure (formyl-NH-terminal-L-residue versus formyl-NH-terminal-D-residue) can form hybrid channels, but with a gap in the channel wall.[18] This imperfection has two consequences: such hybrid channels have low conductances and short durations.[19]

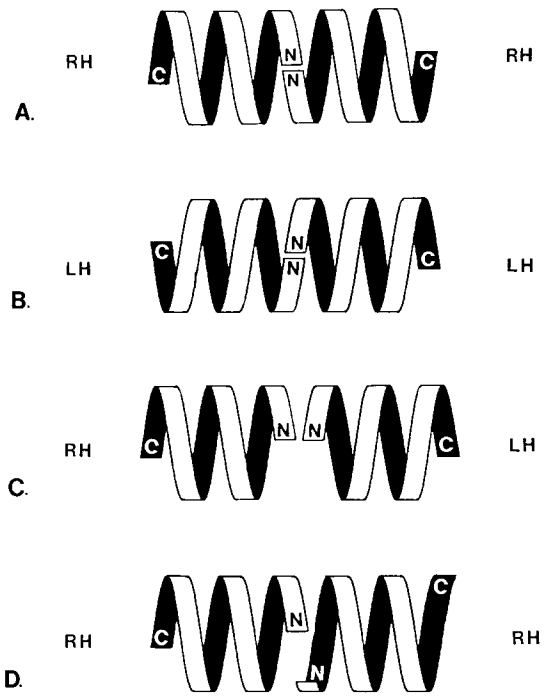

Fig. 1. Schematic representations of channel formation by formyl-NH-to-formyl-NH dimerization of RH and LH gramicidin helices. (**A**) Dimerization of RH helices (with intramolecular formyl H-bonds). (**B**) Dimerization of LH helices (with intermolecular formyl H-bonds). (**C**) Incompatibility (no dimer formation) of a LH monomer opposite a RH monomer. (**D**) Dimerization of RH helices with a gap in the channel wall due to an L,D mismatch of the end residues.

This article reports the results of hybrid channel experiments using gA^+, gM^-, gA^-, and a position-one analogue of gA^+ $[trifluorovaline^1]gA^+$,[16] as well as analogues with a modified amino or carboxyl terminus (Table I), all of which were chosen because their distinctive single-channel conductances allow unambiguous identification of symmetrical and hybrid channels. We show that gM^- and gA^- form

channels that have the same handedness, which is opposite to that of all other molecules tested, and conclude that the handedness is determined primarily by the amino acid sequence.

MATERIALS AND METHODS

Solid-Phase Synthesis

gA^- was synthesized by deprotecting and extending Boc-D-Trp-resin, using a Dupont Model 1000 peptide synthesizer to add the appropriate sequence of BOC-amino acid precursors followed by formyl-D-Val at the last step. BOC-L-Leu was coupled by a direct dicyclohexylcarbodiimide (DCCD) procedure[20]; all other BOC-amino acids and formyl-D-Val were coupled as the symmetric anhydrides,[20] which were preformed and prefiltered to remove dicyclohexylurea.

Following the addition of formyl-D-Val to complete the synthesis of the resin-attached gA^-, the product was released from the resin by ethanolaminolysis at 60°C for 24–48 hr in anhydrous 50:50 ethanolamine:methanol.[21] gA^- was precipitated by addition of four volumes of H_2O, collected by centrifugation, reprecipitated, dried, resuspended in methanol, and purified by preparative reversed-phase HPLC.[22,23] An aliquot of the product was then further purified on an analytical octyl silica HPLC column, collected by hand[24,25] and diluted into 10–100 volumes of ethanol prior to single-channel experiments.

Other Compounds

The total synthesis of gM^- has been described,[26] as has the semisynthesis of $[F_3Val^1]gA.^+$[16,24] Endo-D-Ala^{Oa}-gramicidin A^+ was synthesized by coupling formyl-D-Ala to desformyl-gramicidin A^+.[24,27] The isolation of natural gramicidin K–A ($gK–A^+$), in which a fatty acyl chain is attached to the ethanolamine of gA^+, has been described,[23] as has the synthesis of gramicidin P (gP^+), in which the ethanolamine group is replaced by methylamine.[28]

Single-Channel Measurements

Planar bilayers were formed at 25 ± 1°C from diphytanoylphosphatidylcholine (DPhPC) in *n*-decane (2–3% w/v), and single-channel measurements were done using the bilayer punch,[29] in which a membrane with an area ~1,000 μm^2 is isolated on the tip of a glass pipette. Single-channel activity was monitored as discrete (rectangular) current jumps across the membrane. When assaying for hybrid channel formation, each of the gramicidin analogues in question was added to *each* aqueous phase (on either side of the membrane) to give a reasonable single-channel activity (about 10 pM gA^+ or 50 pM gM^- to give about 1 channel appearance per sec). The currents were measured with symmetrical 1.0 M CsCl solutions and an applied potential of 200 mV. The analysis of the hybrid channel experiments to obtain histograms and survivor plots was as in Durkin et al.[17]

CD Measurements

Samples of gA^+ or gA^- in DMPC (1/20 mol ratio) were prepared as small unilamellar vesicles for CD spectroscopy as described by Sawyer et al.[13] Spectra were recorded on a Jasco Model J-500 spectropolarimeter that had an IF-500 interface to an IBM microcomputer. The CD spectrum of gM^- in DMPC (1/30 mol ratio) was recorded as in Hetiz et al.[15] using a model III Jobin-Yvon dichrograph.

Materials

Ethanolamine (Aldrich Chemical Co., Milwaukee, WI) was distilled in vacuo, sealed in glass, and stored at −20°C until use. BOC-D-Trp resin and BOC-amino acids were from Peninsula Scientific Co. (Belmont, CA). D-Valine (Sigma Chemical Co., St. Louis, MO) was formylated using formic acetic anhydride,[24] and the formyl-D-Val product was recrystallized from ethyl acetate. Trifluoroacetic acid (TFA) for removal of N-terminal BOC groups and diisopropylethylamine for neutralization were from Chemical Dynamics Corp. (South Plainfield, NJ). Indole (1 mg/ml) was added to the TFA to protect against the degradation of Trp side chains during the removal of BOC groups. Solvents for solid-phase synthesis included dichloromethane (HPLC grade from Fisher Scientific Co., Memphis, TN) and dimethylformamide, purchased from Aldrich and stored over 4 Å molecular sieve. Other materials have been previously described.[16,23–25]

RESULTS

Properties of gA^- and gA^+

In symmetrical environments, the mirror-image molecules gA^+ and gA^- behave identically, as evidenced by their behavior during reversed-phase chromatography on (symmetrical) octyl silica, where the two molecules are indistinguishable (Fig. 2A). Functionally, the single channels formed by gA^+ and gA^- are likewise virtually indistinguishable: Figure 2B shows individual current transitions for gA^+ and gA^- channels in DPhPC membranes; Figure 2C shows the distributions of the respective single-channel current transition amplitudes; and Figure 2D shows the duration distributions of gA^+ and gA^- channels. The channels have very similar single-channel conductances, conductance distributions, and average channel durations.

CD Spectra

Figure 3 shows the 200–270 nm CD spectra for gA^+, gA^-, and gM^- dispersed in DMPC. The spectra of the entantiomeric pair of molecules gA^+ and gA^- are mirror images of each other, as they should be if the amino acid sequence were the primary determinant of the helix sense. Nevertheless, the ab-

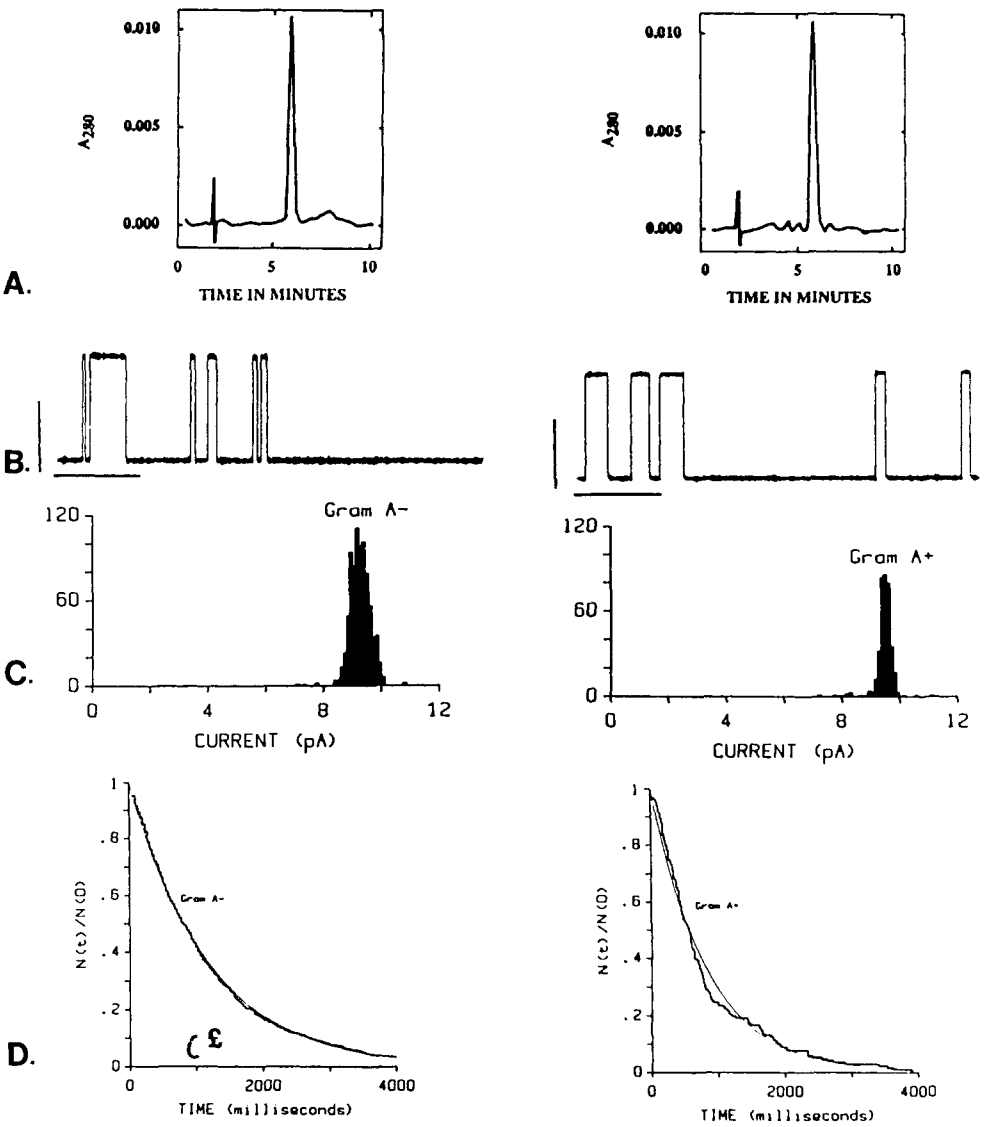

Fig. 2. Comparisons of physical and channel properties of the mirror-image molecules gA^+ and gA^-. (**A**) Chromatographic behavior on a 0.46 × 25 cm column of Zorbax C_8 in 83% methanol–17% water at 1.5 ml/min, 1800 psi, and room temperature. (**B**) Single-channel current traces observed in the presence of gA^- (left) and gA^+ (right). Channel appearances are upward; The calibration bars denote 5 pA (vertically) and 5 sec (horizontally). The traces were filtered at 100 Hz. (**C**) Current transition amplitude histograms for channels observed in the presence of gA^- (left), and gA^+ (right). Channels lasting longer than 14 msec were used to determine the magnitude of the current transitions. For gA^+, the histogram contains 486 transitions, of which 474 (or 98%) are in the main peak at ~9.6 pA. For gA^-, the histogram contains 781 transitions, of which 768 (or 98%) are in the main peak at ~9.6 pA. (**D**) Normalized survivor histograms for channels formed by gA^- (left) and gA^+ (right). The curves depict fits to the populations: for gA^+ channels, τ = 930 msec (N = 280); for gA^- channels, τ = 1160 msec (N = 905). 1.0 M CsCl, 200 mV.

solute helix sense for gA^+ or gA^- cannot be determined from their CD spectra because of overlap of the indole ring dichroism with that of the peptide bonds.

For the assignment of the helix handedness, we turn to gM^-. The CD spectrum of gM^- shows positive ellipticity in the 210–225 nm range, which usually is considered to be characteristic of left-handed helical structures.[30] It is noteworthy that this positive ellipticity is reversed when the four Phe rings are replaced with Trp in going from gM^- to gA^- (Fig. 3, middle), even though the helix handedness does not change (see below). The indole rings of the Trp side chains are contributing more to the ellipticity at 220 nm than are the peptide bonds of gA^- (or gA^+).

With gM^- assigned as left-handed in DMPC vesicles, then gA^- is also left-handed because gA^- channels have the same handedness as gM^- channels (see below). Thus, gA^+ is right-handed in

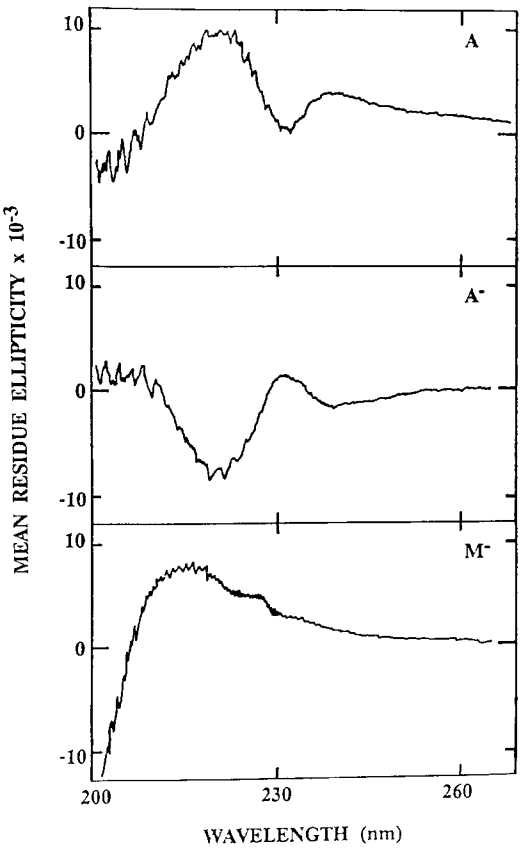

Fig. 3. Circular dichroism spectra of gA^+, gA^-, and gM^- dispersed in small unilamellar DMPC vesicles. Samples were prepared as described by Sawyer et al.[13] or by Heitz et al.[15] The gramicidin:lipid ratio was either 1:20 (gA^+ and gA^-) or 1:30 (gM^-).

DMPC vesicles since it was designed to have opposite chirality as confirmed by CD (Fig. 3).

Hybrid Channel Experiments

The helix sense of gA^+ and gA^- channels *relative* to gM^- channels was determined by testing for the formation of heterodimers between the different gramicidins (cf. Fig. 1). gA^+ and gA^- channels are effectively indistinguishable, however, and one cannot assay directly for hybrid channel formation between these two compounds. We therefore made use of the fact that hybrid channel formation is a transitive property among different gramicidins,[17] and used $[F_3Val^1]gA^+$ as a gA^+ "substitute." $[F_3Val^1]gA^+$ forms homodimeric channels with a lower conductance than gA^+ channels; it also forms distinct hybrid channels with gA^+,[16] such that $[F_3Val^1]gA^+$ channels are structurally equivalent to gA^+ channels.[17] It thus suffices to test for hybrid channel formation between gA^- and $[F_3Val^1]gA^+$, as well as between gA^- and gM^-.

Figures 4 and 5 show that hybrid channels form between gM^- and gA^-, but not between gM^- and gA^+: When gM^- and gA^- are present in the same membrane, the characteristic gM^- and gA^- channels are seen, as well as two hybrid channel types that have intermediate conductances. [Two hybrid channel types are observed because gA^-/gM^- heterodimers occur in two orientations, $gA^-\rightarrow gM^-$ and $gM^-\rightarrow gA^-$. The energy profile for ion movement through the heterodimers will be different for the two orientations. The currents through the $gA^-\rightarrow gM^-$ and $gM^-\rightarrow gA^-$ channels will therefore differ at the potentials used here (cf. ref. 4)].

When gM^- and gA^+ are present in the same membrane, however, *no* hybrid channels are observed. Only channels having the characteristic gM^- and gA^+ single-channel conductances, but no new channel types (having other conductances) are seen in the current traces (Fig. 5a) or the amplitude histograms (Fig. 5b). The survivor plots of channel durations (Fig. 5c) show likewise *no* new channel population with a duration different from that of gM^- or gA^+ channels. This is important because two channel types of similar conductance can be distinguished if their average durations are different.[17,31]

Figures 6 and 7 show that hybrid channels form between $[F_3Val^1]gA^+$ and gA^+, but not between $[F_3Val^1]gA^+$ and gA^-: When gA^+ and $[F_3Val^1]gA^+$ are present in the same membrane, the characteristic $[F_3Val^1]gA^+$ and gA^+ channels are seen as well as hybrid channels with an intermediate conductance. [In this case the asymmetry in the energy profiles of the $[F_3Val^1]gA^+\rightarrow gA^+$ and the $gA^+\rightarrow[F_3Val^1]gA^+$ heterodimers is not sufficient to allow us to discriminate between the two orientations, although they can be distinguished at higher potentials (see ref. 16).]

When gA^- and $[F_3Val^1]gA^+$ are present in the same membrane, however, *no* hybrid channels are observed. Only channels having the characteristic gA^- and $[F_3Val^1]gA^+$ single-channel conductances, but no new channel types (having other conductances) are seen in the current traces (Fig. 7a) or the amplitude histograms (Fig. 7b). The survivor plots of channel durations (Fig. 7c) show likewise *no* new channel population with a duration different from that of gA^- or $[F_3Val^1]gA^+$ channels.

We conclude that hybrid channels do not form between gA^- and $[F_3Val^1]gA^+$, or between gM^- and gA^+, because the helix sense of gA^- and of gM^- is incompatible with that of gA^+ and $[F_3Val^1]gA^+$ (cf. Fig. 1). gA^- channels are structurally equivalent to gM^- channels (see Discussion), which indicates that gA^- and gM^- β-helices have the same handedness. The four Trp→Phe substitutions at positions 9, 11, 13, and 15 have not altered the handedness of the β-helices that form channels in membranes.

Possibility of Minor Conformers

The preceding results show that the predominant gA^- channel type has the same handedness as gM^-

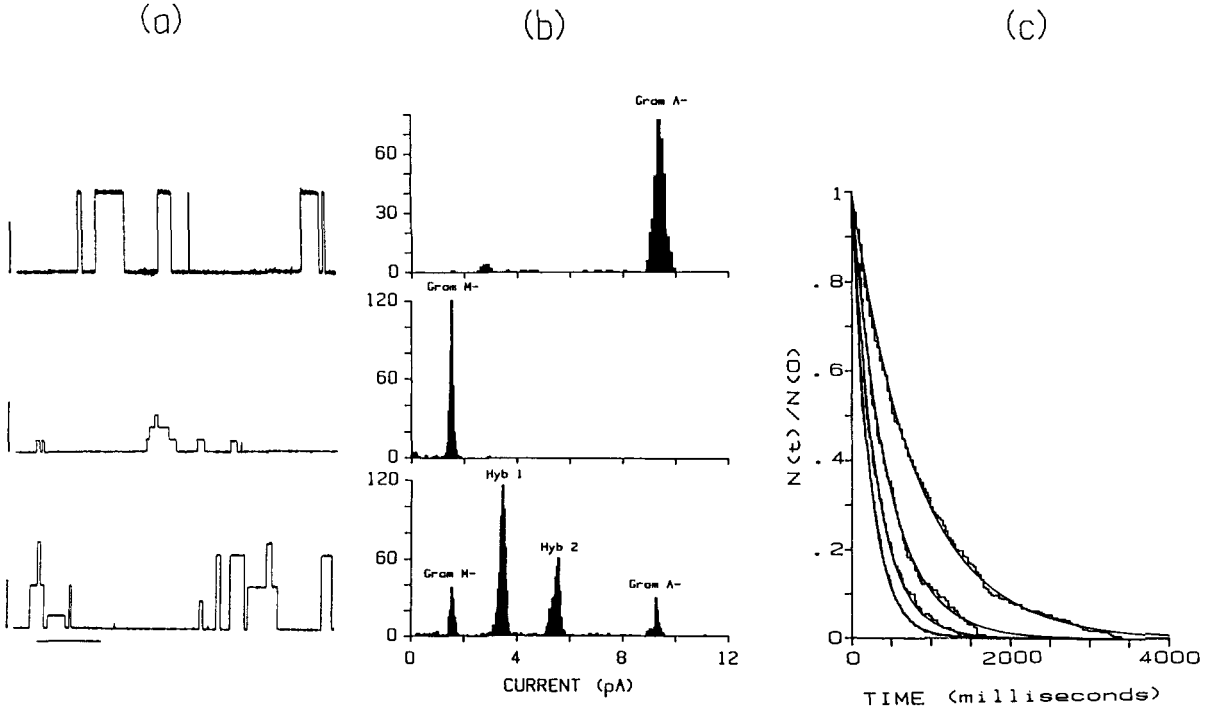

Fig. 4. Hybrid channels form between gM^- and gA^-. (**a**) Single-channel current traces observed in the presence of gA^- (top), gM^- (middle), and their mixture (bottom). Both homodimeric channels appear in the bottom trace, as well as two new channel types (the hybrid channels) that represent the two different orientations of the asymmetrical heterodimer. Channel appearances are upward. The calibration bars denote 2.5 pA (vertically) and 5 sec (horizontally). The traces were filtered at 100 Hz. (**b**) Current transition amplitude histograms for channels observed in the presence of gA^- (top), gM^- (middle), and their mixture (bottom). Channels lasting longer than 50 msec were used to determine the magnitude of the current transitions. The top histogram contains 342 transitions, of which 329 (or 92%) are in the main peak at ~9.2 pA. The middle histogram contains 352 transitions, of which 342 (or 97%) are in the main peak at ~1.5 pA. The bottom histogram contains 1,111 transitions: 98 (or 9%) are in the peak at ~9.2 pA, 130 (or 12%) are in the peak at ~1.5 pA, and 883 (or 79%) are in the two peaks at ~3.4 pA (545 or 49%) and ~5.4 pA (338 or 30%). These new peaks are seen only when both gramicidins are present and correspond to the hybrid channels. (**c**) Normalized survivor histograms for the four channel types identified in transition amplitude histograms. The four curves depict the fits to the different populations. gA^- channels have the longest average duration (τ = 810 msec; N = 323); gM^- channels have the shortest (τ = 320 msec; N = 528). The average durations of the two hybrid channels are intermediate to those of the pure channels: for the channels at ~3.4 pA, τ = 310 msec (N = 169); for the channels at ~5.4 pA, τ = 450 msec (N = 109). 1.0 M CsCl, 200 mV.

channels, which is opposite to that of $[F_3Val^1]gA^+$ or gA^+ channels. The next question is more difficult: could any of the compounds give rise to a minor population of monomers that have the opposite handedness? The small predicted energy difference between LH and RH forms[5,6] suggests that there could be monomers of either handedness in the same membrane. Minor conformers may not be amenable to spectroscopic detection, however, because the major conformers will be expected to dominate the UV, CD, and NMR spectra. Minor conformers may be detected electrophysiologically, however, if a more stringent protocol for hybrid channel detection is followed.

We first note that *if* a fraction of gA^- monomers had the *same* handedness as the major fraction of $[F_3Val^1]gA^+$ monomers, they would still differ at their formyl-NH-termini because gA^- begins with a formyl-D-amino acid while $[F_3Val^1]gA^+$ begins with a formyl-L-amino acid. Hybrid channels that might form between these gramicidins (or between gA^- and gA^+) would therefore be of the type shown in Figure 1D. Such hybrid channels would be destabilized by the absence of a residue at the join. Hybrid channels of this type ("missing residue" hybrid channels) exhibit low conductances and short durations.[19] It is therefore necessary to look specifically for short-lived, low-conductance events.

We next note that since gramicidins are dimers, the distribution of channel types that are formed by two different gramicidins (A and B) can be approximated by the binomial distribution.[17,32] Let p_A and p_B denote the relative channel-forming probabilities of A and B monomers ($p_A + p_B = 1$). The relative appearance rates of the two symmetrical, homodimeric channel types (A–A and B–B) are then approximated by $(p_A)^2$ and $(p_B)^2$, respectively, while the relative appearance of the asymmetrical hybrid channels (A–B and B–A) is approximated by $2\,p_A\,p_B$ (see also ref. 31). A small minority conformer is thus

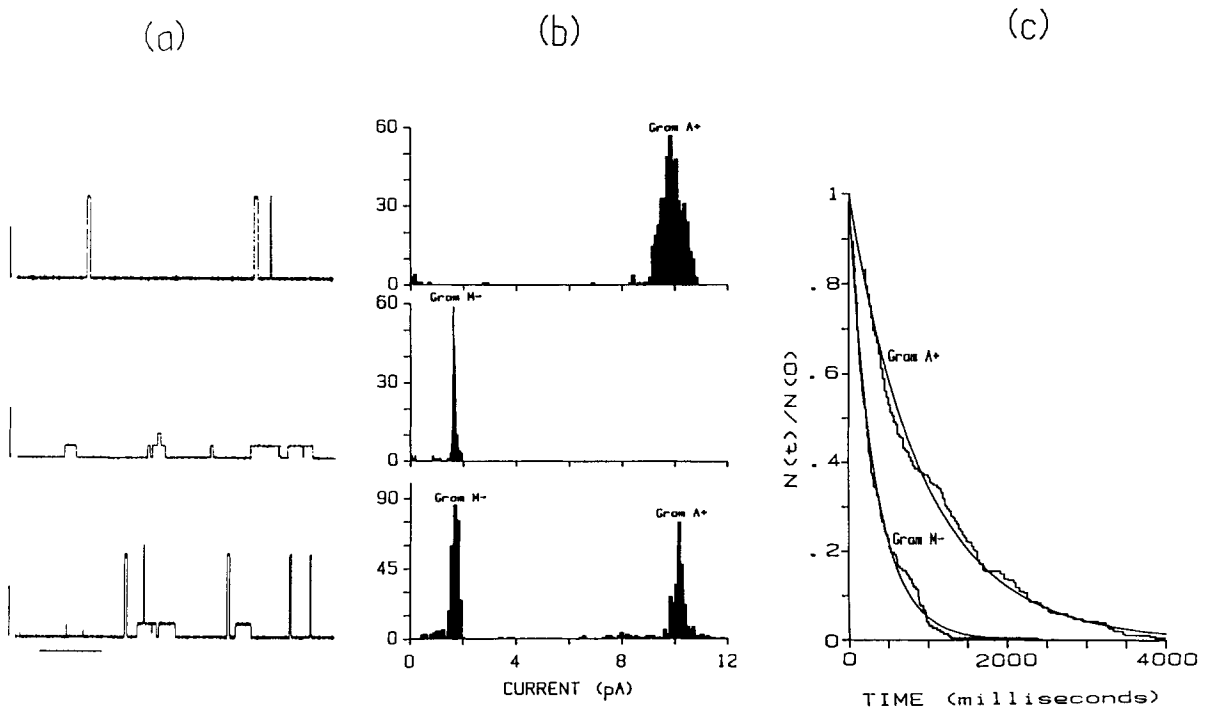

Fig. 5. Hybrid channels do not form between gA^+ and gM^-. (**a**) Single-channel current traces observed in the presence of gA^+ (top), gM^- (middle), and their mixture (bottom). Both types of homodimeric channels appear in the bottom trace, but there is no evidence for hybrid channels. Channel appearances are upward. The calibration bars denote 2.5 pA (vertically) and 5 sec (horizontally). The traces were filtered at 100 Hz. (**b**) Current transition amplitude histograms for channels observed in the presence of gA^+ (top), gM^- (middle), and their mixture (bottom). Channels lasting longer than 50 msec were used to determine the magnitude of the current transitions. The top histogram contains 492 transitions, of which 469 (or 95%) are in the main peak at ~9.6 pA. The middle histogram (recorded at a different gain setting) contains 560 transitions, of which 483 (or 86%) are in the main peak at ~1.6 pA. The bottom histogram contains 602 transitions: 266 (or 44%) are in the peak at ~1.6 pA and 264 (or 44%) are in the peak at ~10.1 pA. There is no evidence for additional peaks. (**c**) Normalized survivor histograms for the two channel types identified in the transition amplitude histograms. The two curves denote fits to each population. gA^+ channels have the longer average duration (τ = 950 msec; N = 295); gM^- channels have the shorter (τ = 340 msec; N = 168). Both distributions are well described by single exponential decays. 1.0 M CsCl, 200 mV.

much easier to detect in hybrid channel experiments. If A and B denote two conformers of a single gramicidin type with $p_B = 0.1\ p_A$, the relative appearance rate of B–B vs. A–A is $(p_B)^2/(p_A)^2$ or 0.01, which is beyond our present resolution. But if a second gramicidin (C), which can form hybrid channels with B, were added to the system, the relative appearance rate of the hybrid channels (B–C and C–B) vs. B–B would be $2\,p_B\,p_C/(p_B)^2 = 2\,p_C/p_B$. If $p_C = p_A$ ($= 10\ p_B$), the relative hybrid channel appearance rate would be $2\,p_B\,p_C/[(p_A)^2 + (p_B)^2 + (p_C)^2] \simeq 0.1$, which should be detectable. No such channels were observed, however, when gA^- and $[F_3Val^1]gA^+$ were added to the same membrane (Fig. 7).

Nevertheless, $[F_3Val^1]gA^+$ channels themselves have relatively short mean durations and low conductance (300 msec and 16 pS in 1.0 M CsCl; ref. 16, and Fig. 6), and it is possible that (rare) hybrid $[F_3Val^1]gA^+/gA^-$ channels could have escaped detection. For this reason, it is appropriate to also look for "missing residue" hybrid channel formation between gA^- and gA^+. The results of experiments of this type are shown in Figure 8. Again, no brief, low-amplitude events are apparent. Within the experimental limits of detection, minor conformers of reversed handedness are not present.

This question was further pursued in experiments where we searched for hybrid channel formation between gA^- and a 16-amino acid analogue, endo-D-Ala^{Oa}–gramicidin A^+ (Table I), which forms channels with the same helix sense as gA^+ channels.[33] In this case, the extra D-Ala would be able to occupy the position of the missing residue (in a gA^-/gA^+ heterodimer). Consequently, a minor population of gA^- monomers with the "opposite" helix sense to the standard gA^- molecules would be readily apparent due to their ability to form conventional (stable) heterodimers with the test compound. No such channels were observed (data not shown). The same result (no hybrid channel formation) was found with gM^- and endo-Gly^{Oa}–gramicidin A^+.[18]

In order to examine whether the ethanolamine blocking group could be important in determining the channels' helix sense,[34,35] we also tested whether gA^- forms hybrid channels with either gP^+, in which the ethanolamine –OH has been

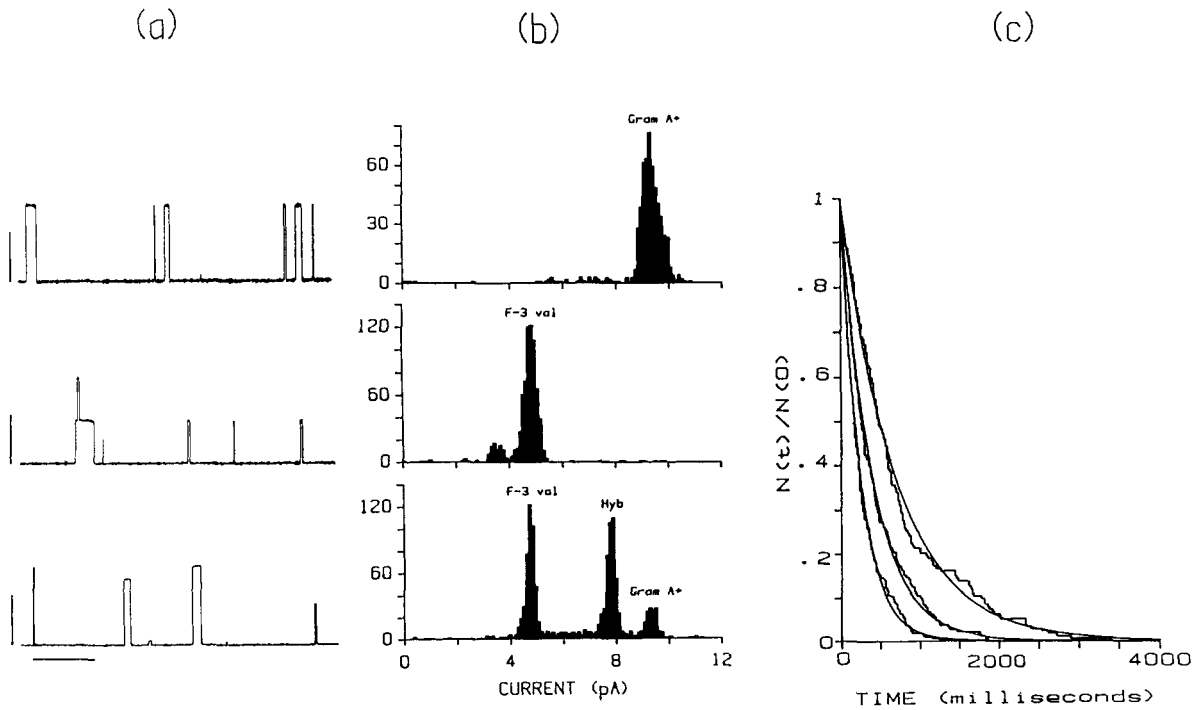

Fig. 6. Hybrid channels form between [Val^1]gramicidin A$^+$ and [F_3Val^1]gramicidin A$^+$. (**a**) Single-channel current traces observed in the presence of gA$^+$ (top), [F_3Val^1]gA$^+$ (middle), and their mixture (bottom). Both types of homodimeric channels appear in the bottom trace, as well as a new channel type (the hybrid channel) that represents the heterodimers. Channel appearances are upward. The calibration bars denote 2.5 pA (vertically) and 5 sec (horizontally). The traces were filtered at 100 Hz. (**b**) Current transition amplitude histograms for channels observed in the presence of gA$^+$ (top), [F_3Val^1]gA$^+$ (middle), and their mixture (bottom). Channels lasting longer than 50 msec were used to determine the magnitude of the current transitions. The top histogram contains 584 transitions, of which 524 (or 90%) are in the main peak at ~9.3 pA. The middle histogram contains 758 transitions, of which 661 (or 87%) are in the main peak at ~4.7 pA. The bottom histogram contains 1,199 transitions: 442 (or 37%) are in the peak at ~4.7 pA, 152 (or 14%) are in the peak at ~9.3 pA, and 461 (or 38%) are in the peak at ~7.7 pA. This new peak is seen only when both gramicidins are present and corresponds to the hybrid channels. (**c**) Normalized survivor histograms for the three channel types identified in transition amplitude histograms. The three curves depict the fits to the different populations. gA$^+$ channels have the longest average duration (τ = 700 msec; N = 154); [F_3Val^1]gA$^+$ channels have the shortest (τ = 250 msec; N = 173). The average durations of the hybrid channels are intermediate to those of the pure channels (τ = 400 msec; N = 144). 1.0 M CsCl, 200 mV.

removed[28] (Fig. 9), or gK–A$^+$, in which a fatty acid has been esterified to the ethanolamine –OH[23] (Fig. 10), For either compound, a change in handedness should again be apparent in the appearance of brief, low-conductance "missing residue" hybrid channels. In the case of gP$^+$, no such channels were observed (Fig. 9). In the case of gK–A$^+$, there is a suggestion that "missing residue" hybrid channels could form (cf. the upper and lower histograms in Fig. 10). The low-conductance channels are, however, too rare to be characterized further, so the issue remains unsettled.

DISCUSSION

The basic $\beta^{6.3}$-helical conformation for gramicidin channels[3] has weathered the tests of time and many experiments.[8,36–46] Because this conformation has been observed only in membranes or micelles, but not in other environments,[12,47–53] it has been difficult to examine more subtle details of the gramicidin channel structure such as the backbone handedness. Several factors contribute to establishing the handedness of gramicidin-like helices for molecules of alternating (L,D) sequence.* First, the chemical identity of each L and each D side chain must somehow influence the overall handedness of the chain. Second, the N- and C-terminal amino acids and blocking groups could influence the handedness, e.g., through intra- or intermolecular hydrogen bonds.[3,7,34,35] Third, the molecular environment around the channels could affect helix handedness. We seek to understand the importance of each of these factors.

Handedness of gM$^-$ and gA$^+$ Channels

gA$^+$ itself cannot serve as the reference molecule for the determination of the channel's helix sense by CD spectroscopy because the strong indole dichro-

*If the alternation of L and D residues is interrupted, as in [D-Val^1]gA$^+$, the channels do not assemble.[24,27]

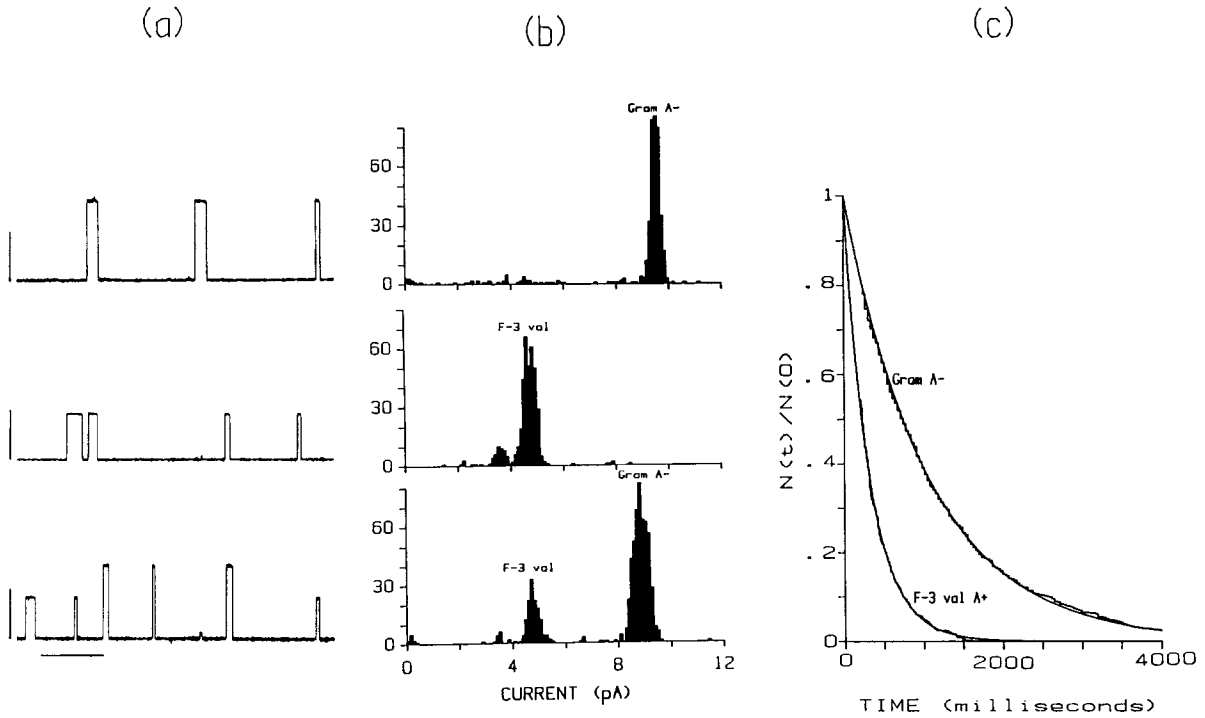

Fig. 7. Hybrid channels do not form between [Val^1]gramicidin A$^-$ and [F_3Val^1]gramicidin A$^+$. (**a**) Single-channel current traces observed in the presence of gA$^-$ (top), [F_3Val^1]gA$^+$ (middle), and their mixture (bottom). Both types of homodimeric channels appear in the bottom trace, but there is no evidence for hybrid channels. Channel appearances are upward. The calibration bars denote 2.5 pA (vertically) and 5 sec (horizontally). The traces were filtered at 100 Hz. (**b**) Current transition amplitude histograms for channels observed in the presence of gA$^-$ (top), [F_3Val^1]gA$^+$ (middle), and their mixture (bottom). Channels lasting longer than 50 msec were used to determine the magnitude of the current transitions. The top histogram contains 410 transitions, of which 358 (or 87%) are in the main peak at ~9.5 pA. The middle histogram contains 404 transitions, of which 347 (or 86%) are in the main peak at ~4.7 pA. [The peak at ~3.5 pA results from an impurity; it contains 43 (or 11%) of the transitions. See also Fig. 6.] The bottom histogram contains 662 transitions: 138 (or 21%) are in the peak at ~4.7 pA, and 503 (or 76%) are in the peak at ~8.8 pA. The peak at ~3.5 pA (14 events, or 2%) corresponds to the extra peak seen with [F_3Val^1]gA$^+$ by itself, and there is no evidence for additional peaks. (**c**) Normalized survivor histograms for the two channel types identified in the transition amplitude histograms. The two curves denote fits to each population. gA$^-$ channels have the longer average duration (τ = 1100 msec; N = 755); [F_3Val^1]gA$^+$ channels have the shorter (τ = 320 msec; N = 207). Both distributions are well described by single exponential decays. 1.0 M CsCl, 200 mV.

ism obscures the peptide bond dichroism.[12] gM$^-$, however, may be able to serve this purpose. The positive ellipticity at 220 nm for gM$^-$ in DMPC[15] (Fig. 3) is generally considered to be characteristic of an LH-helical structure, but this has only been definitely established for α-helices.[30] Given this uncertainty in interpretation,[†] the assignment of an LH motif to gM$^-$ serves to define the helix sense of gA$^-$ channels by virtue of the formation of gA$^-$/gM$^-$ heterodimers (Fig. 4). The helix sense of gA$^+$ channels is thus determined because gA$^-$ and gA$^+$ have opposite handedness (Figs. 3, 6, 7, and 8)—gA$^+$ channels are consequently right-handed. Channels formed by sequence-substituted analogues of gA$^+$ can be related to gA$^+$ channels using the hybrid channel criterion.[17] Reversals in helix sense due to amino acid sequence substitutions would be detected by the absence of hybrid channel formation with gA$^+$ (or a structural equivalent), and by hybrid channel formation with gA$^-$ (or a structural equivalent); see next section.

The preceding arguments depend on an implicit assumption: that one can relate the high gramicidin/lipid (~1/20) conditions used in the spectroscopic measurements to the low gramicidin/lipid conditions used in the single-channel experiments. We believe that such an assumption should be valid in this situation because gA$^+$ and gA$^-$ channels have opposite helix sense based on either type of measurement, and because the CD spectrum of gA$^+$ is essentially invariant to changes in the gramicidin/lipid ratio from 1/15 to 1/350.[48]

[†]An additional consideration is that gM$^-$ may not exhibit a unique conformation in DMPC vesicles. If conformations other than the $\beta^{6.3}$-helix were to contribute significantly to the CD spectrum, the interpretation of the spectra would be even more complicated. For example, double-helical conformers of gM$^-$ may contribute; we note, however, that long-lived channel events that could be double-helical dimers are *not* observed when only gM$^-$ is present in bilayers, whereas they can be observed with other (special) combinations of analogues.[59,60]

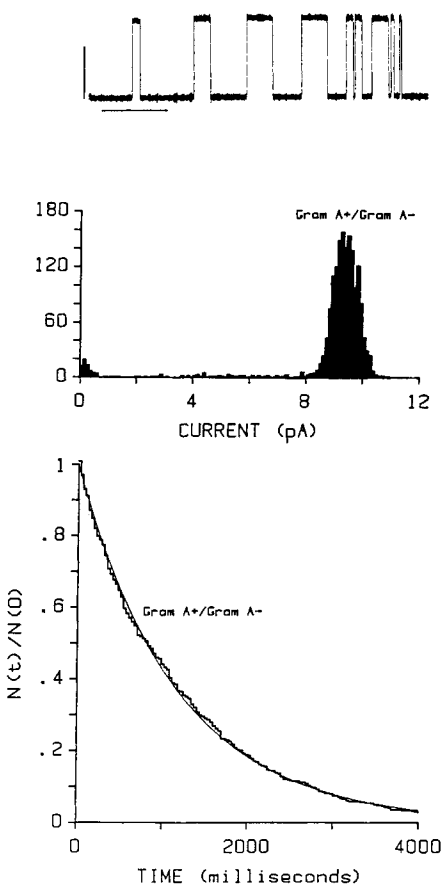

Fig. 8. Hybrid channels do not form between [Val^1]gramicidins A^+ and A^-. The experiment was done by adding equipotent amounts of gA^+ and gA^- symmetrically to the electrolyte solution on both sides of the membrane. This should ensure that there is an approximately equal number of gA^+ and gA^- channels. **Top:** Single-channel current traces observed in the presence of a mixture of gA^+ and gA^-. There is no evidence for channels other than those observed in the presence of either of the gramicidins alone (cf. Figs. 4–7). Channel appearances are upward. The calibration bars denote 5 pA (vertically) and 5 sec (horizontally). The trace was filtered at 150 Hz. **Middle:** Current transition amplitude histograms for channels observed in the presence of a mixture of gA^+ and gA^-. Channels lasting longer than 10 msec were used to determine the magnitude of the current transitions. The histogram contains 875 transitions, of which 815 (or 93%) are in the broad main peak at ~9.5 pA. There is no evidence for additional peaks. **Bottom:** Normalized survivor histogram for the channels identified by the main peak in the transition amplitude histogram. The curve denotes a fit to the population (τ = 880 msec; N = 310). 1.0 M CsCl, 200 mV.

Effects of Substitutions in the Amino Acid Sequence

gA^- forms hybrid channels with gM^- (Fig. 4); these molecules therefore form channels that have the same handedness. Following Durkin et al.,[17] one can use the relative channel appearance rates for the hybrid channels with respect to the two symmetric channels, and the average channel durations, to calculate the activation energy $\Delta\Delta G^{\ddagger}$ see also Table II and the standard free energy $\Delta\Delta G^{\circ}$ for a hybrid channel relative to the corresponding pure channel types. For gM^- and gA^-, $\Delta\Delta G^{\circ}$ is −2.3 kJ/mol (Table II). There is no energetic cost associated with the formation of gA^-/gM^- heterodimers. Actually, there is a preference for hybrid channel formation; we do not understand the molecular basis for this.

The seemingly dramatic substitution of the four D-Trp residues of gA^- with D-Phe in gM^- has no effect on the channel conformation. gM^- channels are "structurally equivalent"[17] to gA^- channels. This result is surprising in view of the dipole moment and hydrogen-bonding capability of the Trp indole rings. The aromatic residues at positions 9, 11, 13, and 15 are within the C-terminal turn of a $\beta^{6.3}$-helix, and are therefore near the membrane–solution interface. Indoles at these positions would be expected to form hydrogen bonds with water and with the phospholipid polar backbone and head groups[54]; these interactions could determine the indole orientations and possibly even the helix handedness. For the D-Phe residues of gM^-, however, such hydrogen bonding interactions are not possible and the helix sense should be dictated by other considerations. At this time, we do not know of local sequence substitutions that can flip the global handedness of a gramicidin helix.

Formyl Group Orientation and End Residue Effects

With a LH $\beta^{6.3}$-helical gA^- structure and a RH $\beta^{6.3}$-helical structure assigned to gA^+ (Fig. 11), the formyl carbonyl oxygen for both compounds is involved in an intramolecular hydrogen bond to HN^7 in that monomer. The six intermolecular hydrogen bonds that stabilize the dimeric channel are $O^1 \cdots N^5$, $O^3 \cdots N^3$, and $O^5 \cdots N^1$ (two of each). At the C-terminal end (the channel entrance) the carbonyl oxygens of $Leu^{10,12,14}$ point toward the aqueous solution. Are these particular end structures a mere consequence of a helix sense that is dictated by the side chains elsewhere in the sequence, or could the particular hydrogen bond patterns within the terminal turns of the helix help to directly specify the helix handedness? First, the helical sense is insensitive to a wide variety of side chain substitutions at position one.[17] Second, neither the insertion nor the deletion of a single amino acid between the formyl and the first amino group of gramicidin alters the predominant helix sense of the channels.[18,19,33] These observations imply that the helix sense is not determined by the formyl group, i.e., whether the formyl C=O forms an inter- or an intramolecular hydrogen bond.[3,7]

In order to assess whether the COOH-terminal ethanolamine –OH could be important,[34] we searched for the formation of "missing residue" hybrid channels, formed between gA^- and either gP^+ (28), which has a methylamine in place of the ethanolamine (Table I), or $gK{-}A^+$,[23,55] which has a fatty

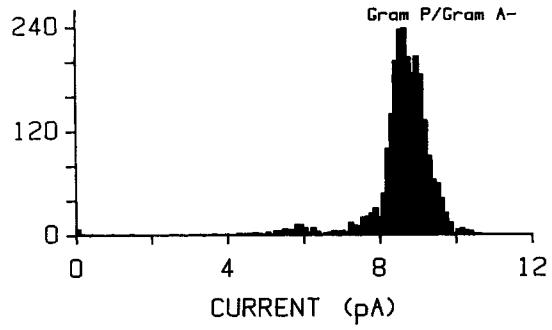

Fig. 9. Hybrid channels do not form between gP^+ and gA^-. The experiment was done by adding equipotent amounts of gP^+ and gA^- symmetrically to the electrolyte solution on both sides of the membrane. This should ensure an approximately equal number of gP^+ and gA^- channels. Channels lasting longer than 10 msec were used to determine the magnitude of the current transitions. The histogram contains 2,470 transitions, of which 2,267 (or 92%) are in the broad main peak at ~9.4 pA. The minipeak contains 107 transitions (4%) at 6.0 pA. 1.0 M CsCl, 200 mV.

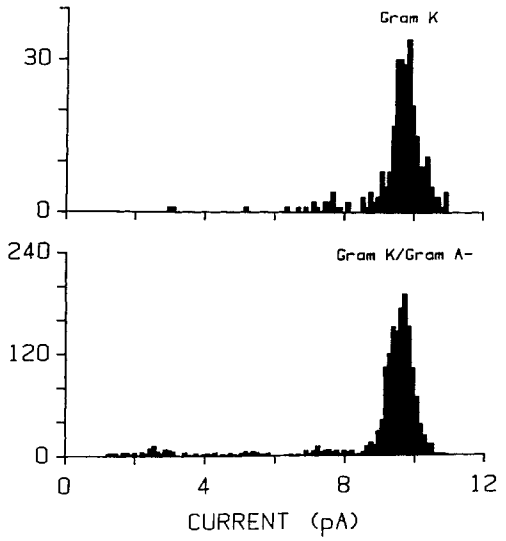

Fig. 10. Hybrid channels do not form between $gK\text{–}A^+$ and gA^-. **Top:** Current transition amplitude histogram for $gK\text{–}A^+$ channels. There are 306 transitions, of which 242 or 79% are in the main peak at 9.6 pA. There are ~30 transitions (10% of total) outside the main peak between 6 and 8 pA. **Bottom:** Current transition amplitude histogram for channels observed in the presence of a mixture of $gK\text{–}A^+$ and gA^-. Channels lasting longer than 10 msec were used to determine the magnitude of the current transitions. The histogram contains 1,941 transitions, of which 1,692 (or 87%) are in the broad main peak at ~9.6 pA. The minipeak contains 110 transitions, or 5% of all transitions, and is located between 2 pA and 8 pA on the histogram. 1.0 M CsCl, 200 mV.

acid esterified to the ethanolamine –OH (Table I). In neither case did we observe hybrid channels (Figs. 9 and 10). These results imply that the helix sense is not determined by the ethanolamine blocking group.

LH ↔ RH Interconversion?

On theoretical grounds, it is not clear why one should not observe *both* LH and RH helical conformations of gramicidin channels within the same membrane.[5,6] A stability difference of 5.7 kJ/mol would correspond to an equilibrium distribution of 10:1, easily within detection limits using the heterodimer assay. However, minor channel conformations that differ in backbone folding have not been observed for gA^+ or any of the analogues (see Figs. 5–9; and refs. 16,17). Only RH channels are observed for gA^+ and $[F_3Val^1]gA^+$, and only LH channels for gA^- and gM^-. There is either a thermodynamic or a kinetic barrier to the formation of channels of the opposite handedness within bilayers: either the RH-helical dimers of gA^+ are energetically favored to an extent that the LH-helices are never seen, or there is a kinetic barrier to the folding of gA^+ into LH-helices.

TABLE II. Energetics of Heterodimer Formation*

Analogue	Relative frequency	τ (msec)	$\Delta\Delta G^{\pm}$ (kJ/mol)	$\Delta\Delta G^0$ (kJ/mol)
gA^-	0.09	980		
gM^-	0.12	230		
Heterodimers	0.79	(320, 470)	−3.3	−2.3
gA^-	0.2	1200		
$[F_3Val^1]gA^+$	0.8	350		
Heterodimers	<0.05		>7	
gA^+	0.5	950		
gM^-	0.5	340		
Heterodimers	<0.05		>7	
gA^+	0.14	770		
$[F_3Val^1]gA^+$	0.42	270		
Heterodimers	0.44	370	0.2	0.8

*The relative channel appearance rates were calculated based on all identifiable channel events in the respective current transition amplitude histograms. For the $[Val^1]gA^-/[F_3Val^1]gA^+$ and the $[Val^1]gM^-/[Val^1]gA^+$ mixtures, less than 5% of events occurred within any discrete peak that was outside the major peaks that had been identified in experiments with only a single compound.

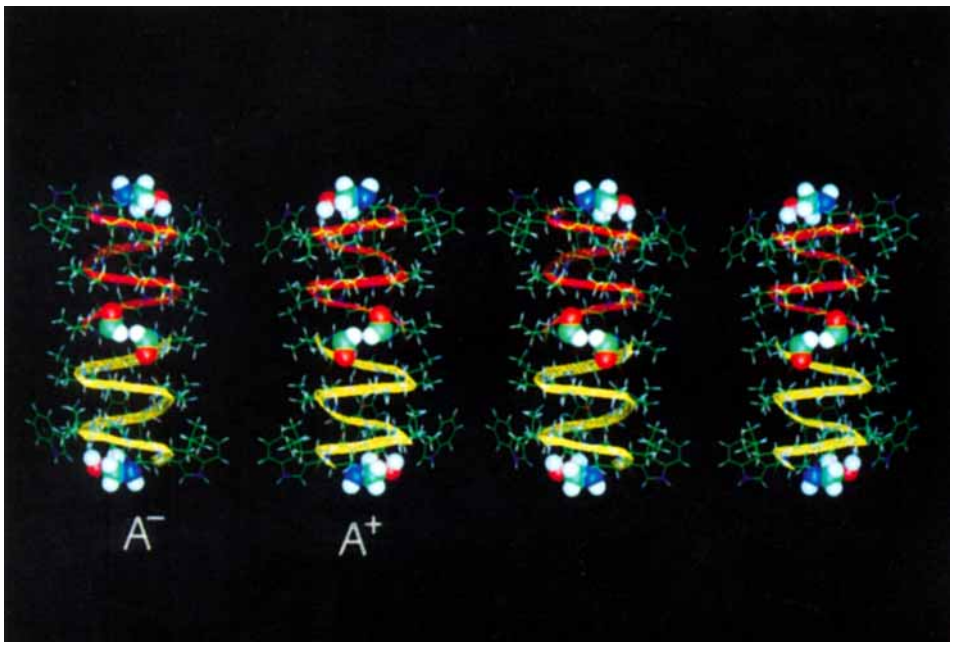

Fig. 11. Stereo view of models for the right-handed gA^+ helical dimer (second and fourth images) and the left-handed gA^- helical dimer (first and third images). The coordinates are from ref. 46. The formyl and ethanolamine groups are represented using space-filling models of 0.75 van der Waals radius.

We finally note the value of using protein (or peptide) analogues with reduced numbers of Trp residues for spectroscopic measurements. The present investigations were based upon removal of all of the Trp residues of gA^+ (and their replacement with Phe). Analogues having only single Trp residues (e.g., at either position 9, 11, 13, or 15) would be useful for single-channel studies of the influence of the Trp dipoles,[56] and for fluorescence studies on the position(s)[44] and motion(s)[57] of the indole side chains of gramicidin channels within membranes.

ACKNOWLEDGMENTS

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