deuterated phospholipid by hydrating lyophilized powders formed from benzene-methanol solutions of lipid and gramicidin A in the molar ratio 15:1. The carbonyl region of the resonance was studied over a range of temperatures spanning the phase transition temperature of a hydrated lipid dispersion. The use of perdeuterated DMPC in combination with cross polarization minimizes the contribution of the natural-abundance ^{13}C signal to the resultant spectrum. Parallel studies were performed using a Hahn echo sequence.

There were three distinct spectral components. They were interpreted as arising from: (*a*) aggregated or undispersed gramicidin A, which results in an essentially rigid lattice powder pattern and is independent of temperature from 273K to 310K; (*b*) mobile gramicidin A dispersed in lipid and undergoing some form of reorientation; and (*c*) signal from the carbonyl ester carbons of the lipid.

By subtracting the temperature-independent spectral component we obtained the spectral series shown in Fig. 2. The subtracted component typically amounted to 20% of the signal intensity. Spectra were recorded at 75.46 MHz and a Hartmann-Hahn matched 90° pulse duration of 5 μs. The double-labeled C_o–C_α glycine gave spectra which reflected the chemical shift anisotropy convoluted with the carbon-carbon dipole interaction. At 75.46 MHz the carbon-nitrogen dipolar interaction produces only a minor broadening of the overall spectrum. By reference to computer simulations based on the approach of Spiess, in reference 2, we make the following observations:

(*a*) At 273K and ~15:1 molar ratio of DMPC to gramicidin A any substantial motion undergone by the backbone of the polypeptide occurs on a timescale which is very much longer than 100 μs.

(*b*) At temperatures between 280K and the phase transition temperature of 289K the gramicidin A undergoes random reorientations about an axis close to the long axis of the helix. The rate of this reorientation progressively increases over this temperaure interval and is fast on the 100 μs timescale before reaching 289K.

(*c*) At temperatures well above the phase transition (310K) both the lipid and the gramicidin A undergo rapid reorientation about the bilayer normal.

(*d*) Using the orientation of the shielding tensor obtained from glycine-glycine (3), in which σ_{22} is approximately parallel to the carbonyl bond, the experimental spectra are consistent with a random fluctuation of the azimuthal angle about an axis parallel to both the bilayer normal and the long axis of the peptide helix. These observations are consistent with any conformation in which the carbonyl C = 0 bond is close to the direction of the reorientation axis. This geometry is found in the $\pi_{LD}^{6.3}$ helix.

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STRUCTURAL INFORMATION FROM FUNCTIONAL MEASUREMENTS

Single-channel Studies on Gramicidin Analogues

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The linear gramicidins form dimeric transmembrane channels permeable to monovalent cations and H_2O (for a recent review see 1). The three-dimensional structure of the membrane channel is known. One can therefore reasonably hope to understand the molecular determinants of channel conductance, lifetime, and ion selectivity by making defined changes in the sequence and measuring the resultant changes in channel function. A fundamental

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gramicidin analogues used in this paper are as follows:

A) Val-A (Valine-gramicidin A)
HCO-L-Val- Gly-
L-Ala-D-Leu-LAla-D-Val-L-Val-D-Val-
(L-Trp-D-Leu)$_3$-L-Trp-NHCH$_2$CH$_2$OH

B) Phe-15-A (Phe^1-gramicidin A)
HCO-L-Phe- Gly- · · · · · ·

C) Gly-15-C (Gly^1-gramicidin C)
HCO- Gly- Gly- · · · · · ·

D) Gly-14-C (des-Val^1-gramicidin C)
HCO- Gly- · · · · · ·

E) Gly-16-C (For-Gly-des-For-gramicidin C)
HCO- Gly-L-Val- Gly- · · · · · ·

F) M$^-$ (gramicidin M$^-$)
HCO-D-Val- Gly-
D-Ala-L-Leu-D-Ala-L-Val-D-Val-L-Val-
(D-Phe-L-Leu)$_3$-D-Phe-NHCH$_2$CH$_2$OH

question in any such study is to what extent does the structure of the analogue channel resemble the structure of the parent compound?

Systematic names and amino acid sequences of the Gramicidin analogues were synthesized by deletion and addition at the NH_2-terminus, using natural gramicidin as the starting material. Analogues synthesized from gramicidin C have a tyrosine residue substituted for the second of the four tryptophane residues. The compounds were purified by analytical HPLC (2). Gramicidin M$^-$ was synthesized de novo. (3)

Val-A channels are NH_2-terminal to NH_2-terminal dimers of left-handed β-helices. Fig. 1 *A* shows the contact between two gramicidin molecules in a NH_2-terminal to NH_2-terminal dimer. The fit is very tight, and hydrogen bonding across the contact is seamless. The quality of this fit depends upon the match between the two peptide backbones. If, therefore, an analogue of gramicidin forms hybrid channels with Val-A (or another analogue of known conformation), the peptide backbone conformations of the two monomers must be very similar. Functional measurements can thus provide structural information.

The experimental approach is illustrated in Fig. 2, which depicts an experiment with Gly-15-C and Phe-15-A. When only one peptide is present, histograms of current transitions have a clear main peak. When both peptides are added, however, the histograms show not only the peaks corresponding to both pure channels but, in addition, a third peak between them. This peak appears only in the presence of both peptides and must represent hybrid channels, formed by two different monomers (4). Both the conductance and average lifetime of the hybrid channels are intermediate to those of the pure channels (Table I),

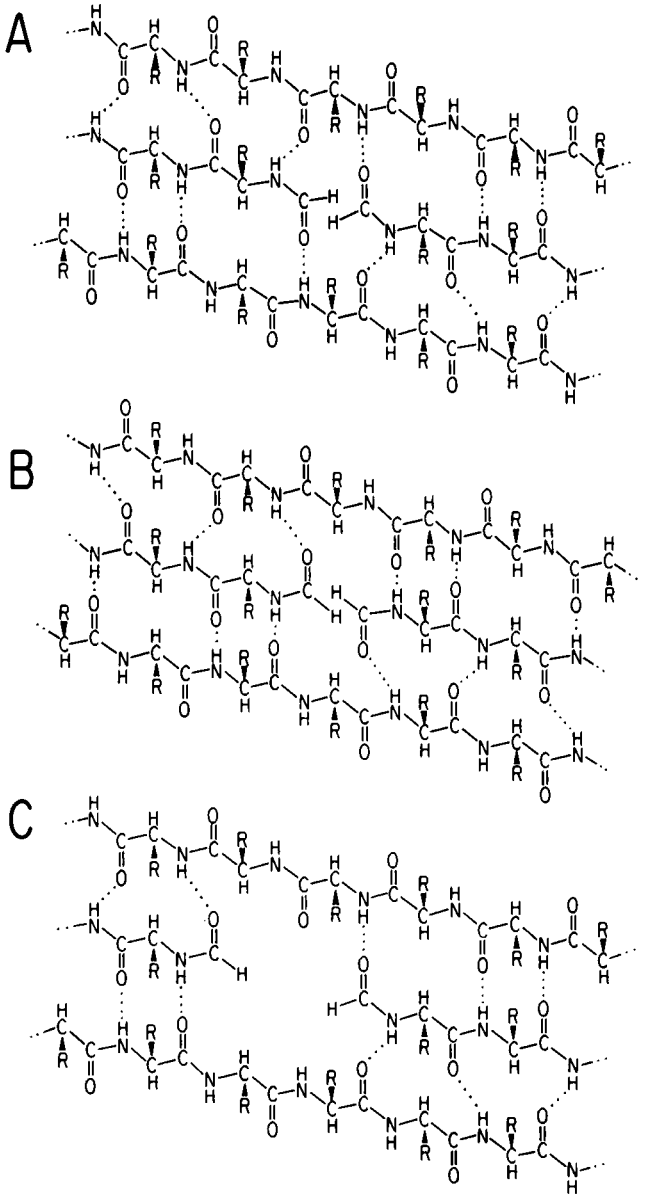

FIGURE 1 (*A*) Hydrogen bonding at the junction between two monomers of Val-A, or any other gramicidin analogues with an L amino acid in position 1, in the NH_2-terminal to NH_2-terminal β-helical dimeric channel. The left-handed helix has been cut parallel to the helix axis and unrolled. (*B*) The same for any analogue of gramicidin with a D amino acid in position 1. (*C*) The same for a left-handed helical channel formed by one L^1 and one D^1 monomer.

and the number of hybrid channels, N_h, is predicted by

$$N_h \simeq 2 \cdot (N_a \cdot N_c)^{0.5}, \quad (1)$$

where N_a and N_c are the number of the two pure channels. This prediction follows from the assumption that the kinetics of dimerization for the hybrid are about the same as for pure channels (2, 4). For Phe-15-A and Gly-15-C, the ratio (number hybrids observed)/(number hybrids

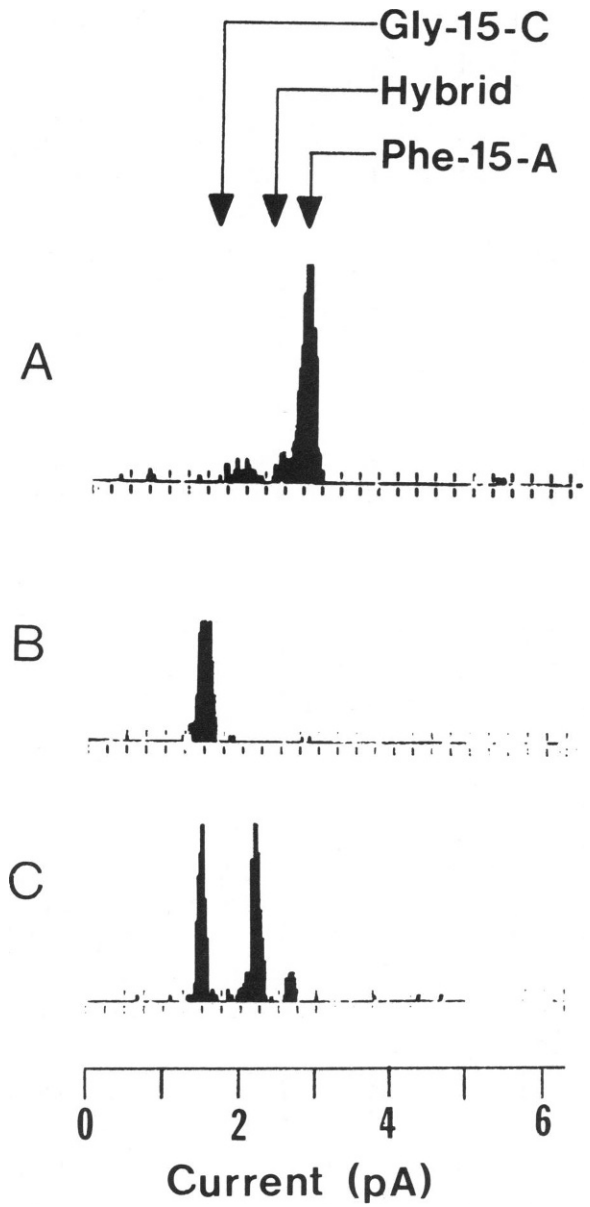

FIGURE 2 Amplitude histograms of single-channel current transitions for (*A*) Phe-15-A, (*B*) Gly-15-C, and (*C*) mixtures of Phe-15-A and Gly-15-C. Planar bilayer membranes were formed, at 25 ± 1°C, from diphytanoylphosphatidylcholine in n-decane, and electrical recordings were made using the bilayer punch (5). 1.0 M NaCl, 200 mV, 200 Hz filter. Channels lasting longer than 10 ms were used for determination of the current transitions.

predicted) was 1.2 ± 0.4 in twelve experiments. Hybrid channels also form in mixtures of Phe-15-A and Val-A, and of Gly-15-C and Val-A (data not shown). We conclude that all three peptides form channels that have the same basic structure, dimers of left-handed β-helices.

Active gramicidin analogues 14 or 16 amino acids long can be made by deletion from or addition to the NH_2-terminus (*D* and *E* in the scheme above). These analogues have a D amino acid in position 1 instead of an L amino acid, but should still be able to form left-handed NH_2-terminal to NH_2-terminal dimers (Fig. 1 *B*). Alternatively, they could be right-handed β-helical dimers. These alternatives can be distinguished by hybrid experiments with Val-A. Monomers of opposite helix handedness should form no hybrids. If hybrids form they would have a mismatch in the hydrogen bonding at the contact (Fig. 1 *C*), because Val-A has an L-amino acid in position 1, and analogues with an even number of amino acids have a D-amino acid.

TABLE I
SUMMARY OF SINGLE-CHANNEL CHARACTERISTICS

Channel type	Conductance (pS)	Average lifetime (ms)
Phe-15-A	13.5 ± 0.4	200
Gly-15-C	7.6 ± 0.2	40
Hybrids	10.9 ± 0.3	70
Val-A	15.0 ± 0.9	500
Gly-16-C	4.7 ± 0.2	300
Hybrids	1.4 ± 0.2	10

1.0 M NaCl, 200 mV. The single-channel conductances are mean ± standard deviation, based on at least four experiments.

Hybrid channels form between Gly-16-C and Val-A at nearly the frequency predicted by Eq. 1. Their conductance is, however, smaller than the pure channel conductances (Table I). This is expected because the center of the hybrid channels lacks one carbonyl (Fig. 1 *C*). The peptide carbonyls coordinate the ion during its passage through the channel. The gap in the channel wall may thus be associated with an energy barrier to ion passage. The lifetime of the hybrid channels is also shorter than either pure channel lifetime (Table I). This is again expected, because only five hydrogen bonds join these hybrids, whereas six join the pure channels (Fig. 1). Channels formed by Gly-16-C therefore have the same structure and handedness as channels formed by Val-A.

The conclusion about handedness can be further tested using Gramicidin M^-, an optically reversed analogue of Val-A, in which phenylalanine residues are substituted for the tryptophane residues of Val-A. This substitution preserves the aromatic-aliphatic repeat of the COOH-terminal half of the molecule. One would a priori expect that M^- channels have the opposite handedness to Val-A. Because M^- has a D amino acid at position 1, however, it would form hybrid channels with Gly-16-C if the two pure channels were of the same handedness. These hybrids would have complete hydrogen bonding across the contact. Fig. 3 shows that no hybrid channels have been detected between Gly-16-C and M^- This experiment was performed in 1.0 M CsCl to increase our ability to detect small and brief channel events (gramicidin single-channel currents are larger for Cs^+ than for any of the other alkali metal cations). Similar results were obtained in three

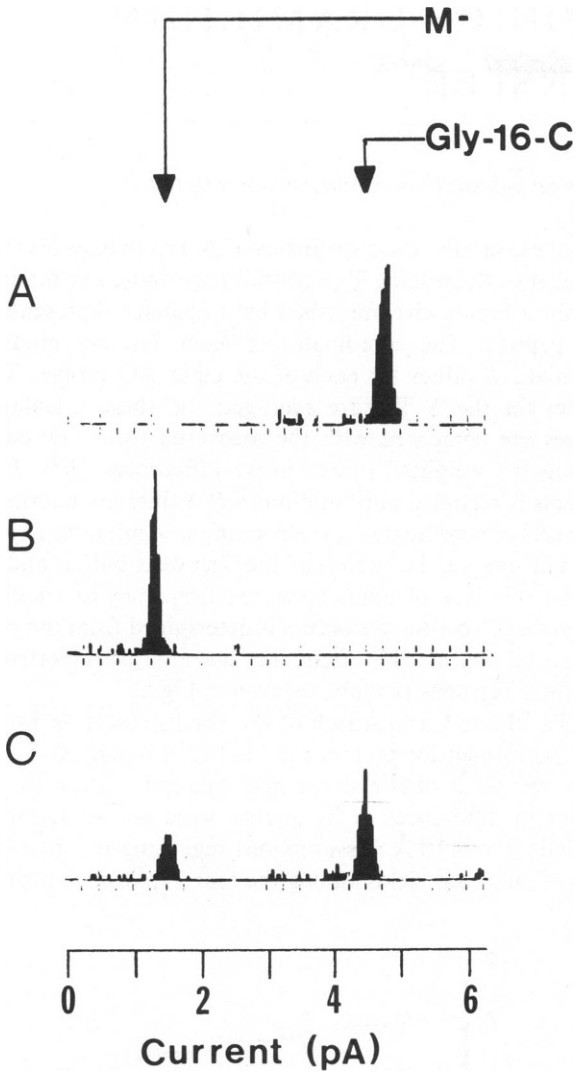

FIGURE 3 Amplitude histograms of single-channel current transitions for (*A*) Gly-16-C, (*B*) M^-, and (*C*) mixtures of Gly-16-C and M^-. 1.0 M CsCl. Other conditions as in Fig. 2.

additional experiments. Hybrid channels would have been detected if their conductance were larger than 5 pS and if they lasted longer than 5 ms.

The analogues discussed in this communication have a variety of modifications at the NH_2-terminus, the crucial contact for dimerization. Gly-15-C has increased flexibility. Phe-15-A has a bulky side chain. Gly-16-C has increased length and reversed configuration at the first amino acid. (Although Gly has no side chain, its position next to L-Val in the strictly alternating L–D sequence aligns its carbonyl group in the same orientation as the D-amino acids.) Despite these perhaps extreme modifications, one can conclude from single-channel measurements that all three have the same channel structure as Val-A, and even the same handedness. Hybrid experiments are a robust tool for examining structural similarity in modified macromolecular assemblies, given the structure of the parent assembly.

The gramicidin analogues used in these studies were produced chemically, but the same logic may also be applied to protein analogues produced genetically, e.g., by site-directed mutagenesis. The technology is now available for expressing mutated membrane channels and measuring their activity (6).

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