# SINGLE-CHANNEL STUDIES ON LINEAR GRAMICIDINS WITH ALTERED AMINO ACID SIDE CHAINS

## Effects of Altering the Polarity of the Side Chain at Position 1 in Gramicidin A

E. W. BARRETT RUSSELL,*‖ L. B. WEISS,*§¶ F. I. NAVETTA,*,** R. E. KOEPPE II,‡ AND O. S. ANDERSEN*

*Department of Physiology and Biophysics, Cornell University Medical College, New York, NY 10021;
‡Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72721;
§Department of Cell Biology, Sherman Fairchild Center, Stanford University School of Medicine, Stanford, CA 94305;‖Department of Medicine, Medical College of Wisconsin, Milwaukee, WI 53226;
¶Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305; and
**Department of Medicine, Duke University Medical Center, Durham, NC 27710

ABSTRACT The modulation of gramicidin A single-channel characteristics by the amino acid side chains was investigated using gramicidin A analogues in which the $NH_2$ terminal valine was chemically replaced by other amino acids. The replacements were chosen such that pairs of analogues would have essentially isosteric side chains of different polarities at position 1 (valine vs. trifluorovaline or hexafluorovaline; norvaline vs. S-methyl-cysteine; and norleucine vs. methionine). Even though the side chains are not in direct contact with the permeating ions, the single-channel conductances for $Na^+$ and $Cs^+$ are markedly affected by the changes in the physico-chemical characteristics of the side chains. The maximum single-channel conductance for $Na^+$ is decreased by as much as 10-fold in channels formed by analogues with polar side chains at position 1 compared with their counterparts with nonpolar side chains, while the $Na^+$ affinity is fairly insensitive to these changes. The relative conductance changes seen with $Cs^+$ were less than those seen with $Na^+$; the ion selectivity of the channels with polar side chains at position 1 was increased. Hybrid channels could form between compounds with a polar side chain at position 1 and either valine gramicidin A or their counterparts with a nonpolar side chain at position 1. The structure of channels formed by the modified gramicidins is thus essentially identical to the structure of channels formed by valine gramicidin A. The polarity of the side chain at position 1 is an important determinant of the permeability characteristics of the gramicidin A channel. We discuss the importance of having structural information when interpreting the functional consequences of site-directed amino acid modifications.

## INTRODUCTION

Transmembrane channels formed by the linear gramicidins have been extensively used to study physico-chemical aspects of ion movement through membrane-bound channels (e.g. Bamberg et al., 1978; Hladky et al., 1979; Finkelstein and Andersen, 1981; Andersen, 1984; Hinton and Koeppe, 1985). The gramicidins are well suited for such studies because their primary structures are known, and because there exists a generally accepted channel structure, the $\beta$-helical dimer proposed by Urry (1971). The structure of valine gramicidin A is (Sarges and Witkop, 1965):

Formyl-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-Ethanolamine.

The channel is formed by monomeric $\beta$-helices joined by hydrogen bonds at their $NH_2$ terminal regions. The site of dimerization is located at the center of the channel—in the middle of the membrane. Support for these general features of the channel has been obtained through single-channel and spectroscopic investigations of appropriately modified gramicidins (Urry et al., 1971; Bamberg and Janko, 1977; Bamberg et al., 1977; Veatch and Stryer,

Please address all correspondence to O. S. Andersen, Department of Physiology and Biophysics, Cornell University Medical College, 1300 York Avenue, New York, New York 10021

1977; Szabo and Urry, 1979; Weinstein et al., 1979, 1980; Urry et al., 1982; Urry et al., 1983; Boni et al., 1986).

The dimeric $\beta^{6.3}$-helix (with 6.3 amino acid residues per turn) is ideally suited to be a transmembrane channel. There is a central cavity with a radius ~ 2 Å (Urry, 1972; Finkelstein and Andersen, 1981) along the axis of the helix which is lined by the polar groups in the peptide backbone, while the hydrophobic side chains project from the exterior surface. The nonpolar exterior permits the channel to be incorporated into the hydrophobic core of a lipid bilayer and span the membrane. The peptide groups that line the lumen solvate the permeating ions and water molecules, and decrease the energy barrier for ion movement through the channel (across the bilayer).

The magnitude of the single-channel conductance depends critically upon the existence of the central solvation path (Parsegian, 1975; Finkelstein, 1975; Levitt, 1978*a*; Jordan, 1982). Given the central solvation path, one finds also that the channel conductance varies when the amino acid sequence is altered (Bamberg et al., 1976; Morrow et al., 1979; Heitz et al., 1982; Mazet et al., 1984), even though the side chains are located at the channel's exterior surface and not in direct contact with the permeating ions. The variations in single-channel conductances reflects changes in the energy barriers for ion and water movement through the channel, and possibly also changes in the ion and water mobility in the channel. These changes could arise from steric restrictions of conformational motions essential for ion movement (Morrow et al., 1979); from electrostatic interactions between the side chain and the permeating ion, either directly through ion-dipole interactions (Tredgold et al., 1977; Morrow et al., 1979; Mazet et al., 1984) or indirectly through inductive shifts in the electron densities of the peptide carbonyl oxygens; or from a combination of these factors.

We have attempted to assess the importance of the electrostatic characteristic (the polarity) of the side chain at position 1 on the permeability behavior and stability of gramicidin channels through single-channel studies on semi-synthetically modified gramicidins. Valine at position 1 was replaced with amino acids that were chosen to produce pairs of gramicidin A analogues having different polarities, but essentially isoteric hydrophobic side chains at position 1. Fig. 1 illustrates the side chains that were investigated. Our results show that the electrostatic characteristics of the side chains can play an important role in modulating the permeability behavior of the gramicidin A channel. Some of this material has appeared in preliminary form (Barrett et al., 1981; Andersen et al., 1984).

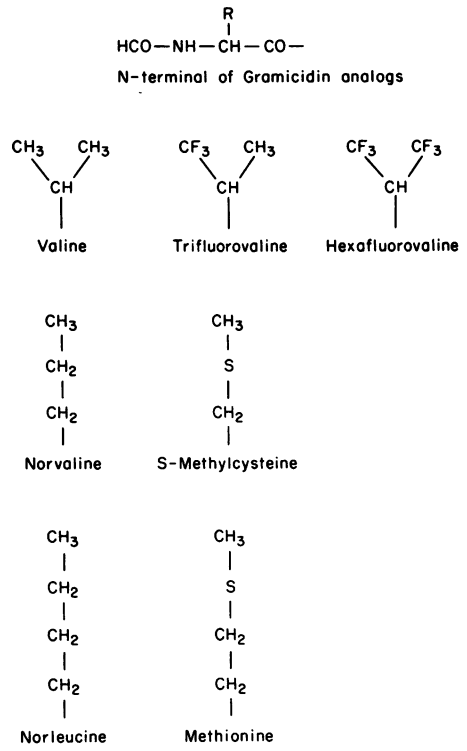


FIGURE 1 *Top:* The N-terminal end of the gramicidin molecule, to indicate the position of the side chain that is varied. *Bottom:* The chemical structure of the amino acid side chains we have investigated. The leftmost column denotes the nonpolar reference compounds. The horizontal rows denote the three different set of essentially isosteric side chains.

## METHODS AND MATERIALS

### Chemical Modification of Gramicidin A

A summary of the synthetic procedures is given here. More details of the chemistry have been presented elsewhere (Weiss and Koeppe, 1985). Desformyl gramicidin (A, B, and C) was prepared from commercial gramicidin according to the method of Sarges and Witkop (1965), except that acetic acid was not included in the reaction mixture. The product was purified by ion exchange chromatography (Morrow et al., 1979). Des(formyl-valyl) gramicidin was prepared by reacting the desformyl peptide with phenyl isothiocyanate as described by Morrow et al. (1979).

The *N*-formyl amino acids were prepared using formic-acetic anhydride (Mehlenbacher, 1953; Sheehan and Yang, 1958). The products were recrystallized from ethyl acetate. A purified *N*-formyl amino acid and an aliquot of the des(formyl-valyl) gramicidin were coupled using diphenylphosporyl azide as described by Shiori et al. (1972) and Yamada et al. (1975). These coupling reactions produced gramicidin analogues of high purity in nearly quantitative yields. Absence of racemization was demonstrated by the appearance of <0.1% formyl-L-valine gramicidin A in a preparation of formyl-D-valine gramicidin A. (The contamination with the L-valine compound was monitored by the appearance of formyl-L-valine gramicidin A channels when formyl-D-valine gramicidin A was added to the aqueous phases. This latter compound does not appear to form active transmembrane channels (Morrow et al., 1979). The concentration of the D-valine compound had to be $10^3$ times larger than the concentration of the L-compound before we began to notice the formyl-L-valine gramicidin A channels.) The gramicidin A analogues were collected in the effluent following separation of the reaction mixture by high-performance liquid chromatography, HPLC, in 80–85% methanol, 15–20% water on a 4.6 × 250 mm Zorbax-C8 column (1.5 ml/min, 1800 PSI).

Some of the resulting gramicidin A analogues were contaminated with small amounts of the corresponding gramicidin C (from the trailing edge of the gramicidin C peak in the HPLC effluent) or by valine gramicidin A (presumably material that was absorbed to the effluent tubing of the column and exchanged with the pure peptides coming from the column). Most of the samples were therefore further purified by running repeated

samples of the same substance through the HPLC column, pooling the material under the maxima of the peaks of the ultraviolet absorbance curves, concentrating and rechromatographing the pooled material (Weiss and Koeppe, 1985). The resulting compounds showed sharp, clean peaks in the HPLC traces (not shown) and single-channel conductance amplitude histograms (see Figs. 6, 7, and 9).

## Single-channel Studies

Planar lipid bilayer membranes were formed by the pipet techniques of Szabo et al. (1969) across a hole (area ~1.6 $mm^2$) in a Teflon partition separating two Teflon chambers containing 5 ml of the appropriate unbuffered electrolyte solution. The membrane-forming solution was diphytanoyl-phosphatidylcholine dissolved in *n*-decane, 2–3% wt/vol. The temperature was maintained at 25 ± 1°C with a circulating water bath. A small aliquot (2–30 μl) of an ethanolic dilution of the appropriate gramicidin A was added to each aqueous phase, sufficient to give a channel opening rate of ~1/s.

Single-channel measurements were made using the bilayer-punch technique and channel recognition procedure described in Andersen (1983*a*). For each analogue, the conductance data at a given salt concentration were based on more than 300 current transitions, generally obtained at 25 and 50 mV applied potential. The data at each potential were based on at least four measurements, two at each polarity. The final conductances are given as mean ±SEM, based upon a weighted average of the data from the individual measurements using the number of channels as the weight function.

## Data Analysis

The single-channel conductances in NaCl were analyzed in terms of a single-ion-occupancy model for ion movement through the channel (Läuger, 1973; Hladky, 1974; Andersen and Procopio, 1980; Finkelstein and Andersen, 1981).[1] In this approximation, the concentration dependence of the small-signal single-channel conductance, *g*, is expressed as:

$$g = g_{max}\, a/(K_g + a), \qquad (1)$$

where $g_{max}$ is the maximal, small-signal single-channel conductance, *a* is the aqueous activity of the permeating ion (from Robinson and Stokes [1965], Table 8.10), and $K_g$ is the activity for half-maximal conductance. Estimates for $K_g$ and $g_{max}$ were obtained by fitting Eq. 1 to the conductance data using a nonlinear least-squares search based upon a combination of the grid search and Marquardt-Levenberg algorithms (e.g., Bevington [1969], pp. 204–246). The weight function was 1/(standard error of mean for the conductances). All analogues were studied at 0.1, 0.2, 0.5, and 1.0 M NaCl. Where technically feasible, measurements were also done at 0.05 M to better define the conductance in the concentration range around $K_g$. No attempts were made to correct for interfacial polarization effects (Andersen, 1983*b*); they should be negligible at the salt concentrations and applied potentials used here. The quality of the fit of Eq. 1 to the data was expressed as the reduced chi-square, chi-square/*n*, where *n* is the number of conductance values used in the fit (e.g. Bevington [1969], 187–190).

In the absence of aqueous diffusion limitations, $K_g$ will be equal to *K*, the equilibrium dissociation constant for the channel and $Na^+$. In the presence of aqueous diffusion limitations one should be able to approximate *K* as (Andersen, 1983*c*):

$$K = K_g - 2\,(kT/e^2)\, g_{max}/p_a, \qquad (2)$$

where *k* is the Boltzmann's constant, *T* is temperature in Kelvin, *e* is the elementary charge, and $p_a$ is the aqueous convergence permeability to the channel entrance. The convergence permeability for $Na^+$ was not determined directly. It was assumed to be equal to $p_a$ for valine gramicidin A: ~1.1 $10^8$ liter $mole^{-1}$ $s^{-1}$ (Andersen, 1983*c*). This assumption is supported by measurements of single-channel currents in 0.1 M CsCl (see Results). At 25°C Eq. 2 can thus be rewritten as: $K = K_g - 2.92\ 10^{-3}\ g_{max}$, where *K* and $K_g$ are in *m* and $g_{max}$ is in pS.

Average single-channel lifetimes were estimated from lifetime histograms assembled from stripchart recordings obtained at 200 mV applied potential. The average lifetimes for the different analogues were estimated by fitting a single exponential decay to the data using the nonlinear least-squares search described above. The final average lifetime estimates (mean ± SEM) were obtained from data on several different membranes.

The channel-forming potency of each analogue was estimated from the aqueous concentration of an analogue necessary to achieve a single-channel frequency of approximately one opening/s relative to the concentration of valine gramicidin A necessary to obtain a comparable activity. (The channel-forming potency thus estimated reflects the adsorption of the compounds to the membrane as well as the rate constant for channel formation from the membrane-bound monomers. We have not attempted to separate these contributions.) No corrections were made for changes in pipette capacitance (membrane area).

The chromatographic behavior of each analogue was quantitated as the relative retention time, $R_t$, a measure of the overall polarity of the molecules, with valine gramicidin A as the reference, on a Zorbax-C8 column (Dupont Analytical Instruments, Wilmington, DE) eluted with methanol: $H_2O$ (80:20 vol/vol) at room temperature.

Dipole moments of the polar side chains were estimated along the α-β bonds from standard bond angles, group moments (proximal to distal) of −0.4 D for ⇒C-H and $-CH_3$, +1.95 D for $-CF_3$ and +1.40 D for $-SCH_3$, and a moment angle of 57° for $-SCH_3$ (Smyth [1955], pp. 228–280). The group moment for $-CF_3$ was chosen based on measurements on $CF_3 - CF_3$ ($\mu$ = 2.35 D; Smyth [1955], pp. 271–273). The estimates for methionine and S-methyl-cysteine were made assuming free rotation about all the side chain bonds. Restricted rotation would increase the components of the dipole moment along the α-β (and β-γ) bonds, especially for methionine.

## Materials

Linear gramicidin (gramicidin-Dubos) was obtained from ICN Nutritional Biochemicals (Cleveland, OH) or from Sigma Chemical Company (St. Louis, MO). Phenylisothiocyanate pyridine, triethylamine, dimethyl formamide, and 4 N HCl in dioxane were from Pierce Chemical Company (Rockford, IL), while diphenylphosphorylazide was from Sigma Chemical Company. The amino acids were from Sigma Chemical Company, except for trifluorovaline and hexafluorovaline which were from Fairfield Chemical Company (Blythewood, SC).

Diphytanoylphosphatidylcholine was from Avanti Polar Lipids (Birmingham, AL). It gave a single spot on thin layer chromatograms. The lipid was further cleaned by ion exchange chromatography as described in Andersen (1983*a*). *n*-Decane was 99.9% pure from Wiley Organics (Columbus, OH), it was used without further purification.

NaCl and CsCl were Suprapur grade from E. Merck, Darmstadt (through MCB, Cincinnati, OH). Before use they were roasted for at least 24 h at 550–600°C and stored in an evacuated dessicator over

[1]This model was chosen because ion tracer flux studies show no evidence for interaction among $Na^+$ in valine gramicidin A channels in diphytanoylphosphatidylcholine/*n*-decane membranes (Procopio and Andersen, 1979), and because the shapes of single-channel current-voltage characteristics vary as a function of $[Na^+]$ (Andersen and Procopio, 1980). These findings suggest that at most one $Na^+$ occupies a gramicidin channel at attainable $[Na^+]$. Other alkali metal cations ($K^+$ through $Cs^+$) exhibit clearcut evidence for multiple-ion occupancy, at least two ions can simultaneously occupy the channel (Urban et al., 1980; Eisenman and Sandblom, 1983; Schagina et al., 1983; Procopio, Haspel, and Andersen, manuscript in preparation). The $Na^+$ data can also be fit assuming multiple ion occupancy (e.g., Eisenman and Sandblom, 1983). If this were the case for channels in diphytanoylphosphatidylcholine/*n*-decane bilayers, it would not affect our general conclusions.

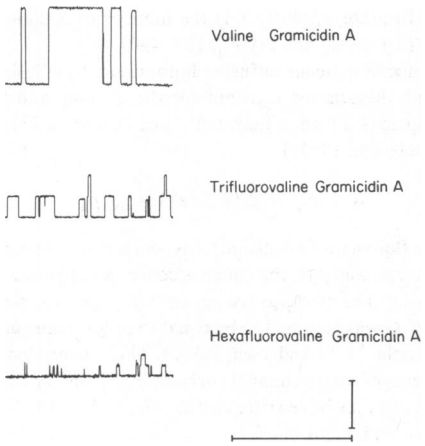


FIGURE 2 Comparison of single channel events observed with different gramicidin A analogues. The three traces were all obtained at the same amplification and chart speed. The calibration bars denote 2 pA (vertically), and 5 s (horizontally). 1.0 M NaCl, 200 mV.

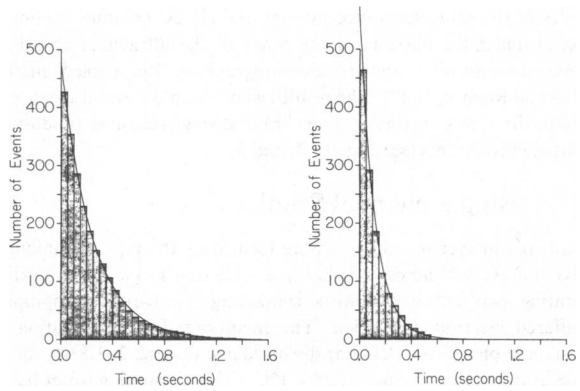


FIGURE 3 Survivor plots of single-channel durations. *Left:* For 427 norleucine gramicidin A channels. *Right:* For 464 methionine gramicidin A channels. The curves describe the best fit of a single exponential decay to the data: $N(t) = N(0) \exp(-t/\tau)$, where $N(t)$ denotes the number of channels remaining at time $t$, and $\tau$ is the average single-channel lifetime. The width of the sampling intervals was 0.05 s, and the curves have been offset by 0.025 s as suggested by Colquhoun and Sigworth (1983). For norleucine gramicidin A, $\tau = 0.244$ s; for methionine gramicidin A, $\tau = 0.117$ s. 1.0 M NaCl, 200 mV.

NaOH. All other chemicals were reagent grade. The water was deionized Millipore Milli-Q water (Bedford, MA).

## RESULTS

Alterations of the side chain at position 1 in gramicidin A will generally alter the characteristics of the channels formed by the new compounds. An example of these changes is illustrated in Fig. 2, where channels formed by valine gramicidin A are compared with channels formed by trifluorovaline and hexafluorovaline gramicidin A. The polarity (dipole moment) of the side chain at position 1 increases from valine to trifluorovaline to hexafluorovaline (vide infra). The increased polarity of the side chains is in this case associated with a decreased single-channel conductance.

Changes in side chain polarity affect not only the permeability characteristics, but also the stability (average channel lifetime) of the channels. Fig. 3 depicts survivor plots for norleucine and methionine gramicidin A. The increased polarity of the methionine side chain is associated with a decreased channel stability.

Similar modulations of the single-channel characteristics by changes in side chain polarity were observed with each pair of analogues, although the quantitative changes varied among the compounds. The polarities of the side chains were quantitated either as the estimated dipole moment along the $\alpha$-$\beta$ bond in the side chains (which is approximately perpendicular to the channel axis), or as the $pK_a$ of the free amino acids in aqueous solutions (e.g. Edsall and Wyman, 1958). These estimates are listed in Table I together with our results on the HPLC behavior, channel-forming potencies, and average single-channel lifetimes for channels formed by the analogues. Table II summarizes our data on the single-channel conductances in NaCl. The single-channel conductances are less for the channels formed by the more polar compound in each set. The relative conductance decrements decrease with decreasing salt concentrations. This shows that the changes in the polarity of the side chain at position 1 affect not only $g_{max}$ but also $K_g$, the $Na^+$ activity for half-maximal conductance of the channel.

Qualitatively similar variations in the small-signal conductance were observed with $Cs^+$ as the permeant ion (Table III), although the relative conductance changes were less than with $Na^+$. Gramicidins analogues with polar side chains at position 1 thus form channels that have increased selectivity among alkali metal cations relative to their nonpolar counterparts. Interestingly, the $Cs^+$ conductances of trifluorovaline gramicidin A channels are less than the conductances of hexafluorovaline gramicidin A channels, contrary to what one might have predicted on the

TABLE I
CHARACTERISTICS OF THE MODIFIED GRAMICIDIN A's

| Amino acid at position 1 | Estimated dipole moment | $pK_a$ | Relative $R_f$ | Channel-forming potency | Average lifetime |
|---|---|---|---|---|---|
| | *Debye* | | | | *s* |
| Valine | −0.4 | 2.29* | 1.0 | 1.0 | 0.56 ± 0.06 |
| Trifluorovaline | +0.8 | 1.54* | 1.20 | 0.3 | 0.19 ± 0.02 |
| Hexafluorovaline | +1.6 | N.A. | 0.86 | 0.1 | 0.07 ± 0.01 |
| Norvaline | −0.4 | 2.32‡ | 1.05 | 1.0 | 0.16 ± 0.01 |
| S-methyl-cysteine | +0.3 | 1.99‡ | 0.92 | 0.3 | 0.16 ± 0.01 |
| Norleucine | −0.4 | 2.33‡ | 1.20 | 0.3 | 0.28 ± 0.02 |
| Methionine | +0.1 | 2.20§ | 1.02 | 0.1 | 0.10 ± 0.02 |

The dipole moments are estimated along the $\alpha$–$\beta$ bonds as described in Methods and Materials.
*From Walborsky and Lang (1956).
‡From Martell and Smith (1982).
§From Martell and Smith (1972).

TABLE II
SINGLE-CHANNEL CONDUCTANCES IN NaCl

| Amino acid at position 1 | NaCl 0.05 M (0.0411 *m*) | 0.1 M (0.0778 *m*) | 0.2 M (0.148 *m*) | 0.5 M (0.344 *m*) | 1.0 M (0.670 *m*) |
|---|---|---|---|---|---|
| Valine | 3.51 ± 0.08 | 5.32 ± 0.12 | 7.96 ± 0.30 | 11.53 ± 0.24 | 12.37 ± 0.20 |
| Trifluorovaline* | | 1.01 ± 0.03 | 1.47 ± 0.02 | 1.79 ± 0.05 | 1.93 ± 0.02 |
| Hexafluorovaline‡ | | 1.06 ± 0.04 | 1.30 ± 0.03 | 1.32 ± 0.05 | 1.42 ± 0.04 |
| Norvaline | 3.54 ± 0.15 | 5.66 ± 0.14 | 9.58 ± 0.05 | 12.30 ± 0.11 | 14.69 ± 0.15 |
| S-methylcysteine | 3.12 ± 0.03 | 4.16 ± 0.10 | 6.51 ± 0.07 | 8.38 ± 0.11 | 9.90 ± 0.11 |
| Norleucine | 4.25 ± 0.04 | 6.34 ± 0.08 | 8.81 ± 0.06 | 11.94 ± 0.06 | 14.74 ± 0.14 |
| Methionine | 2.38 ± 0.03 | 3.70 ± 0.03 | 5.38 ± 0.08 | 7.14 ± 0.08 | 8.28 ± 0.13 |

The numbers in parentheses denote the mean ion activity in *m*.
Conductances in pS, mean ± SEM.
*Data at 50 mV only.
‡Data at 100 mV.

TABLE III
SINGLE-CHANNEL CONDUCTANCES IN CsCl

| Amino acid at position 1 | CsCl 0.1 M | 0.5 M | 1.0 M | 2.0 M |
|---|---|---|---|---|
| Valine | 17.92 ± 0.23 | 44.50 ± 0.27 | 50.40 ± 0.82 | 50.03 ± 0.50 |
| Trifluorovaline | 9.74 ± 0.12 | 15.78 ± 0.19 | 16.03 ± 0.25 | 15.17 ± 0.24 |
| Hexafluorovaline | 10.63 ± 0.24 | 18.66 ± 0.34 | 18.77 ± 0.18 | 15.88 ± 0.48 |
| Norleucine | 18.11 ± 0.12 | | 49.42 ± 0.34 | |
| Methionine | 16.88 ± 0.23 | | 38.19 ± 0.54 | |

Conductances in pS, mean ± SEM.

basis of the results in NaCl. Both channels have their maximal conductances ~1.0 M CsCl, as evidenced by experiments in 0.5 and 2.0 M CsCl.

At high potentials there was little discernible difference between the single-channel currents observed in 0.1 M CsCl among the different channels, and the currents leveled off toward similar, approximately voltage-independent values. Fig. 4 presents data for valine, trifluorovaline and hexafluorovaline gramicidin A channels. (The finite slopes of the current-voltage characteristics at high potentials are at least in part the result of interfacial polarization [Andersen, 1983*b*].) The current-voltage characteristics for valine and trifluorovaline gramicidin A channels are almost indistinguishable at 450–500 mV, while the characteristic for hexafluorovaline gramicidin A channels crosses that for trifluorovaline gramicidin A channels to become slightly lower at high potentials. It is possible that the voltage-independent limiting current is smaller for hexafluorovaline gramicidin A channels than for the other channels. If this were the case, it would not affect our use of a single estimate for $p_a$ to correct $K_g$ (see Eq.2), as $g_{max}$ for hexafluorovaline gramicidin A channels is so low that $K_g$ and $K$ in any case will be very similar. Data similar to those in Fig. 4 were observed with norleucine and methionine gramicidin A, where the currents at 450–500 mV were 2.84–2.88 pA and 2.72–2.78 pA, respectively. The similarity in the single-channel currents at high potentials were also observed in NaCl for norvaline and norleucine gramicidin A relative to valine gramicidin A. In 0.05 M NaCl, the single-channel currents at 450–500 mV were 0.96–0.99 pA and 0.94–1.01 pA for norvaline and valine gramicidin A, respectively; in 0.1 M NaCl, the corresponding currents were 1.72–1.83 pA and 1.62–1.71 pA for norleucine and valine gramicidin A, respectively. The currents through hexafluorovaline gramicidin A channels were again less, 1.00–1.14 pA in 0.1 M NaCl, than for the other channels.

From the magnitudes of the limiting currents in CsCl we can estimate that the voltage-independent rate constant for

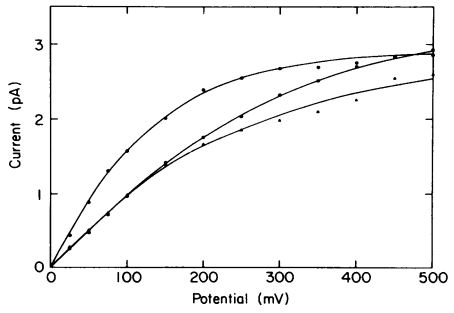


FIGURE 4 Single-channel current-voltage characteristics. (●): valine gramicidin A channels; (■): trifluorovaline gramicidin A channels; and (▲): hexafluorovaline gramicidin A channels. The solid curves have no theoretical significance. 0.1 M CsCl.

$Cs^+$ entry into each of the different channels should be similar to the rate constant previously determined for valine gramicidin A, ~1.5 $10^8$ 1 $mol^{-1}$ $s^{-1}$ (Andersen, 1983*c*). This rate constant is mainly determined by $p_a$, the bimolecular rate constant for diffusion-controlled ion access to the channel entrance, and its variation among the alkali metal cations is proportional to the aqueous diffusion coefficient of the ions (Andersen, 1983*c*). The similar estimates for $p_a$ for $Cs^+$ among the different analogue channels, taken together with the similarity in the high-voltage $Na^+$ currents, argue that $p_a$ for $Na^+$ should be ~1.1 $10^8$ 1 $mol^{-1}$ $s^{-1}$ for the channels studied here.

The conductance data in Table II were used to obtain estimates for $g_{max}$, $K_g$ and $K$ according to Eqs. 1 and 2. These estimates are summarized in Table IV. Eq. 1 provides a reasonable prediction of the conductance vs. activity relation, although the fit is noticeably worse for norvaline and norleucine gramicidin A channels than for the other channels. While this is a cause for concern, we will for the present use the simple model to analyze the data. Both $g_{max}$, and $K_g$ vary as the side chain at position 1 is altered. The major effect is on $g_{max}$, and a substantial part of the changes in $K_g$ occur as consequences of the changes in $g_{max}$; see Eq. 2. The variations in $K$, the $Na^+$ affinity of the channels, are less pronounced than the changes in $K_g$, indicating that the depths of the energy wells forming the ion binding sites vary little with changes in the side chain at position 1 (except for hexafluorovaline gramicidin A). It appears that the $NH_2$ terminal side chains exert their major effect locally, upon the central barrier for ion translocation through the channel.

A decrease in $g_{max}$ could result from an increase in the height of the energy barrier (a decrease of the rate constant) for ion translocation through the channel interior. If this were the case, the single-channel current-voltage characteristics should be more superlinear for the channels with the lower conductances. The voltage-dependence of the normalized single-channel conductance, $g(V)/g$, should be steeper for channels formed by analogues with polar side chains at position 1. This general pattern was observed; see Fig. 5. Channels formed by valine gramicidin A are almost Ohmic; $g(V)/g$ varies little with $V$, whereas channels formed by trifluorovaline and hexafluorovaline gramicidin A have quite voltage-dependent conductances. Similar data were obtained with S-methyl-cysteine vs. norvaline and methionine vs. norleucine gramicidin A, where $g(200)/g$ in 1.0 M NaCl was 1.28 vs. 1.15, and 1.35 vs. 1.12, respectively. The same pattern was observed at lower [NaCl] (data not shown) and with $Cs^+$ as the permeating ion (0.1 M CsCl, e.g., Fig. 4, and 1.0 M CsCl).

TABLE IV
PERMEABILITY CHARACTERISTICS OF MODIFIED GRAMICIDIN A'S

| Amino acid at position 1 | $g_{max}$ | $K_g$ | $K$ | Red. chi-square |
|---|---|---|---|---|
| | *pS* | *m* | *m* | |
| Valine | 15.3 ± 0.3 | 0.137 ± 0.006 | 0.09 | 0.75 |
| Trifluorovaline | 2.2 ± 0.1 | 0.077 ± 0.004 | 0.07 | 1.57 |
| Hexafluorovaline | 1.48 ± 0.04 | 0.027 ± 0.005 | 0.02 | 0.58 |
| Norvaline | 17.5 ± 0.2 | 0.130 ± 0.004 | 0.08 | 6.72 |
| S-methyl-cysteine | 11.3 ± 0.1 | 0.111 ± 0.003 | 0.08 | 2.35 |
| Norleucine | 16.4 ± 0.1 | 0.121 ± 0.002 | 0.07 | 4.25 |
| Methionine | 9.9 ± 0.1 | 0.129 ± 0.003 | 0.10 | 0.15 |

$Na^+$ is the permeating ion.
Mean ± SEM.

The variations in single-channel conductances illustrated in Tables II and III do not seem to result from significant changes in channel structure. The average lifetimes for the different channels are, for example, fairly similar; see Table I. The channel stability is determined, among other factors, by the stability of the hydrogen-bonded connection(s) at their abutting ends. The similar lifetimes are thus indicative of a fairly similar structure of the formyl-$NH_2$-terminal regions in the different channels.

This conclusion is strengthened by the results of experiments where pairs of compounds having different single-channel conductances (e.g., valine and trifluorovaline gramicidin A) were added simultaneously to the aqueous phases. In these experiments we observed new channel types, with conductances that were intermediate between the conductances seen with channels formed by the respective pure compounds; see Fig. 6. These new channels represent hybrid channels formed by two chemically dissimilar monomers.

Hybrid channels come in two flavors: with trifluorovaline gramicidin A toward the positive aqueous phase, and

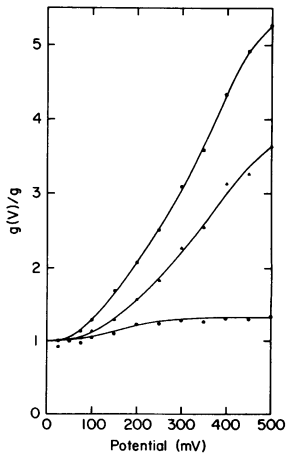


FIGURE 5 Plot of the normalized voltage-dependence of single-channel current-voltage characteristics, $g(V)/g$. (●): valine gramicidin A channels; (■): trifluorovaline gramicidin A channels; and (▲): hexafluorovaline gramicidin A channels. The solid curves have no theoretical significance. 1.0 M NaCl.

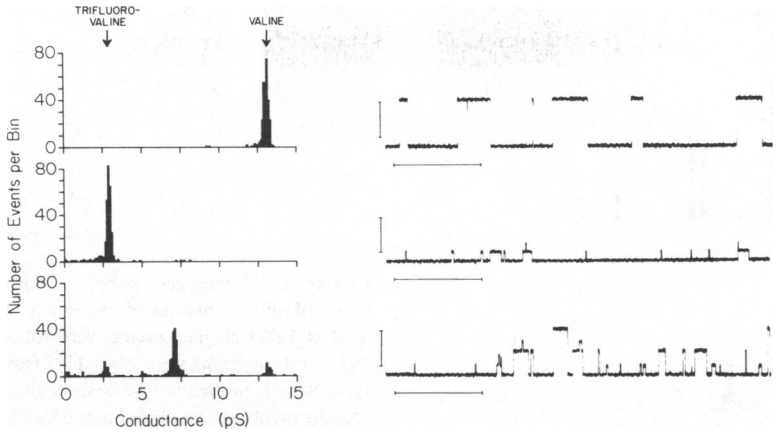


FIGURE 6 Amplitude histograms of single-channel current steps and current tracings obtained with mixtures of valine and trifluorovaline gramicidin A. *Top:* Histogram and current tracing obtained with valine gramicidin A alone, the single-channel conductance was 13.02 ± 0.17 pS (mean ± SD). There were 293 channel transitions in the histogram, of which 283 (or 96%) were in the major peak. *Middle:* Histogram and current tracing obtained with trifluorovaline gramicidin A alone, the single-channel conductance was 2.84 ± 0.12 pS. There were 296 channel transitions in the histogram, of which 250 (or 84%) were in the major peak. *Bottom:* Histogram and current tracing obtained with a mixture of valine and trifluorovaline gramicidin A. The tracing shows the existence of a new channel type that has an intermediate single-channel current, the hybrid channel. The new peak between the two peaks that were observed with the pure compounds represent hybrid channels. The average conductances for the three peaks were, from left to right: 2.57 ± 0.15 pS (37); 7.02 ± 0.22 pS (173); and 13.09 ± 0.17 pS (37). The numbers in parentheses denote the number of channels in each population. There was a total of 298 channel transitions in the histogram; we can account for 83% of the transitions. Note how the peak representing the hybrid population has a larger standard deviation than the peaks representing the pure channels. Channels lasting longer than 40 ms were used in the analysis. The calibration bars for the current tracings denote 1 pA (vertically) and 5 s (horizontally). The records were obtained at 100 Hz cutoff. 1.0 M NaCl, 100 mV.

with trifluorovaline gramicidin A toward the negative aqueous phase. The energy profile for ion movement through the hybrid channels (from the positive to the negative aqueous phase) will, in general, be different for these two orientations of the hybrid channels. The currents through the hybrid channels should consequently differ (slightly) for the two orientations. This asymmetry in the energy profiles will for thermodynamic reasons not be visible in the limit of zero applied potential. At higher potentials the asymmetry will begin to be reflected as an increase in the width of the hybrid peak compared to the width of the peaks representing the two pure channel types; see Fig. 6. As the potential is increased further the hybrid peak will split up into two peaks; see Fig. 7. This split should become more pronounced as a function of applied potential, the relative split was for example ~9% at 200 mV and ~12% at 300 mV.

Not only the conductance, but also the average lifetime of the hybrid channels was intermediate between the lifetimes of the pure channel types. Fig. 8 depicts survivor plots for channels seen with mixtures of valine and trifluorovaline gramicidin A. The rate of decay of the hybrid channel population is intermediate between the decays of the two pure channel populations. We can thus conclude that the stability of the hybrid channels must be comparable to the stability of the pure channels. The conformation of the two chemically dissimilar abutting formyl-$NH_2$-termini must be very similar in the hybrid dimers.

Results similar to those in Fig. 6 were also observed with mixtures of norvaline or norleucine and trifluorovaline or S-methyl-cysteine or methionine gramicidin A, and with mixtures of S-methyl-cysteine or methionine and valine gramicidin A. The conductances of the hybrid channels were in all cases in between the conductances of the two pure channel types. For channels formed between trifluorovaline and any of the three nonpolar analogues, the split in the hybrid peaks at 200 mV were comparable—between 7 and 9% of the average conductance for the hybrid channels. The average lifetimes of these hybrid channels were comparable to those of the pure channels, but they were not quantitated to the same extent as those of the hybrid channels formed between trifluorovaline and valine gramicidin A.

Hybrid channels formed between hexafluorovaline and valine gramicidin A differ qualitatively from the other hybrid channels: their conductance and average lifetime were *less* than the conductance and average lifetime of either of the pure channels. Fig. 9 contains analog tracings and amplitude histograms obtained with valine gramicidin A, with hexafluorovaline gramicidin A, and with a mixture of both. The experiments were done in 1.0 M CsCl, as we were unable to resolve the hybrid channels in NaCl because of their small amplitudes and short durations. Fig. 10 depicts survivor plots for hexafluorovaline, valine and hybrid channels. The rate of decay of the hybrid channel populations is much faster than the rate of decay of either of the pure channel types.

The frequency of the hybrid channels should be related to the frequencies of the pure channels (Veatch and Stryer, 1977; Mazet et al., 1984). If the monomers form helices with identical structures at their joining ends, the frequency of hybrid channels, $f_H$, should be related to the

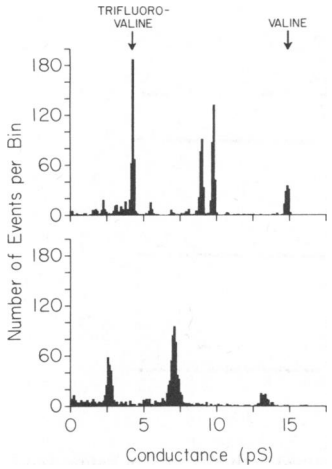


FIGURE 7 Amplitude histograms of single-channel current steps obtained with mixtures of valine and trifluorovaline gramicidin A. *Bottom:* Histogram obtained at 100 mV applied potential. The data represent four separate experiments consisting of 1,091 channel transitions. The average conductances for the three peaks were, from left to right: 2.57 ± 0.24 pS (250); 7.02 ± 0.31 pS (519); and 13.23 ± 0.26 pS (87). 1.0 M NaCl, 100 mV. The numbers in parentheses denote the number of channels in each population; we can account for 78% of the transitions. The range of the average conductances in the individual experiments were, from left to right: 2.51–2.61 pS; 6.95–7.12 pS; and 13.07–13.40 pS. *Top:* histogram obtained at 200 mV. The data represent four separate experiments consisting of 1,244 channel transitions. Note how the hybrid peak seen at 100 mV has split into two peaks, representing the two different orientations of the hybrid channels. The average conductances for the four peaks were, from left to right: 4.21 ± 0.11 pS (356); 8.91 ± 0.12 pS (235); 9.73 ± 0.09 pS (285); and 14.78 ± 0.11 pS (111). The numbers in parentheses denote the number of channels in each population; we can account for 79% of transitions. The range of the average conductances in the individual experiments were, from left to right: 4.16–4.22 pS; 8.84–8.94 pS; 9.63–9.74 pS; and 14.60–14.84 pS. 1.0 M NaCl.

frequencies of the pure channels, $f_a$ and $f_b$, by (Mazet et al., 1984):

$$f_H \geq 2\,(f_a f_b)^{0.5}, \qquad (3)$$

where the equality holds if the two orientations of the hybrid channels in the bilayer (a–b and b–a) occur with equal probability (see Mazet et al. [1984] for further discussion). The frequency of hybrid channels did indeed conform to the predictions of Eq. 3; see Table V. For hybrids formed between hexafluorovaline gramicidin A and valine gramicidin A or valine gramicidin B (which has a phenylalanine instead of a tryptophane at position 11), the number of hybrid channels in the amplitude histograms was significantly less than predicted by Eq. 3, 0.37 ± 0.09 and 0.28 ± 0.07, respectively. This resulted from the loss of channels in the sampling procedure, because of the short duration of the hybrid channels, the average lifetime is ~11 ms for channels formed between valine and hexafluorovaline gramicidin A. This is comparable to the minimum duration of channels accepted for the amplitude analysis, 11 ms. Assuming a single exponential decay, the number of transitions in the hybrid population can be corrected for the ensuing loss, and the observed/predicted ratio becomes ~1.0, consistent with predictions based on Eq. 3.

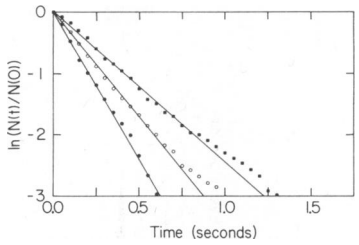


FIGURE 8 Normalized survivor plots of single-channel durations observed with a mixture of valine and trifluorovaline gramicidin A. A total of 1,093 channel events were collected on stripchart records, and duration histograms were assembled from these. The different channels types were identified on the basis of their amplitudes. Multiple channel opening events were included in the histogram, where channel openings of different amplitudes were connected with their respective closing events, and several openings of the same amplitude were dealt with as described in Mazet et al. (1984). The straight lines denote the logarithmic transform of the best fit of a single exponential decay, $N(t)/N(0) = \exp(-t/\tau)$, to the duration histograms for each of the three channel types. ■: Data for 259 valine gramicidin A channels, $\tau = 0.405$ s; ●: Data for 366 trifluorovaline gramicidin A channels, $\tau = 0.209$ s; ○: Data for 468 hybrid channels, $\tau = 0.290$ s. The plot is truncated at three time constants. 1.0 M NaCl, 200 mV.

## DISCUSSION

The molecular characteristics of gramicidin A channels depend upon the structure and polarity of their side chain at position 1. Qualitatively similar results were obtained with $Na^+$ and $Cs^+$ as the permeating ions, and with chemically very different side chains, and we will conclude that the major effects correlate with the changes in polarity of the side chains: An increase in polarity between two essentially isosteric side chains is associated with a decrease in the single-channel conductances for both $Na^+$ and $Cs^+$, and (less pronounced) with a decrease in the average channel lifetime. The changes in conductances and lifetimes could reflect substantial changes in the structure of the polypeptide backbone, alternatively they could signify that the side chains are important modifiers of the channel behavior even though the basic channel structure is unaltered.

We will first deal with the question of the channel structure and conclude that the modified gramicidins form channels that are structurally very similar to those formed by valine gramicidin A. We will then consider the changes in the energy profile induced by the changes in the side chains, as well as the changes in single-channel lifetimes and channel-forming potencies. We will finally discuss some unresolved problems that may have general implications for structure-function studies on channels in biological membranes.

### The Channel Structure

There is no evidence that the basic channel structure is affected by the modifications we have imposed upon the

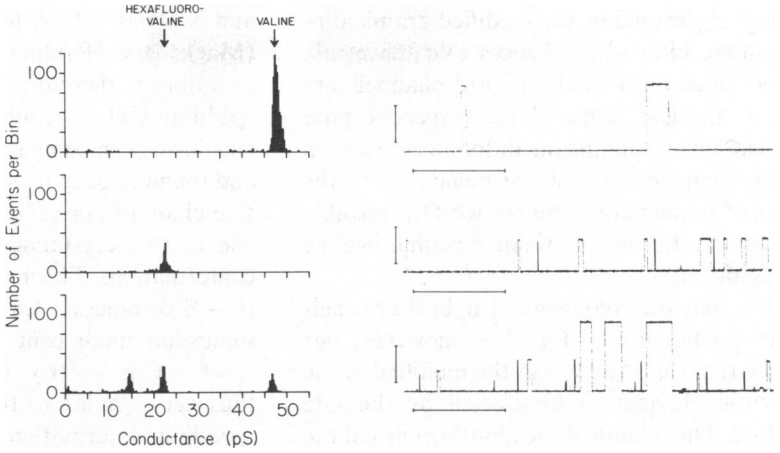


FIGURE 9 Amplitude histograms of single-channel current steps and current tracings obtained with mixtures of valine and hexafluorovaline gramicidin A. *Top:* Histogram and current tracing obtained with valine gramicidin A alone, the single-channel conductance was 47.72 ± 0.71 pS (mean ± SD). There were 821 channel transitions in the histogram, of which 772 (or 94%) were in the major peak. *Middle:* Histogram and current tracing obtained with hexafluorovaline gramicidin A alone, the single-channel conductance was 22.39 ± 0.66 pS. There were 173 channel transitions in the histogram, of which 143 (or 83%) were in the major peak. *Bottom:* Histogram and current tracing obtained with a mixture of valine and hexafluorovaline gramicidin A. The current tracing shows the existence of a new channel type (mostly visible as brief current spikes) that has a single-channel current less than that of hexafluorovaline gramicidin A channels, the hybrid channel. The break in the record denotes a different membrane to demonstrate the rare appearance of a hybrid peak that lasts long enough to be well resolved by the recorder. The new peak that appears to the left of the hexafluorovaline peak in the histogram represents hybrid channels. The average conductances for the three peaks were, from left to right: 14.32 ± 0.45 pS (96); 21.90 ± 0.45 pS (136); and 46.61 ± 0.71 pS (86). The numbers in parentheses denote the number of channels in each population. There was a total of 391 channel transitions in the histogram; we can account for 81% of the transitions. Channels lasting longer than 11 ms were used in the analysis. The calibration bars for the current tracings denote 5 pA (vertically) and 2 s (horizontally). The records were obtained at 140 Hz cutoff. 1.0 M CsCl, 100 mV.

gramicidin A molecule, and the available data suggest strongly that the channel structure is invariant with respect to the sequence changes we have imposed. This conclusion is primarily based upon the observations of hybrid channels formed between two chemically dissimilar monomers (Figs. 6 and 9). The existence of hybrid channels shows that two different monomers, and especially their folding at their adjoining ends, can adapt to each other to form the hydrogen-bonded dimer. The mere observation of hybrid channels does not, however, tell us whether the conformation(s) of the monomers in the hybrid channels correspond(s) to their conformation(s) in the pure channels, or whether channels formed by the modified gramicidins conform to the head-to-head $\beta^{6.3}$-helical dimeric theme. That the latter is the case can be deduced from the data on the average lifetimes of pure and hybrid channels (Table I, Figs. 8 and 10 and the adjacent text), and the hybrid channel frequencies (Table V).

The similarity of the lifetimes among the pure channels indicates that the dimeric channel structures are stabilized by the same number of hydrogen bonds as the $\beta^{6.3}$-helical

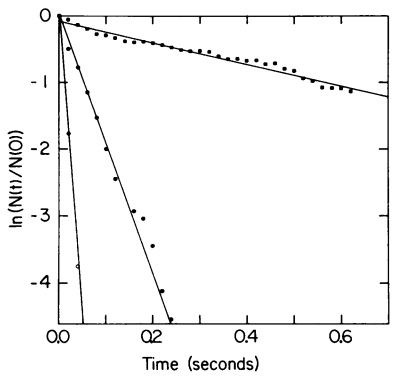


FIGURE 10 Normalized survivor plots of single-channel durations observed with a mixture of valine and hexafluorovaline gramicidin A. A total of 375 channel events were collected on stripchart records, and duration histograms were assembled from these. The different channels types were identified on the basis of their amplitudes. Multiple channel opening events were included in the histogram, as described in the legend to Fig. 8. The straight lines denote the logarithmic transform of the best fit of a single exponential decay, $N(t)/N(0) = \exp(-t/\tau)$, to the duration histograms for each of the three channel types. ■: Data for 59 valine gramicidin A channels, $\tau$ = 0.629 s; ●: Data for 187 hexafluorovaline gramicidin A channels, $\tau$ = 0.51 s; ○: Data for 129 hybrid channels, $\tau$ = 0.011 s. 1.0 M CsCl, 200 mV.

TABLE V
FREQUENCY OF OCCURRENCE OF HYBRID CHANNELS

| | Trifluorovaline | S-methyl-cysteine | Methionine |
|---|---|---|---|
| Valine | 1.19 ± 0.11 | 1.30 ± 0.30 | 1.11 ± 0.04 |
| Norvaline | 3.00 ± 0.31 | 1.09 ± 0.15 | 1.20 ± 0.23 |
| Norleucine | 1.36 ± 0.14 | 1.08 ± 0.15 | 1.08 ± 0.10 |

Mean ± SEM of the ratio of observed/predicted hybrid channel frequencies. The predicted frequency was calculated from the observed frequencies of the pure channels using Eq. 3.
1.0 M NaCl, 200 mV.

dimers.[2] The channels formed by the modified gramicidins are not, for example, intertwined helices (Veatch et al., 1974). Moreover, since most of the hybrid channels are about as stable as the less stable of the respective pure channels, there cannot be significant differences between the folding of the abutting ends of the monomers in the hybrid channels and in the pure channels, with the possible exception of channels formed between hexafluorovaline and valine gramicidin A.

The observation that the frequency of hybrid channels conforms to the predictions of Eq. 3 is, however, our strongest evidence that the structure of the modified of the modified gramicidin channels is unaffected by the side chain modifications. The folding of the abutting ends of the different monomers must be very similar as they join to form the dimers for the rate of channel formation to correspond to the prediction of Eq. 3. These results indicate also that all the compounds dimerize at the same end, i.e., at their formyl-$NH_2$-termini.

The split at 200 mV in the hybrid peak in amplitude histograms (Figure 6) indicates that the hybrid channels must have some asymmetry. But the split is quite small compared to that found in channels that have been modified towards their COOH-terminal regions. Veatch and Stryer (1977) thus found that the hybrid peak in amplitude histograms with (valine) *p*-phenylazobenzene-sulfonyl gramicidin *C* and valine gramicidin A exhibited a split hybrid peak at 100 mV, while Apell et al. (1977) found a 20–25% split of the hybrid peak in amplitude histograms with (valine) gramicidin A and (valine) O-pyromellityl gramicidin A at 50 mV. The magnitude of the split in the hybrid peaks reflects channel asymmetry towards the COOH-terminal regions, and the small split seen in our experiments may just reflect the asymmetric positions of the polar side chains in the hybrid channels. This asymmetry does not affect our major conclusion that alterations in the amino acid side chains, which are at the channel exterior and not in direct contact with the permeating ion, can modify the permeability characteristics of gramicidin channels without producing significant changes in the channel structure.

The lack of significant structural effects may in part be a consequence of our choice of amino acid substitions. We chose to compare pairs of compounds where the $NH_2$ terminal amino acid side chains occupy similar positions and volumes in space, to minimize the possibility of perturbing the polypeptide backbone structure of the channel. Fluorine is generally regarded as a good structural analogue of hydrogen in organic compounds (Goldman, 1969). It has a van der Waal's radius ~1.35 Å (Pauling, 1960) which should be compared with the effective van der Waal's radius of hydrogen, which varies from 1.06–1.34 Å and is about 1.29 Å for the hydrogens in a methyl group (Mack, 1932; Pauling, 1960). The $CF_3-$ group in trifluorovaline is therefore only a little larger than the corresponding $CH_3-$ group in valine. Methionine and norleucine have been compared directly by x-ray crystallography and found to be quite similar (Torii and Iitaka, 1973). The side chain of norleucine is fully extended and identical to one of two crystallographically independent methionine conformations. The methionine side chain is slightly longer (C—S distances ~1.8 Å vs. ~1.5 Å for C—C), and has somewhat more bent angles (C—S—C is ~100°, while C—C—C is ~110°). The sulphur-containing side chain is thus very similar to the hydrocarbon side chain. Corresponding information is not available for S-methylcysteine and norvaline, but the van der Waal's radius of sulphur is comparable to that of a methylene group, ~2.0 Å, which in conjunction with the similar bond distances and angles argues that these side chains also should be very similar. Perfect isosteric replacement is probably not attainable (except for H/D substitutions), and the pairs we have chosen are probably as isosteric as can be achieved.

The modulations of the single-channel conductances that we observe within each of the three sets of isosteric amino acid substitutions, as well as with phenylalanine-to-tyrosine substitions at position 1 (Mazet et al., 1984), can thus to a first approximation be regarded as consequences of the changes in polarity of the amino acid chains. Recent calculations on the energy profile for ion movement through gramicidin A channels (Etchebest and Pullman, 1985; see also Mazet et al., 1984), show also that the amino acid side chains could be important modulators of the rate of ion movement through gramicidin A channels.

## Ion Permeation

The decreases in the maximal single-channel conductance and the changes in the $Na^+$ activity for half-maximal conductance, are most simply interpreted in terms of a three-barrier-two-site model (Hladky, 1974; Levitt, 1978*b*; Urban and Hladky, 1979) using the single-ion-occupancy approximation, and with the incorporation of aqueous diffusion-limitations (Andersen, 1983*c*). It is assumed that ion movement through the channel itself can be divided into three discrete steps: Ion association with the channel; translocation through the channel interior; and dissociation from the channel

$$I' + G \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} IG \underset{l''}{\overset{l'}{\rightleftharpoons}} GI \underset{k''_1}{\overset{k'_{-1}}{\rightleftharpoons}} G + I'',$$

Scheme I

where $I'$ and $I''$ represent ions at the channel entrances in the left and right aqueous phases, $G$, $IG$, and $GI$ denote a channel without ions, or with an ion in the left or right ion binding site (free energy minimum), and $k_1$, $l$, and $k_{-1}$ denote the rate constants for association, translocation, and dissociation, respectively. The rate constants will be func-

[2]The Gibbs free energy for formation of a )N—H – O=C( hydrogen-bond is about −13 kJ/mol (Schulz and Schirmer, 1979, Table 3-1). The making or breaking of a single hydrogen-bond could thus change the channel stability more than 100-fold.

tions of the applied membrane potential. They will not be the same for transitions from left to right as for transitions from right to left. This bias is indicated by the superscripts, ′ and ″.

The maximal conductance, $g_{max}$, is

$$g_{max} = (e^2/kT)\,(k_{-1}\,l)/[2(2\,l + k_{-1})] \quad (4)$$

and the dissociation constant between channel and ion, $K$, is

$$K = k_{-1}/(2\,k_1), \quad (5)$$

while the activity for half-maximal conductance is given by Eq. 2. Changes in $g_{max}$ and $K$ (or $K_g$) result from changes in one or more of the rate constants in the above scheme. It is usually assumed that changes in the rate constants reflect changes in the height of the energy barriers or in the depth of the energy wells as described by Eyring's Transition State theory (Läuger, 1973). We will adhere to this convention.

We first note that $g_{max}$ decreases as we go from a nonpolar to an isosteric polar side chain at position 1, and that a similar trend is observed for $K_g$, while $K$ is comparatively constant among the different channels, except for hexafluorovaline gramicidin A. The constancy of $K$ implies that the depth of the energy well that forms the ion binding sites is insensitive to the changes we have made in the gramicidin molecule. This invariance is not surprising since the ion binding sites are close to the channel entrances (Andersen et al., 1981; Urry et al., 1982). The differences between $K$ and $K_g$ serve additionally to emphasize the need to incorporate the effects of access limitations into the analysis (see also Andersen, 1983*c*).

A decrease in $g_{max}$ reflects a decrease in either $l$, $k_{-1}$, or both. Attempts to distinguish among these possibilities and to quantitate changes in $l$ and $k_{-1}$ are model-dependent. Generally, however, a decrease in $k_{-1}$ would occur if the height of the energy barrier an ion must traverse when dissociating from the channel were increased, most likely because of an increased depth of the energy well forming the ion binding site, a decrease in $K$. The invariance of $K$ and the rather small splits in the hybrid peaks in amplitude histograms indicates thus that a polar group outside the channel proper, but close to the permeation pathway in the middle of the membrane, has little effect on the rate constants for ion entry/exit.[3] The increased voltage-dependence of the single-channel current-voltage characteristics for channels formed by the polar analogues (Fig. 5) argues further that the height of the central barrier is increased relative to the height of the barrier for ion exit. The decrease in $g_{max}$ is most likely the predominant result of a decrease in $l$ resulting from an increase in the height of the energy barrier for translocation through the channel interior due to unfavorable interactions between the polar side chains and an ion close to the center of the channel. These interactions could be either inductive electron withdrawal from the carbonyl oxygen or unfavorable ion-dipole interactions between the ion and the side chains. The dipoles along the $\alpha$-$\beta$ bonds of the side chain have their positive ends pointing toward the channel lumen. Either possibility, or a combination, would be consistent with our observations.[4] (Our data cannot distinguish between these possibilities. But other studies have shown that ion-dipole interactions can be important modulators of the channels' permeability characteristics [Koeppe et al., 1984].)

The results with $Cs^+$ as the permeating ion show qualitatively the same features as the $Na^+$ data (vide supra), although the relative conductance changes are much smaller. It should, in particular, be noted that the currents in 0.1 M CsCl at 450–500 mV are comparable for all compounds tested, which indicates that the kinetics of the ion entry step (and the structure of the channel entrances) should be comparable. At least two different effects may be responsible for the smaller decreases in the $Cs^+$ small-signal conductances. First, the intrinsic permeability of gramicidin A channels relative to the access permeability could be higher for $Cs^+$ than for $Na^+$. This is the case, as indicated by the relative changes in small-signal conductances and high-potential currents at low permeant ion concentrations. A given decrease in the intrinsic channel permeability will therefore result in a larger conductance change when $Na^+$ is the permeating ion than when $Cs^+$ is. Second, both the inductive electron withdrawal and the unfavorable ion-dipole interactions that were proposed to account for the decrease in $l$ for $Na^+$ would act to decrease the magnitude of the electrostatic field at the carbonyl oxygen at position 1. This decrease in field strength will have larger effect on the interactions with $Na^+$ than with $Cs^+$ (e.g., Eisenman, 1962; Eisenman and Horn, 1983). One would thus expect that $l$ should be affected less for $Cs^+$ than for $Na^+$.

The diminished small-signal $Cs^+$ conductances seen with trifluorovaline compared to hexafluorovaline gramicidin A channels are not readily interpretable because it is necessary to consider the consequences of multiple ion occupancy and possible changes in $Cs^+$ affinity. The results are nevertheless remarkable as they break the pattern observed with the other compounds, particularly in light of the crossover of the currents in 0.1 M CsCl (Fig. 4)

[3]The presence of a polar group outside the channel proper, but fairly close to the permeation pathway in the middle of the membrane, could mimic an increased dielectric constant of the channel surroundings. This should decrease the electrostatic repulsion of ions out of the channel, as may be inferred from the calculations of Levitt (1978*a*) and Jordan (1982). Experiments with channels in membranes formed by diphytanoylphosphatidylcholine/*n*-chlorodecane, which have a specific capacitance ~0.73 $\mu$F/cm$^2$ and a more polar membrane interior than diphytanoylphosphatidylcholine/*n*-decane membranes (Dilger et al., 1979), show, however, that an increase in dielectric constant is associated with an increase in single-channel conductances (O. S. Andersen et al., manuscript in preparation).

[4]Both the ion-dipole interactions and the inductive electron withdrawal will also lead to a decrease in $pK_a$ for the free amino acids (Edsall and Wyman, 1958).

and the smaller voltage dependence of the current-voltage characteristics in hexafluorovaline compared with trifluorovaline gramicidin A channels (Fig. 8). This latter finding indicates that the ratio $l/k_{-1}$ is less in hexafluorovaline gramicidin A, which most likely is a reflection of the higher $Na^+$ affinity (and the presumably smaller value for $k_{-1}$).

## Channel-forming Potencies and Single-channel Lifetimes

It is energetically unfavorable to transfer dipolar groups into low dielectric media (Bell, 1931), although factors other than the dipole moment may be significant for determining the partition energy. The variations in channel-forming potencies and average single-channel lifetimes are qualitatively consistent with the predictions of such a simple electrostatic argument. We expected, however, that the average lifetime for channels formed by S-methyl-cysteine gramicidin A should have been decreased relative to their norvaline gramicidin A counterparts. Other factors, such as dipole-dipole interactions between the side chains in the monomers or the electrostatic consequences of the neighboring polar permeation pathway, could also be important.

Our results do not address the importance of the bulk of the side chains for channel behavior, although the higher channel-forming potency, longer lifetimes, and reduced conductances seen with valine (and isoleucine, data not shown) at position 1 as compared to norvaline or norleucine could reflect steric effects. The peptide backbone should be more rigid when amino acid 1 is valine (or isoleucine), than when it is a linear amino acid because of the branch at the $\beta$-carbon in valine (Schulz and Schirmer, 1979, p. 11). This effect may be important because the side chains of glycine and alanine, at positions 2 and 3, have little bulk. The Val-Gly-Ala sequence is close to the junction where the formyl groups abut, any rigidity conferred to the backbone by the valine could thus be important for channel behavior.

## Present Problems and Their Possible Implications for Structure-function Studies

The relative changes in $g_{max}$ (and $K$) are qualitatively consistent with those expected from the relative changes in the side chain polarities, although the data show unexpected, or at least unexplained, features for some channels: those formed by hexafluorovaline or methionine gramicidin A.

Given the apparently complete structural equivalence of valine and trifluorovaline gramicidin A channels as judged from hybrid channel frequencies, conductances, and average lifetimes, it is not clear why this equivalence does not extend fully to hexafluorovaline gramicidin A channels. These channels satisfy the criteria for head-to-head $\beta^{6.3}$-helical dimers. But we do not understand why the conductance and average single-channel lifetimes for hybrid channels formed between hexafluorovaline and valine gramicidin A are so much less than for hexafluorovaline gramicidin A channels. The increased $Na^+$ affinity of hexafluorovaline gramicidin A channels indicates furthermore that replacement of the remaining methyl group at position 1 by a trifluorocarbon group increases the depth of the energy wells that form the ion binding sites, while the smaller voltage-dependence seen with hexafluorovaline compared to trifluorovaline gramicidin A channels (Fig. 5) indicates that this replacement decreases the rate constant for ion exit more than the rate constant for ion translocation through the channel interior. We can in some cases produce fairly global changes in the channels, but we do not understand the underlying mechanism(s). Resolution of these questions will likely depend on much more detailed information about the channel structure, and side chain and backbone dynamics than is presently available.

It is likewise not clear why the relative changes in $K$ and $g_{max}$ are comparable for norvaline vs. S-methyl-cysteine and norleucine vs. methionine. The more distant location of the polar group in the side chain in methionine compared to S-methyl-cysteine, the smaller estimated dipole moment along the $\alpha$-$\beta$ bond (and the difference in $pK_a$ for the free amino acids) all suggest that the relative conductance changes should be less for the norleucine/methionine pair. This was not observed, possibly because of side chain flexibility and the different positions of the polar group with respect to the channel lumen. The long, flexible methionine side chain may thus reorient in the presence of a transmembrane potential difference, to change the component of the dipole pointing towards the channel lumen. Changes in the energy profile along the channel interior may thus occur at different positions in S-methyl-cysteine and methionine gramicidin A channels, which should be reflected as different splits of the hybrid peaks in amplitude histograms. The difference was, however, very small—and in the opposite direction to that expected: 4% for S-methyl-cysteine/valine vs. 3% for methionine/valine gramicidin A hybrid channels (200 mV, 1.0 M NaCl). Again the question cannot be resolved in the absence of more structural information.

The above examples illustrate the importance of having as much structural information as possible before interpreting chemical modification (or site-directed mutagenesis) experiments. The importance of having the structural information is further underlined by the fact that one, in the absence of information about the structure of the gramicidin A channel, could have used the present data to build a convincing case for a channel structure where the $NH_2$-terminal amino acid side chain is an important element in the wall of the permeation pathway. This conclusion is clearly inappropriate for the present situation (see also Mazet et al., 1984).[5] But the correct interpretation may not be as straightforward with other proteins.

[5]Note, however, that the side chains contribute to the characteristics of the selectivity filter of the channels even though they do not form part of the wall of the permeation path proper.

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## REFERENCES

Andersen, O. S. 1983*a*. Ion movement through gramicidin A channels. Single-channel measurements at very high potentials. *Biophys. J.* 41:119–133.

Andersen, O. S. 1983*b*. Ion movement through gramicidin A channels. Interfacial polarization effects on single-channel current measurements. *Biophys. J.* 41:135–146.

Andersen, O. S. 1983*c*. Ion movement through gramicidin A channels. Studies on the diffusion-controlled association step. *Biophys. J.* 41:147–165.

Andersen, O. S. 1984. Gramicidin channels. *Annu. Rev. Physiol.* 46:531–548.

Andersen, O. S., E. W. Barrett, and L. B. Weiss. 1981. On the position of the alkali metal cation binding sites in gramicidin channels. *Biophys. J.* 33(2, Pt. 2):63*a* (Abstr.)

Andersen, O. S., R. E. Koeppe II, J.-L. Mazet, E. W. B. Russell, and L. B. Weiss. 1984. Single-channel studies on linear gramicidins with altered amino acid sequences. *Hoppe-Seyler's Z. Physiol. Chem.* 365:225. (Abstr.)

Apell, H. -J., E. Bamberg, H. Alpes, and P. Läuger. 1977. Formation of ion channels by a negatively charged analog of gramicidin A. *J. Membr. Biol.* 31:171–188.

Bamberg, E., and K. Janko. 1977. The action of a carbonsuboxide dimerized gramicidin A on lipid bilayer membranes. *Biochim. Biophys. Acta.* 465:486–499.

Bamberg, E., K. Noda, E. Gross, and P. Läuger. 1976. Single-channel parameters of gramicidin A, B and C. *Biochim. Biophys. Acta.* 419:223–228.

Bamberg, E., H. -J. Apell, and H. Alpes. 1977. Structure of the gramicidin A channel: discrimination between the $\pi_{L,D}$ and the $\beta$-helix by electrical measurements with lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 74:2402–2406.

Bamberg, E., H. -J. Apell, H. Alpes, E. Gross, J. L. Morell, J. E. Harbaugh, K. Janko, and P. Läuger. 1978. Ion channels formed by chemical analogues of gramicidin A. *Fed. Proc.* 37:2633–2638.

Barrett, E. W., L. B. Weiss, O. S. Andersen, and L. Stryer. 1981. Single-channel studies on gramicidin A analogs. *Biophys. J.* 33(2, Pt. 2):63*a* (Abstr.)

Bell, R. P. 1931. The electrostatic energy of dipole molecules in different media. *Trans. Faraday Soc.* 27:797–802.

Bevington, P. R. 1969. Data reduction and error analysis for the physical sciences. McGraw-Hill Inc., NY.

Boni, L. T., A. J. Connolly, and A. M. Kleinfeld. 1986. Transmembrane distribution of gramicidin by tryptophan energy transfer. *Biophys. J.* 49:122–123.

Colquhoun, D., and F. J. Sigworth. 1983. Statistical analysis of records. *In* Single-channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., NY. 191–263.

Cox, D. R., and P. A. W. Lewis. 1966. The statistical analysis of series of events. Methuen, London. 1–16.

Dilger, J. P., S. G. A. McLaughlin, T. J. McIntosh, and S. A. Simon. 1979. The dielectric constant of phospholipid bilayers and the permeability of membranes to ions. *Science* (*Wash. DC*). 206:1196–1198.

Edsall, J. T., and J. Wyman. 1958. Biophysical Chemistry. Vol. I. Academic Press Inc., NY. 457–463.

Eisenman, G. 1962. Cation selective glass electrodes and their mode of operation. *Biophys. J.* 2(2, Pt. 2):259–323.

Eisenman, G., and Horn, R. 1983. Ionic selectivity revisited: the role of kinetic and equilibrium processes in ion permeation through channels. *J. Membr. Biol.* 76:197–225.

Eisenman, G., and J. P. Sandblom. 1983. Energy barriers in ionic channels: Data for gramicidin A interpreted using a single-file (3B4S″) model having 3 barriers separating 4 sites. *In* Physical Chemistry of Transmembrane Ion Motions. G. Spach, editor. Elsevier, Amsterdam. 329–347.

Etchebest, C., and A. Pullman. 1985. The effect of the amino-acid chains on the energy profiles for ion transport in the gramicidin A channel. *J. Biomol. Struct. Mech.* 2:859–870.

Finkelstein, A., and O. S. Andersen. 1981. The gramicidin A channel: a review of its permeability characteristics with special reference to the single-file aspect of transport. *J. Membr. Biol.* 59:155–171.

Heitz, F., F. Spach, and Y. Trudelle. 1982. Single channels of 9, 11, 13, 15-destryptophyl-phenylalanyl-gramicidin A. *Biophys. J.* 40:87–89.

Goldman, P. 1969. The carbon-fluorine bond in compounds of biological interest. *Science* (Wash. DC). 164:1123–1130.

Hinton, J. F., and R. E. Koeppe II. 1985. The complexing properties of gramicidins. *In* Metal Ions in Biological Systems. 19: H. Sigel, editor. Marcel Dekker, NY. 173–206.

Hladky, S. B. 1974. Pore or carrier? Gramicidin A as a simple pore. *In* Drugs and Transport Processes. B. A. Callingham, editor. MacMillan and Company, London. 193–210.

Hladky, S. B., B. W. Urban, and D. A. Haydon. 1979. Ion movements in the gramicidin pore. *In* Membrane Transport Processes. Vol 3. C. F. Stevens and R. W. Tsien, editors. Raven Press, New York. 89–103.

Jordan, P. C. 1982. Electrostatic modelling of ion pores. Energy barriers and electric field profiles. *Biophys. J.* 39:157–164.

Koeppe, R. E. II, O. S. Andersen, and J. -L. Mazet. 1984. Distinction between dipolar and inductive effects of substituted phenylalanine side chains in modulating the single-channel conductances of gramicidin analogues. *8th Int. Biophys. Congr.* 273 (Abstract).

Läuger, P. 1973. Ion transport through pores: A rate-theory analysis. *Biochim. Biophys. Acta.* 311:423–441.

Levitt, D. G. 1978*a*. Electrostatic calculations for an ion channel. I. Energy and potential profiles and interactions between ions. *Biophys. J.* 22:209–219.

Levitt, D. G. 1978*b*. Electrostatic calculations for an ion channel. II. Kinetic behavior of the gramicidin A channel. *Biophys. J.* 22:221–248.

Mack, E. 1932. The spacing of non-polar molecules in crystal lattices. The atomic domain of hydrogen. A new feature of the structure of the benzene ring. *J. Am. Chem. Soc.* 54:2141–2165.

Martell, A. E., and R. M. Smith. 1974. Critical Stability Constants. Vol. 1. Plenum Publishing Corp., NY

Martell, A. E., and R. M. Smith. 1982. Critical Stability Constants. Vol. 5. Plenum Publishing Corp., NY

Mazet, J. -L., O. S. Andersen, and R. E. Koeppe II. 1984. Single-channel studies on linear gramicidins with altered amino acid sequences. A comparison of phenylalanine, tryptophane, and tyrosine substitutions at positions 1 and 11. *Biophys. J.* 45:263–276.

Mehlenbacher, V. C. 1953. Determination of hydroxyl groups. *In* Organic Analysis. 1. J. Mitchell, Jr., I. M. Kolthoff, E. S. Proskauer, and A. Weissberger, editors. Interscience, New York. 1–65.

Morrow, J. S., W. R. Veatch, and L. Stryer. 1979. Transmembrane channel activity of gramicidin A analogs: effects of modification and deletion of the amino-terminal residue. *J. Mol. Biol.* 132:733–738.

Pauling, L. 1960. The Nature of the Chemical Bond. 3rd ed. Cornell University Press, Ithaca, NY. 260–262.

Procopio, J., and O. S. Andersen. 1979. Ion tracer fluxes through gramicidin A modified lipid bilayers. *Biophys. J.* 25(2, Pt. 2):8*a* (Abstr.)

Sarges, R., and B. Witkop. 1965. Gramicidin A. V. The structure of valine- and isoleucine-gramicidin A. *J. Am. Chem. Soc.* 87:2011–2020.

Schagina, L. V., A. E. Grinfeldt, and A. A. Lev. 1983. Concentration dependence of bidirectional flux ratio as a characteristic of transmembrane ion transporting mechanism. *J. Membr. Biol.* 73:203–216.

Schulz, G. E., and R. H. Schirmer. 1979. Principles of Protein Structure. Springer-Verlag, NY. 314 pp.

Sheehan, J. C., and D. -D. H. Yang. 1958. The use of N-formyl amino acids in peptide synthesis. *J. Am. Chem. Soc.* 80:1154–1158.

Shiori, T., K. Ninomiya, and S. Yamada. 1972. Diphenylphosphoryl azide. A new convenient reagent for a modified Curtius reaction and for the peptide synthesis. *J. Am. Chem. Soc.* 94:6203–6205.

Smyth, C. P. 1955. Dielectric Behavior and Structure. McGraw-Hill Inc., NY. 441 pp.

Szabo, G., G. Eisenman, and S. Ciani. 1969. The effects of the macrotetralide actin antibiotics on the electrical properties of phospholipid bilayer membranes. *J. Membr. Biol.* 1:376–382.

Szabo, G., and D. W. Urry. 1979. N-acetyl gramicidin: single-channel properties and implications for channel structure. *Science* (*Wash. DC*). 203:55–57.

Torii, K., and Y. Iitaka. 1973. Crystal structures and molecular conformations of L-methionine and L-norleucine. *Acta Cryst.* B29:2799–2807.

Urban, B. W., and S. B. Hladky. 1979. Ion transport in the simplest single-file pore. *Biochim. Biophys. Acta.* 554:410–429.

Urban, B. W., S. B. Hladky, and D. A. Haydon. 1980. Ion movements in gramicidin pores. An example of single-file transport. *Biochim. Biophys. Acta.* 602:331–354.

Urry, D. W. 1971. The gramicidin A transmembrane channel: a proposed $\pi_{(L,D)}$ helix. *Proc. Natl. Acad. Sci. USA.* 68:672–676.

Urry, D. W. 1972. Protein conformation in biomembranes: optical rotation and absorption of membrane suspensions. *Biochim. Biophys. Acta.* 265:115–168.

Urry, D. W., M. C. Goodall, J. D. Glickson, and D. F. Mayers. 1971. The gramicidin A transmembrane channel: characteristics of head-to-head dimerized $\pi_{(L,D)}$ helices. *Proc. Natl. Acad. Sci. USA.* 68:1907–1911.

Urry, D. W., T. L. Trapane, and K. U. Prasad. 1983. Is the gramicidin A transmembrane channel single-stranded or double-stranded helix? A simple unequivocal determination. *Science* (*Wash. DC*). 221:1064–1067.

Urry, D. W., J. T. Walker, and T. L. Trapane. 1982. Ion interactions in (1-$^{13}$C) D-Val$^{8}$ and D-Leu$^{14}$ analogues of gramicidin A, the helix sense of the channel and location of ion binding sites. *J. Membr. Biol.* 69:225–231.

Veatch, W. R., E. T. Fossel, and E. R. Blout. 1974. The conformation of gramicidin A. *Biochemistry.* 13:5249–5256.

Veatch, W., and L. Stryer. 1977. The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. *J. Mol. Biol.* 113:89–102.

Walborsky, H. M., and J. H. Lang. 1956. Effects of the trifluoromethyl group. IV. The pK's of $\omega$-trifluoromethyl amino acids. *J. Am. Chem. Soc.* 78:4314–4316.

Weinstein, S., B. A. Wallace, E. R. Blout, J. S. Morrow, and W. Veatch. 1979. Conformation of gramicidin A channel in phospholipid vesicles: A$^{13}$C and $^{19}$F nuclear magnetic resonance study. *Proc. Natl. Acad. Sci. USA.* 76:4230–4234.

Weinstein, S., B. A. Wallace, J. S. Morrow, and W. R. Veatch. 1980. Conformation of the gramicidin A transmembrane channel: A $^{13}$C nuclear magnetic resonance study of $^{13}$C-enriched gramicidin in phosphatidylcholine vesicles. *J. Mol. Biol.* 143:1–19.

Weiss, L. B., and R. E. Koeppe II. 1985. Semisynthesis of linear gramicidins using diphenyl phosphorazidate (DPPA). *Int. J. Pept. Prot. Res.* 26:305–310.

Yamada, S., N. Ikota, T. Shiori, and S. Tachibana. 1975. Diphenyl phosphorazidate (DPPA) and diethyl phosphorocyanidate (DEPC). Two new reagents for solid phase peptide synthesis and their application to the synthesis of porcine motilin. *J. Am. Chem. Soc.* 97:7174–7175.