SINGLE-CHANNEL STUDIES ON LINEAR GRAMICIDINS WITH ALTERED AMINO ACID SEQUENCES

A Comparison of Phenylalanine, Tryptophane, and Tyrosine Substitutions at Positions 1 and 11

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ABSTRACT The relation between chemical structure and permeability characteristics of transmembrane channels has been investigated with the linear gramicidins (A, B, and C), where the amino acid at position 1 was chemically replaced by phenylalanine, tryptophane or tyrosine. The purity of most of the compounds was estimated to be >99.99%. The modifications resulted in a wide range of conductance changes in NaCl solutions: sixfold from tryptophane gramicidin A to tyrosine gramicidin B. The conductance changes induced by a given amino acid substitution at position 1 are not the same as at position 11. The only important change in the Na^+ affinity was observed when the first amino acid was tyrosine. No major conformational changes of the polypeptide backbone structure could be detected on the basis of experiments with mixtures of different analogues and valine gramicidin A (except possibly with tyrosine at position 1), as all the compounds investigated could form hybrid channels with valine gramicidin A. The side chains are not in direct contact with the permeating ions. The results were therefore interpreted in terms of modifications of the energy profile for ion movement through the channel, possibly due to an electrostatic interaction between the dipoles of the side chains and ions in the channel.

INTRODUCTION

The linear gramicidins are small polypeptides able to form channels that span lipid bilayer membranes. Gramicidin channels are ideally selective for small monovalent cations (Myers and Haydon, 1972; Urban et al., 1980). They exhibit selectivity among these cations (Myers and Haydon, 1972; Eisenman et al., 1976; Urban et al., 1980), as well as single-file flux-coupling effects (Levitt et al., 1978; Rosenberg and Finkelstein, 1978 *a*, *b*; Schagina et al., 1978; Procopio and Andersen, 1979) and mole-fraction–dependent permeability phenomena (Andersen, 1975, 1978; Neher, 1975; Eisenman et al., 1977; McBride and Szabo, 1978). In addition, the gramicidins can be chemically modified by semisynthetic procedures, allowing the substitution of a great variety of amino acids at position 1 (Morrow et al., 1979; Barrett et al., 1981).[1] These factors make studies on gramicidin channels relevant for understanding the molecular basis for the function of channels in biological membranes.

The primary structure of naturally occurring gramicidis is (Sarges and Witkop, 1965 *a*, *b*, *c*):

Formyl-L—XXX—Gly—L—Ala—D—Leu—L—Ala—
1 2 3 4 5

D—Val—L—Val—D—Val—L—Trp—D—Leu—L—YYY—
6 7 8 9 10 11

D—Leu—L—Trp—D—Leu—L—Trp—Ethanolamine,
12 13 14 15

where XXX can be either valine or isoleucine, while YYY is tryptophane, phenylalanine or tyrosine in gramicidin A, B9, and C, respectively. The alternating *L*- and *D*-sequence permit the molecules to form β-helical structures in which the polar peptide groups line a central cavity, while the nonpolar side chains project from the exterior surface (Urry, 1971; Veatch et al., 1974). This arrangement permits the molecules to be incorporated into lipid bilayer membranes and form water-permeable cation-

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selective channels (Hladky and Haydon, 1970; Krasne et al., 1971; Finkelstein, 1974).

The generally accepted structure for the channel consists of two left-handed β-helical monomers that hydrogen-bond at their formylated NH_2-terminal regions to form an antiparallel dimer (Urry, 1971). The site of dimerization is thus located at the center of the symmetrical channel. Very strong support for these features of the channel has been obtained through single-channel and spectroscopic investigations of appropriately modified gramicidins (Urry et al., 1971; Veatch et al., 1975; Bamberg and Janko, 1977; Bamberg et al., 1977; Veatch and Stryer, 1977; Szabo and Urry, 1979; Weinstein et al., 1979, 1980; Urry et al., 1982 *b*). Permeability considerations show that the channel must have a diameter of ~4 Å (Finkelstein, 1974), which argues that the channel is formed by β^6-helices with 6.3 residues per turn (e.g., Urry, 1971; Koeppe and Kimura, 1983). Fig. 1 shows the polypeptide backbone of this structure, and the intramolecular and intermolecular hydrogen bonds that stabilize it.

The dimensions of the β^6-helical dimer (length 26 Å, luminal diameter 4 Å) are consistent with those determined from x-ray diffraction patterns of gramicidin A crystallized in the presence of KSCN or CsSCN (Koeppe et al., 1978, 1979). There are two crystallographic cation-binding sites per gramicidin A dimer (Koeppe et al., 1979). X-ray, NMR and single-channel investigations are all consistent with ion binding sites located 2.5 Å from each end of a 26 Å-long dimer (Koeppe et al., 1979; Andersen et al., 1981; Urry et al., 1982 *a*, *b*). There are no clearly distinctive groups that can function as ion-binding sites. Ions in the channel are located in energy minima whose positions are determined by the superposition of long-range electrostatic forces from image potentials and ion-ion interactions (Parsegian, 1969; Levitt, 1978 *a*; Jordan, 1982), and local interactions between ion and channel wall (e.g., Renugopalakrishnan and Urry, 1978; Eisenman and Horn, 1983), although other factors also must be important because cations also bind to the COOH-termini of gramicidin dimers in organic solvents (Hinton et al., 1981).

Fig. 2 shows the channel lumen viewed from the COOH-

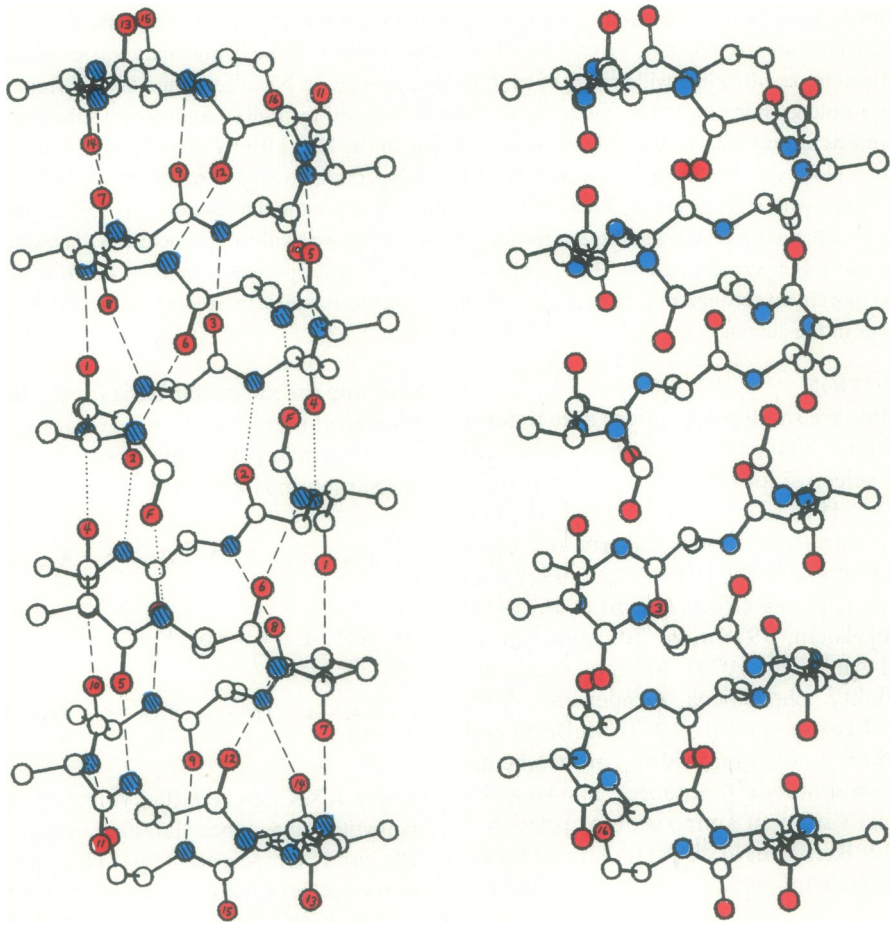

FIGURE 1 Stereoscopic Oak Ridge Thermal Ellipsoid Program (ORTEP) representation of the peptide backbone in the antiparallel β-helical gramicidin dimer. The backbone coordinates are taken from Table I of Koeppe and Kimura (1983). Intramolecular (---) and intermolecular (· · ·) hydrogen bonds are indicated. The atoms are coded as follows: oxygens are red, *F* is the formyl oxygen; other oxygens are numbered; nitrogens are blue; carbons are left unmarked. The model shown has 6.3 residues per turn and a pitch of 4.85 Å per turn.

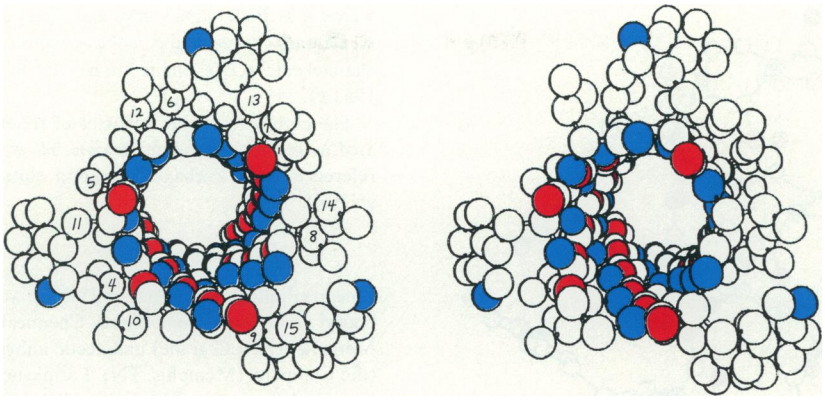

FIGURE 2 ORTEP drawing, without hydrogens, of the cylindrical channel lumen viewed from the end. This figure also illustrates the channel entrance (minus the surrounding lipid molecules). The coordinates of the backbone atoms are from Table I of Koeppe and Kimura (1983). The side chains of the upper monomer have been placed in arbitrary positions, with the restriction that there be no unfavorable steric contacts. The atomic radii are arbitrary. For convenience and clarity, all leucine side chains have been represented as valine. The atoms are coded as follows: oxygens are red; nitrogens are blue selected β-carbons of the upper monomer are numbered. Note that the carbonyl oxygens of residues 11, 13, and 15 protrude from the end of the channel. In this representation (and in Fig. 1) the terminal -OH of the ethanolamine group has been arbitrarily made to hydrogen bond to the peptide nitrogen of 11.

terminus of the β^6-helical dimer to illustrate how the very uniform lumen, which is lined by the polar groups of the peptide backbone, is not in direct contact with the amino acid side chains.

The energy profile for ion movement through gramicidin channels, and thus the ion permeability, is primarily determined by the characteristics of the central solvation path formed by the peptide groups. But the side chains exert significant secondary effects on the ion permeability even though they reside at the exterior surface of the channel (Bamberg et al., 1976; Morrow et al., 1979; Heitz et al., 1982).[1] This modulation is, at least in part, related to the polarity (dipole moment and polarizability) of the side chains.[1] An increase in polarity between two essentially isosteric side chains at position 1 is associated with a decrease in the maximal single-channel conductance and dissociation constant for Na^+. The opposite pattern has been observed with the naturally occurring gramicidins A, C, and B, where the conductance is larger with tryptophane or tyrosine at position 11 than with phenylalanine at that position (Bamberg et al., 1976). The permeability changes induced by a given amino acid substitution may thus depend on the position in the sequence where the substitution occurs. To address this question we have replaced semisynthetically the amino acid at position 1 in each of the three naturally occurring gramicidins by phenylalanine, tryptophane or tyrosine, and we have compared the single-channel conductances of the nine resulting polypeptides. Fig. 3 illustrates the peptide backbone with tryptophanes at positions 1 and 11. Some of this material has been reported in preliminary form (Andersen et al., 1983; Mazet et al., 1983).

METHODS AND MATERIALS

Chemical Modification

Gramicidin B was separated from commercial gramicidin (A, B, and C) by preparative high-performance liquid chromatography, HPLC (Koeppe and Weiss, 1981; Turner et al., 1983). The purified gramicidin B, or the mixture of gramicidins A and C, was then modified as described elsewhere.[1]

Each of the resulting semisynthetic gramicidins was purified by analytical HPLC in 80–85% methanol, 15–20% water on a 4.6 × 250 mm Zorbax-C8 column. The gramicidin B compounds were purified twice, while the gramicidin A and C compounds were separated first and, in some cases, further purified by a second passage through the column. (Some of our analogues, especially those purified by only a single run through the HPLC column, tend to be contaminated with small amounts of valine gramicidin A. This compound is probably absorbed in the effluent tubing of the column and may thus exchange with the pure peptides coming from the column. The contamination can usually be brought down to less than ~0.01% by running repeated samples of the same material through the HPLC column, pooling the material under the peaks of the absorbance maxima, concentrating and rechromatographing the pooled material.) The resulting materials show clean sharp peaks in the HPLC traces.

Single-Channel Studies

Planar lipid bilayer membranes were formed at 25 ± 1°C by the pipette technique of Szabo et al. (1969) across a hole (area ~1.6 mm^2) in a Teflon partition separating two Teflon chambers containing the appropriate electrolyte solution unbuffered (0.1 or 1.0 M NaCl, or 1.0 M CsCl). The membrane-forming solution was diphytanoylphosphatidylcholine dissolved in *n*-decane, 2–3% wt/vol. A small aliquot (5–20 μl) of the stock solution or an ethanolic dilution of the appropriate gramicidin was added to each aqueous phase (5 ml) after membrane formation.

The single-channel measurements were done using the pipette technique described by Andersen (1983 *a*). The final conductance data are generally based on >300 single-channel current transitions measured at 25 and 50 mV applied potential, and the conductances are listed as mean

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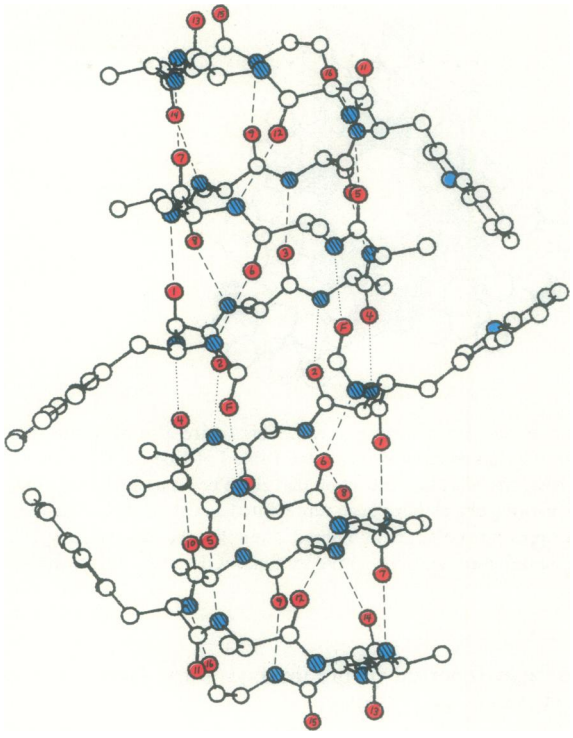

FIGURE 3 ORTEP drawing of the peptide backbone with tryptophanes drawn at positions 1 and 11. Note that the indole group at position 1 in one monomer can come quite close to the indole at position 11 in the other monomer. In this illustration the orientations of all of the indole rings with respect to the backbone atoms have been arbitrarily chosen to be identical. The figure represents one of only a small number of sterically favorable orientations of the indole rings (Kimura and Koeppe, unpublished results).

± SD of the aggregate amplitude histogram based on at least eight measurements (two at each polarity and at each potential). For some analogues, the single-channel current steps were so short-lived that they could not be assayed at these potentials in 0.1 M salt. Tyrosine gramicidin C was studied using many-channel current-voltage characteristics, as described in Andersen (1983 *a*). The conductance at 25 mV was estimated by scaling the normalized many-channel conductance-voltage characteristic to the single-channel conductances measured at 100, 150 and 200 mV. Tryptophane gramicidin B was not sufficiently free of valine gramicidin A to allow this procedure, and the conductance data are based on measurements at 100 mV.

Data Analysis

The single-channel conductances in 0.1 and 1.0 M NaCl were analyzed in terms of a single-ion–occupancy model for ion movement through the channel (Hladky, 1974; Andersen, 1983 *b*). According to this model, the concentration dependence of the small-signal single-channel conductance, g, is

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

where g_{max} is the maximal single-channel conductance, a is the aqueous activity of the permeating ion, and K_g is the activity for half-maximal conductance. The relation between K_g and the equilibrium dissociation constant, K, for the gramicidin A channel and Na^+ is, in the presence of aqueous diffusion limitations, given by (Andersen, 1983 *b*)

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

where k is 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 (estimated to be $\sim 1.1 \times 10^8$ liter/(mole · s), Andersen, 1983 *b*).

The chromatographic behavior of the different analogues was evaluated as the relative retention time, R_f, with valine gramicidin A as the reference, on a Zorbax-C8 column eluted with methanol:H_2O (81:19 vol/vol).

Materials

Linear gramicidin (gramicidin-Dubos), amino acids, and diphenylphosphoryl azide were from Sigma Chemical Company (St. Louis, MO). Methanol (HPLC grade) and acetic anhydride were from Fisher Scientific Company (Memphis, TN). Formic acid, 95–97%, was from Aldrich Chemical Company (Milwaukee, WI). Phenyl isothiocyanate pyridine, dimethyl formamide, triethylamine, and 4N HCl in dioxane were all "Sequanal" grade from Pierce Chemical Company (Rockford, IL).

The analytical HPLC octyl-silica column was "Zorbax-C8" from Dupont Corporation (Wilmington, DE). Preparative HPLC columns were packed with a 37–44 micron mesh phenyl-silica packing, "Chromosorb-LC-5", from Johns-Manville (through Alltech Associates, Deerfield, IL).

Diphytanoylphosphatidylcholine was from Avanti Polar Lipids (Birmingham, AL). It 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.

The micropipette glass was borosilicate glass from Corning Glass Works (Corning, NY). Trioctylsilane was from Petrarch Chemical Company (Bristol, PA).

NaCl was Suprapur grade from E. Merck Darmstadt (through MCB, Cincinnati, OH). Before use it was roasted for at least 24 h at 550–600°C and stored in an evacuated dessicator over NaOH. The water was deionized Millipore Corp. Milli-Q water (Bedford, MA).

RESULTS

The single-channel characteristics of gramicidin channels depend upon the positions of the amino acids in the sequence. This is illustrated in Fig. 4, where we compare channels formed by tyrosine gramicidin B and phenylalanine gramicidin C. These two compounds have the same amino acid composition, but differ in the location (position 1 and 11, respectively) of the phenolic -OH group. This alteration in sequence affects not only the single-channel characteristics, but also the chromatographic behavior of the peptides (Fig. 5).

Similar sequence specificity of the single-channel characteristics was observed with other pairs of analogues where the amino acids at position 1 and 11 were interchanged. Table I summarizes our data on the single-channel conductances in 0.1 and 1.0 NaCl for the nine analogues studied. An amino acid substitution at either position 1 or position 11 may induce substantial changes in the conductance. A particular characteristic of the native valine gramicidin A is, however, preserved in all the analogues. It is the tendency of the conductance to saturate at high electrolyte concentration, as reflected by the less than threefold increase in conductance observed with a 10-fold increase in NaCl concentration.

The conductances observed with tryptophane gramicidin A are essentially indistinguishable from those observed with valine gramicidin A. This raises the question

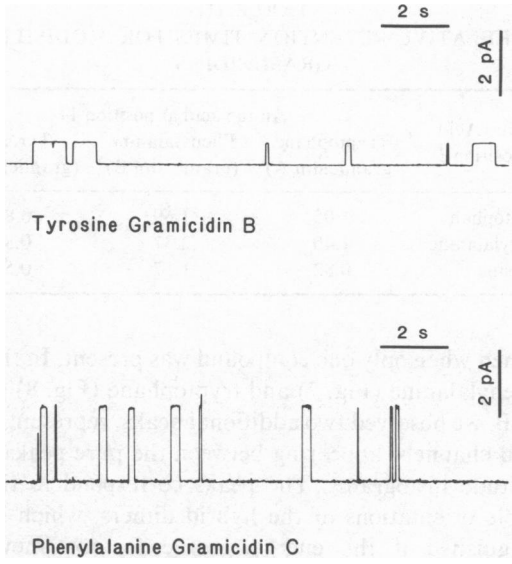

FIGURE 4 Comparison of single-channel events observed with tyrosine gramicidin B and phenylalanine gramicidin C. 1.0 M NaCl, 200 mV, 25 °C. Note that both traces were obtained at the same amplification and chart speed.

of whether these single-channel events, in fact, represent tryptophane gramicidin A channels. To examine this question we analyzed the single-channel lifetimes, Fig. 6. There are indeed several components in the lifetime histogram. We believe that the channels with the short lifetimes represent tryptophane gramicidin A channels, while the population with long lifetimes represent hybrid channels, formed by a tryptophane and a valine gramicidin A, and some valine gramicidin A channels. (The hybrid and valine gramicidin A channels could not be eliminated from the tryptophane gramicidin A activity because the channel-forming potency of this analogue is very low, ~0.003 that

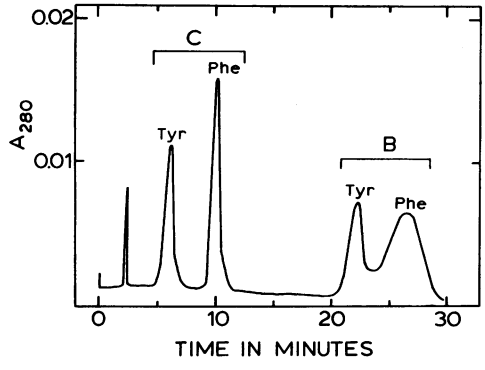

FIGURE 5 Comparisons of the HPLC chromatographic properties of tyrosine and phenylalanine gramicidins B and C. Previously purified samples were rechromatographed in separate runs on the same day and then overlaid to give the comparison indicated. The elution solvent was 83% methanol and 17% water at 1.5 ml/min and 1,800 PSI. Note that peaks which are retained longer on the column are broadened. In practice we collect the phenylalanine gramicidin B samples in 85% methanol to reduce both the retention time and the broadening.

TABLE I
SINGLE-CHANNEL CONDUCTANCES OF MODIFIED GRAMICIDINS

Amino Acid at position 1	[NaCl]	Amino acid at position 11 Tryptophane (gramicidin A)	Phenylalanine (gramicidin B)	Tyrosine (gramicidin C)
	M			
Tryptophane	0.1	5.60 ± 0.56	3.63 ± 0.39	4.86 ± 0.44
	1.0	12.6 ± 0.5	7.18 ± 0.41	10.2 ± 0.6
Phenylalanine	0.1	4.65 ± 0.77	2.42 ± 0.32	3.66 ± 0.31
	1.0	10.2 ± 1.1	4.63 ± 0.40	7.39 ± 1.00
Tyrosine	0.1	2.33 ± 0.26	1.46 ± 0.23	2.17
	1.0	3.94 ± 0.32	2.10 ± 0.19	3.41 ± 0.70

Conductances in pS, mean ± SD.

of valine gramicidin A, and because its HPLC behavior is very close to that of valine gramicidin A.) The conductances are, therefore, biased. The bias is, however, most likely smaller than the standard deviation of the mean since the conductance of the analogue and valine gramicidin A channels only differ by ~3% (see Legend to Fig. 6).

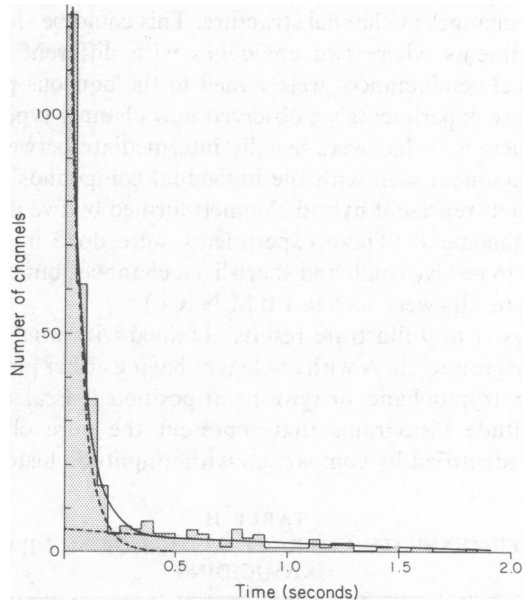

FIGURE 6 Lifetime histogram of single-channel events seen with tryptophane gramicidin A. A total of 312 channels were collected on a stripchart recorder, and the histogram was assembled from these. Three channels, with durations >2.0 s, are not illustrated in the histogram. Multiple channel openings were included in the histogram, using a simple random number generator (a die) to determine which of the several open channels closed in a closing event. The solid line represents the best fit of two exponential decay curves to the data, $N(t) = N_1 \exp(-t/\alpha_1) + N_2 \exp(-t/\alpha_2)$, where $N(t)$ is the predicted number of channels at time t, and α_1 and α_2 are the average lifetimes of the rapidly and slowly decaying events. $N_1 = 123 \pm 0.4$, $\alpha_1 = 0.058 \pm 0.004$ s, $N_2 = 5.2 \pm 0.7$, $\alpha_2 = 0.83 \pm 0.14$ s. The two interrupted lines depict the fast and slow components. The amplitudes of the single-channel currents for events lasting <100 ms were 2.952 ± 0.009 pA (mean ± SEM), 3% less than the single-channel currents for events lasting longer than 500 ms, 3.041 ± 0.011 pA. 1.0 M NaCl, 200 mV.

The conductance data in Table I were used to obtain estimates for g_{max}, K_g, and K according to Eqs. 1 and 2. These estimates are summarized in Table II. Both g_{max} and K vary as a function of the amino acid sequence, and the relative effect of a given amino acid interchange depends upon where in the sequence the interchange is made. At position 11 we find that g_{max} decreases when the comparatively polar tryptophane or tyrosine is replaced by the nonpolar phenylalanine, while K is essentially unaffected. At position 1 we observe a more complex pattern: g_{max} and K are much smaller when the first amino acid is tyrosine than when it is phenylalanine or tryptophane.

The chromatographic behavior of the polypeptides varies with changes in the amino acid sequence. Table III summarizes the relative retention times for the nine analogues. The variations in the HPLC behavior indicate that the sequence alterations affect the binding of the polypeptide to the chromatographic resin. These differences in binding could be due to the juxtaposition of particular functional groups, or alternatively to local changes in structure.

The variations in permeability characteristics summarized in Tables I and II are most likely not the result of gross changes in channel structure. This could be shown in experiments where two analogues with different single-channel conductances were added to the aqueous phases. In these experiments we observed new channel types with conductances that were usually intermediate between the conductances seen with the individual compounds. These channels represent hybrid channels formed by two dissimilar monomers. (These experiments were done in 1.0 M CsCl to resolve small and short-lived channels, but comparable results were seen in 1.0 M NaCl.)

Figs. 7 to 9 illustrate results obtained with mixtures of valine gramicidin A with analogues having either phenylalanine, tryptophane, or tyrosine at position 1. Peaks in the amplitude histograms that represent the pure channels were identified by comparison with amplitude histograms

TABLE II
PERMEABILITY CHARACTERISTICS OF MODIFIED GRAMICIDINS

Amino Acid at position 1	Amino acid at position 11		
	Tryptophane (gramicidin A)	Phenylalanine (gramicidin B)	Tyrosine (gramicidin C)
Tryptophane			
g_{max}(pS)	15	8.2	12
$K_g(m)$	0.13	0.099	0.11
$K(m)$	0.088	0.075	0.078
Phenylalanine			
g_{max}(pS)	12	5.2	8.5
$K_g(m)$	0.13	0.087	0.10
$K(m)$	0.090	0.072	0.079
Tyrosine			
g_{max}(pS)	4.3	2.2	3.7
$K_g(m)$	0.067	0.041	0.054
$K(m)$	0.055	0.035	0.043

TABLE III
RELATIVE RETENTION TIMES FOR MODIFIED GRAMICIDINS

Amino Acid at Position 1	Amino acid at position 11		
	Tryptophane (gramicidin A)	Phenylalanine (gramicidin B)	Tyrosine (gramicidin C)
Tryptophane	1.05	1.69	0.80
Phenylalanine	1.40	2.31	0.87
Tyrosine	0.82	1.57	0.59

obtained when only one compound was present. In the case of phenylalanine (Fig. 7) and tryptophane (Fig. 8) gramicidin B, we observed two additional peaks, representing the hybrid channels, appearing between the pure peaks in the amplitude histograms. The peaks correspond to the two possible orientations of the hybrid dimers, which can be distinguished if the energy profile for ion movement through the hybrid channel is asymmetrical (e.g., Jack et al., 1975). The difference in the heights of the peaks representing the hybrid channels is a reproducible feature of the histograms. The highest peak corresponds to the

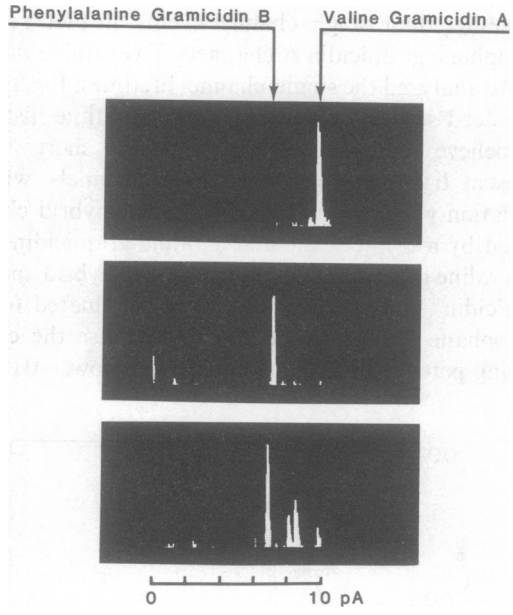

FIGURE 7 Amplitude histogram of single-channel current steps observed with a mixture of phenylalanine gramicidin A and valine gramicidin A. *Top*, histogram of pure gramicidin A. *Middle*, histogram for phenylalanine gramicidin B alone. *Bottom*, histogram for the mixture. The pure peaks were identified by comparison with histograms for the pure compounds. The two peaks appearing between the pure peaks represent hybrid channels, presumably with opposite orientations. The average conductances, mean ± SD (no. of steps), of the channels represented by the peaks are, from left to right: 33.4 ± 0.4 pS (142), 39.6 ± 0.3 pS (46), 41.7 ± 0.5 pS (78), 48.0 ± 0.4 pS (23). The total number of transitions in the hybrid population, 124, is not much different from the minimum number predicted from Eq. 3, 114. The ratio observed/predicted was 1.7 ± 0.4 (mean ± SD) in four independent measurements. 1.0 M CsCl, 200 mV; channels lasting longer than 23 ms were used in the analysis.

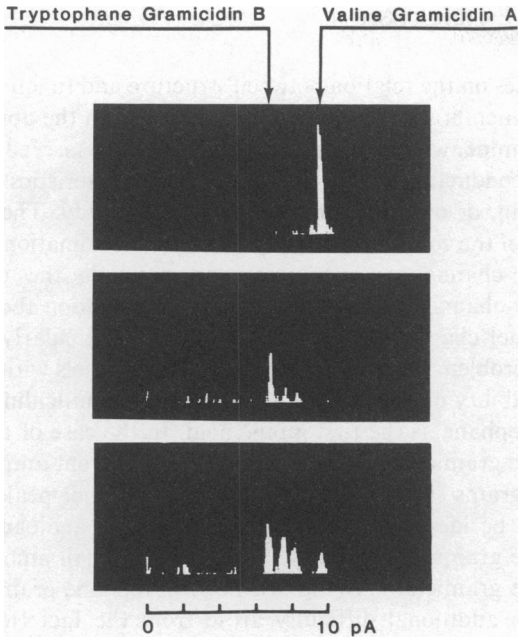

FIGURE 8 Amplitude histogram of single-channel current steps observed with a mixture of tryptophane gramicidin B and valine gramicidin A. *Top*, histogram of pure gramicidin A. *Middle*, histogram for tryptophane gramicidin B alone. Note that the histogram is not quite clean, as there are two peaks to the right of the main peak, in the positions of the hybrid channels. These are attributed to a contamination with about 0.02 % valine gramicidin A (see text). *Bottom*, histogram for the mixture. The pure peaks were identified by comparison with histograms for the pure compounds. The two peaks appearing between the pure peaks represent hybrid channels, presumably with opposite orientations. The average conductances, mean ± SD (no. of steps) of the channels represented by the peaks are, from left to right: 33.1 ± 0.6 pS (111), 37.1 ± 0.6 pS (90), 40.2 ± 0.7 pS (72), 47.5 ± 0.6 pS (39). The total number of transitions in the hybrid population, 162, is not much different from the minimum number predicted from Eq. 3, 132. The ratio observed/predicted was 1.5 ± 0.2 (mean ± SD) in eight independent measurements made in CsCl (or NaCl). 1.0 M CsCl, 200 mV; channels lasting longer than 23 ms were used in the analysis.

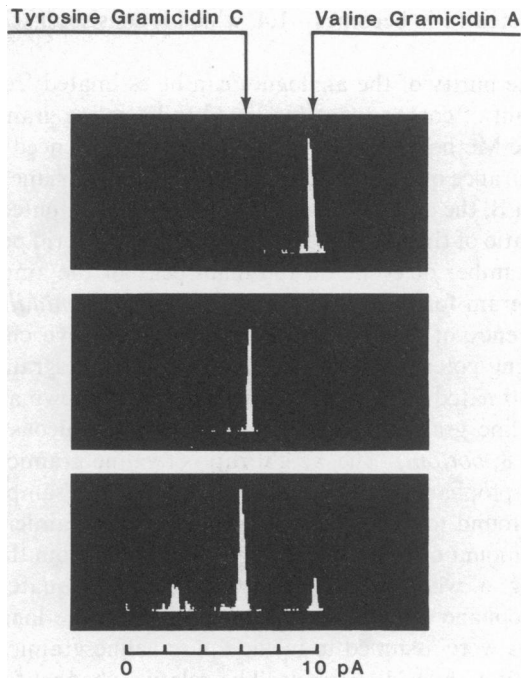

FIGURE 9 Amplitude histogram of single-channel current steps observed with a mixture of tyrosine gramicidin C and valine gramicidin A. *Top*, histogram of pure gramicidin A. *Middle*, histogram for tyrosine gramicidin C alone. *Bottom*, Histogram for the mixture. The pure peaks were identified by comparison with histograms for the pure compounds. The peak appearing on the far left represents the hybrid population. Note that this peak is not split, nor is the standard deviation increased compared to the two pure channel populations. The average conductances, mean ± SD (no. of steps), of the channels represented by the peaks are, from left to right: 12.4 ± 0.7 pS (58), 29.7 ± 0.7 pS (230), 48.5 ± 0.5 (42). The total number of transitions in the hybrid population, 58, is significantly lower than the minimum number predicted from Eq. 3, 197. This is in a large part the result of our inability to determine the single-channel current amplitudes for very short-lived channels. The ratio observed/predicted was 0.42 ± 0.13 (mean ± SD) in 10 independent measurements. 1.0 M CsCl, 200 mV; channels lasting longer than 4.4 ms were used in the analysis.

orientation with the larger conductance for the case with phenylalanine at position 1 (Fig. 7), and the orientation with the smaller conductance for the case with tryptophane at position 1 (Fig. 8). In the case of tyrosine gramicidin C (Fig. 9), only one additional peak was seen, far to the left in the amplitude histogram. This peak represented very short-lived channels. These channels were seen only in the mixture of these two compounds, and not when either of the two compounds was added alone; they must, therefore, represent hybrid channels. Similar results were observed with mixtures of tyrosine gramicidin C and valine gramicidin B (data not shown).

The frequency of the hybrid channels should be related to the frequencies of the pure channels (e.g., Veatch and Stryer, 1977). In the simplest case, where the two monomers form helices with identical structure at their NH_2-termini, one would expect that the number of hybrid channels, n_H, observed in an experiment should be larger than predicted from the binomial distribution (see Appendix):

$$n_H \geqq 2\,(n_a\, n_b)^{0.5} \qquad (3)$$

where n_a and n_b denote the numbers of the two pure channel types observed. The hybrid channel occurrence was consistent with the predictions of Eq. 3 for hybrids of valine gramicidin A with phenylalanine gramicidin B (Fig. 7) and with tryptophane gramicidin B (Fig. 8). When the amino acid at position 1 is a tyrosine, the number of channels in the hybrid peak is less than predicted from Eq. 3. This results from the loss of a substantial number of channels in the sampling procedure because of the very short average lifetime of the hybrid channels, ~3.7 ms (based on 34 channels with lifetimes longer than ~4 ms), which should be compared to the minimum duration of the channels accepted by the computer for the amplitude analysis, 4.4 ms. When the number of transitions in the hybrid population is adjusted for this loss, the observed/

predicted ratio becomes ~1.4, which is consistent with Eq. 3.

The purity of the analogues can be estimated from the amount of contaminant (assumed to be valine gramicidin A, see Methods) found in the samples, as evidenced by the appearance of the hybrid channels. For tryptophane gramicidin B, the amount of contamination was computed from the ratio of the number of events in the two hybrid peaks to the number of events in the main peak of the amplitude histogram for the "pure" compound (Fig. 8, *middle*), the frequency of hybrid channels, and the relative channel-forming potency of the analogue and of valine gramicidin A, estimated from experiments in which a known amount of valine gramicidin A was added to the aqueous phase (Fig. 8, *bottom*). The weight ratio of valine gramicidin A to tryptophane gramicidin B in the analogue sample was thus found to be ~0.02%. For tryptophane gramicidin A, the amount of contamination was estimated from the data in Fig. 6, where the short-lived events were equated with tryptophane gramicidin A channels, while the long-lived events were assumed to include pure valine gramicidin A as well as hybrid channels. The relative channel-forming potency of the analogue was estimated from the amounts of the two compounds that had to be added to the aqueous phases to achieve comparable channel frequencies, and the observed/predicted ratio for the hybrids was assumed to be 1.5. We could then estimate that there was ~0.06% valine gramicidin A in the tryptophane gramicidin A sample. No hybrid channels, or valine gramicidin A channels, were reproducibly detected with the other analogues. We, therefore, estimate the sample purity to be ~99.99% or better for these compounds.

The hybrid experiments also provided some information on the conductances in 1.0 M CsCl. Table IV summarizes the available conductance data for phenylalanine gramicidin B, tryptophane gramicidin B, and tyrosine gramicidin C, as well as for valine gramicidin A, B, and C. There is a substantial variation in the conductance ratios, and the increased selectivity for Cs^+ over Na^+ reflects a decrease in Na^+ conductance rather than an increase in the Cs^+ conductance.

TABLE IV
SINGLE-CHANNEL CONDUCTANCES IN CsCl AND NaCl

Gramicidin A analogue	g_{Na^+}	g_{Cs^+}	g_{Cs^+}/g_{Na^+}
Valine gramicidin A	15.4 ± 1.0	49.5 ± 2.3	3.2
Valine gramicidin B*	8.2 ± 0.5	38.8 ± 1.3	4.7
Phenylalanine gramicidin B	6.8 ± 0.2	34.4 ± 1.0	5.1
Tryptophane gramicidin B	9.9 ± 0.5	33.0 ± 0.9	3.3
Valine gramicidin C*	15.5 ± 0.8	48.1 ± 1.2	3.1
Tyrosine gramicidin C	4.8 ± 0.4	30.3 ± 1.4	6.3

1.0 M salt, 200 mV, Mean ± SD.
*Data from E. W. B. Russell et al., manuscript in preparation.

DISCUSSION

Studies on the relation between structure and function of a transmembrane channel depend critically on the ability to determine, without ambiguity, whether an observed channel (conductance event) reflects the characteristics of the protein, or other channel-former, under study. The presence of the contaminants may bias the determination of the single-channel conductances, and preclude the use of many-channel experiments to obtain information about the channel characteristics. This may be a particularly difficult problem for compounds that form channels with a low probability of opening, as is the case for gramicidins with tryptophane as the first amino acid. In the case of tryptophane gramicidin B, the single-channel current amplitude histograms showed the existence of two minor peaks that could be identified as hybrids between the analogue and valine gramicidin A by the addition of a small amount of valine gramicidin A (Fig. 8). For tryptophane gramicidin A, an additional difficulty arose from the fact that the conductance of the contaminant was identical to that of the analogue. The presence of valine gramicidin A could therefore only be detected on the basis of the different lifetimes for the hybrid and valine gramicidin A channels compared to the tryptophane gramicidin A channels (Fig. 6). The relatively high contamination, ~0.06%, of the tryptophane gramicidin A is probably related to its chromatographic similarity with valine gramicidin A. This similarity precludes obtaining the optimal benefit of the second HPLC purification step.

The permeability characteristics of gramicidin channels depend upon the precise amino acid sequences of the peptides forming the channels. The effects of sequence modifications are large, e.g., sixfold changes in conductance between tryptophane gramicidin A and tyrosine gramicidin B. These conductance changes result from differential effects of having a polar side chain at positions 1 and 11. Substitutions at position 1 decreases the conductance in the order tryptophane, phenylalanine, then tyrosine, while substitutions at position 11 decrease the conductance in the order tryptophane, tyrosine, then phenylalanine. In either case the order is unaffected by the nature of the amino acid at the other position. Note that the conductance in either case is highest with tryptophane. The maximal conductance seen with tryptophane gramicidin B is, in fact, larger than the maximal conductance seen with valine gramicidin B, 6.4 pS (E. W. B. Russell et al., manuscript in preparation).

The conductance changes are not clearly related to the changes in HPLC behavior, where the relative retention times increase in the order tyrosine, tryptophane, then phenylalanine, independent of position.

The permeability changes we observe could possibly be due to substantial changes in the polypeptide backbone structure of the channels, or alternatively they could result

from long-range interactions between the permeating ions and the side chains. There is little evidence for substantial changes in channel structure, with the possible exception of the compounds with tyrosine as the first amino acid. The major evidence against structural changes is the observation of hybrid channels when two different compounds (usually an analogue and valine gramicidin A) are added to the aqueous phases. This argues that the two halves of the channel, in particular their NH_2-termini, can adapt to each other to form the hydrogen bond-stabilized dimer. But it does not tell whether the conformation(s) of the NH_2-termini of the hybrid channels correspond(s) to their conformation(s) in the pure channels. It is, therefore, important to analyze the frequencies of the hybrid events. If we assume that a given monomer cannot discriminate between different analogues when forming a channel with a second monomer, which is equivalent to assuming that the NH_2-terminal regions of the helices formed by the different analogues are very similar, then the frequency of hybrid channel occurrence, f_H, will be related to the pure channel frequencies, f_a and f_b, as (see Appendix)

$$f_H \cong 2\,(f_a\,f_b)^{0.5}. \tag{4}$$

A characteristic prediction of this equation is that if the total frequency of hybrid channels, f_H, is not equal to the predictions of the binomial distribution, then the frequencies of each of the two orientations of the hybrid must be different (see Appendix).

These arguments pertain in particular to analogues with phenylalanine or tryptophane at position 1, where the hybrid channels have conductances between those seen with the pure channels. The splitting of the hybrid peaks indicates, of course, some asymmetry of these channels. But this need not imply that the polypeptide backbone structure is asymmetric. It is most likely the result of an asymmetry in the interactions between a permeating ion and the valine and (tryptophane or phenylalanine) side chains at position 1 in the different monomers. Due to their considerable bulk and steric interactions with the rest of the polypeptide, the aromatic side chains will probably be located asymmetrically with respect to the channel center (see for example Fig. 3). The split in the hybrid peak could also have been due to the different amino acids at position 11. We do not believe this contribution to be important, as we have not been able to resolve clearly two peaks in the hybrid population of channels seen in mixtures of valine gramicidin A and valine gramicidin B (data not shown).

The tyrosine analogues pose a more difficult problem, as it is difficult to reconcile the observation of only a single hybrid channel peak with the single-channel conductance that is not in between the conductances of the pure channels. Conductances that are not in between the conductances of the pure channels could arise if a single side chain produced highly asymmetric alterations in the energy profile: an increase in the energy barriers in one half of the channel and a decrease in the other half. This should lead to asymmetric, strongly rectifying, current-voltage characteristics for the hybrid channels. We observe, however, only a single hybrid peak. One explanation could be that one of the orientations of the hybrid channels is not detected in our system. But we cannot exclude that channels with tyrosine at position 1 could be very different from the normal β-helical head-to-head dimers.

The changes in permeability characteristics are most simply analyzed in terms of the simple three-barrier-two-site model (Hladky, 1974; Levitt, 1978 *b*; Urban and Hladky, 1979; Andersen 1983 *a*). If only one ion can occupy the (symmetric) channel, one can depict the ion movement as:

$$I' + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} IG \underset{l}{\overset{l}{\rightleftharpoons}} GI \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} G + I''$$

where I' and I'' represents an ion 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, and k_1, l, k_{-1} denote the respective rate constants for association, translocation, and dissociation. Our phenomenological parameters, g_{max} and K (Table II), are related by

$$1/g_{max} = (2\,kT/e^2)\,(1/l + k_1/K), \tag{5}$$

where

$$K = k_{-1}/(2\,k_1). \tag{6}$$

Eq. 5 implies that g_{max} and K should vary together. This was indeed observed, but the changes in g_{max} were in all cases larger than predicted from the changes in K, assuming that k_{-1} was the only rate constant that varied. This means that one, or both, of the other two rate constants, l and k_1, also must be affected by the alterations of the polypeptide sequence. The magnitudes of the rate constants reflect the heights of the energy barriers that the ion must traverse as it moves between the aqueous phases and the ion-binding sites, or between the binding sites in the channel. A decrease in a rate constant corresponds, in the conventional Eyring rate theory description, to an increase in the associated energy barrier for ion movement (e.g., Läuger, 1973; Hille, 1975). The magnitude of K reflects the depth of the energy wells that form the ion binding sites. A major effect of altering the amino acid side chains is thus an alteration of at least one of the peak energy barriers in the energy profile for ion movement through the channel. Since the range of gramicidin sequence modifications made and studied up to now is fairly limited, it is not known whether this is a general result.

The effect of a given amino acid interchange on the energy barriers and wells is quite position dependent. Changes at position 11, close to the ion-binding sites

(Andersen et al., 1981; Urry et al., 1982 *a*, *b*), leave the Na^+ affinity (well depth) essentially unchanged, while the peak barrier heights vary. Changes at position 1, far from the ion binding sites, also alter the barrier heights, but now we see substantial changes in the Na^+ affinity with the tyrosine compounds. This may be the result of electrostatic interactions between an ion in the energy well and the dipole associated with the phenolic -OH group. Inspection of CPK models of gramicidins with tyrosine at position 1 indicates that the phenolic -OH may be quite close to the ion binding sites in the opposite monomer, and that the negative end of the dipole associated with this group may point toward the binding site.

The ion selectivity of channels is primarily determined by the peak energy barriers in the energy profile (Hille, 1975), although equilibrium ion binding also may be important in multiply-occupied channels (Kohler and Heckmann, 1980). It is therefore not surprising that sequence alterations in the gramicidins affect the single-channel conductances differently for different permeant ions. This problem is still under investigation, but a comparison of the conductance variations in 1.0 M CsCl (Table IV) shows that the modified gramicidins tend to be more selective for Cs^+ over Na^+ than valine gramicidin A.

The observed changes in conductance result from alterations outside the channel lumen proper, and it is reasonable to assume that they occur with minimal changes in the polypeptide backbone structure. This may have implications for other channels, for example for the interpretations of subconductance states in "flickering" channels (Nelson and Sachs, 1979; Barrett et al., 1982; Sigworth, 1982; Auerbach and Sachs, 1983), and possibly for channel gating per se. If a macromolecular transmembrane channel is composed of a number of α-helical segments that span the membrane, only some of which participate in forming the channel lumen (Devillers-Thiery et al., 1983; Noda et al.,1983), it is possible that the energy profile for ion movement through the channel (and thus the channel conductance) would depend upon the precise arrangement of the "extra" helical segments or other parts of the structure which are exterior to the channel-forming segments.

We finally note that the *de novo* or semisynthetic sequence alterations that can be made with the linear gramicidins (and other small polypeptides of known structure, e.g., alamethicin (Hall et al., 1984) make these molecules powerful tools to study molecular mechanisms of channel-mediated ion permeation (and gating). These chemical techniques allow us to extend structure-function relations beyond what is feasible with "conventional" molecular biology techniques: insertions or deletions in the sequence, and point mutations among the 20 amino acids that mRNA can code for. The possibility of introducing any amino acid into the sequence permits us to investigate the interactions between permeating ions and transmembrane channels in molecular detail, because we can obtain series of graded permeability changes that can be related to the physico-chemical characteristics of the side chains. This should, in combination with molecular dynamics simulations of the channel and structural investigations by x-ray diffraction and spectroscopic techniques, make the gramicidin channels almost uniquely powerful tools for studying mechanisms of ion permeation.

APPENDIX

We shall derive the frequency of hybrid channel formation in an asymmetrical membrane, where the asymmetry in our case is the result of an applied potential difference across the membrane. We assume that the rate of channel formation from a given analogue is the same, irrespective of which analogue forms the other half of the dimeric channel. This implies that the monomer cannot recognize the identity of the monomer with which it is going to form the channel. It should in this case be possible to separate an observed channel frequency, f, into contributions associated with each monomer

$$f_a = f'_a f''_a \tag{A1}$$

where the subscript refers to analogue species, and the superscripts refer to monomers in the left, ', and, '', right half of the bilayer. That is, we assume for simplicity that the two monomers come from different halves of the bilayer. If two different analogues, *a* and *b*, are present the hybrid channel frequencies for each of the two orientations, h_{ab} and h_{ba}, are

$$h_{ab} = f'_a f''_b \tag{A2}$$

and

$$h_{ba} = f'_b f''_a. \tag{A3}$$

The combined hybrid channel frequency, f_H, is then obtained as

$$f_H = ((h_{ab}/h_{ba})^{0.5} + (h_{ba}/h_{ab})^{0.5})\,(h_{ab}h_{ba})^{0.5}. \tag{A4}$$

It follows that

$$f_H \geq 2\,(f_a f_b)^{0.5}. \tag{A5}$$

A large discrepancy between f_H and $2\,(f_a f_b)^{0.5}$, f_H being larger than the expected value, must, therefore, be associated with a large difference in the frequencies of occurrence for the two orientations of the hybrid channels.

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REFERENCES

Andersen, O. S. 1975. Ion-specificity of gramicidin A channels. Fiftieth International Biophysics Congress, Copenhagen. 112. (Abstr.)

Anderson, O. S. 1978. Ion transport across simple membranes. *In* Renal Function. G. H. Giebisch, and E. F. Purcell, editors. Josiah Macy, Jr. Foundation. New York. 71–99.

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. Studies on the diffusion-controlled association step. *Biophys. J.* 41:147–165.

Andersen, O. S., E. W. Barrett, and L. B. Weiss. 1981. On the position of 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. 1983. Single-channel studies on linear gramicidins with altered amino acid sequences. *Hoppe-Seyler's Zeitschr. Physiol. Chem.* In press. (Abstr.)

Auerbach, A., and F. Sachs. 1983. Flickering of nicotinic ion channel to a subconductance state. *Biophys. J.* 42:1–10.

Bamberg, E., H. -J. Apell, and H. Alpes. 1977. Structure of the gramicidin A channel: discrimination between the $\pi_{L,D}$ and the β-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, and P. Läuger. 1978. Ion channels formed by chemical analogues of gramicidin A. *Fed. Proc.* 37:2633–2637.

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.* 449:223–228.

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

Barrett, J. N., K. L. Magleby, and B. S. Pallotta. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* (*Lond.*). 331:211–230.

Devillers-Thiery, A., J. Giraudat, M. Bentaboulet, and J. -P. Changeux. 1983. Complete mRNA coding sequence of the acetylcholine binding α-subunit of *Torpedo marmorata* acetylcholine receptor: a model for the transmembrane organization of the polypeptide chain. *Proc. Natl. Acad. Sci. USA.* 80:2067–2071.

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

Eisenman, G., S. Krasne, and S. Ciani. 1976. Further studies on ion selectivity. *In* International Workshop on Ion Selective Electrodes and on Enzyme Electrodes in Biology and Medicine. M. Kessler, L. Clark, D. Lubbers, I. Silver, and W. Simon, editors. Urban and Schwarzenberg, Munich. 3–21.

Eisenman, G., J. Sandblom, and J. Hägglund. 1983. Electrical behavior of single-filing channels. *In* Structure and Function of Excitable Cells. W. Adelman, D. Chang, R. Leuchtag, and I. Tasaki, editors. Plenum Publishing Corp., New York. 383–411.

Eisenman, G., J. Sandblom, and E. Neher. 1977. Ionic selectivity, saturation, binding, and block in the gramicidin A channel: a preliminary report. *In* Metal-Ligand Interactions in Organic Chemistry and Biochemistry. B. Pullman and N. Goldblum, editors. D. Reidel Publishing Co., Dordrecht-Holland. 2:1–36.

Finkelstein, A. 1974. Aqueous pores created in thin lipid membranes by the antibiotics nystatin, amphotericin B, and gramicidin A: implications for pores in plasma membranes. *In* Drugs and Transport Processes. B. A. Callingham, editor. McMillan and Co., London. 241–250.

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.

Fischer, W., J. Brickman, and P. Läuger. 1981. Molecular dynamics study of ion transport in transmembrane protein channels. *Biophys. Chem.* 13:105–116.

Hall, J. E., I. Vodyanoy, T. M. Balasubramanian, and G. R. Marshall. 1984. Alamethicin: a rich model for channel behavior. *Biophys. J.* 45:233–247.

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

Hille, B. 1975. Ion selectivity, saturation, and block in sodium channels. *J. Gen. Physiol.* 66:535–560.

Hinton, J. F., G. L. Turner, and F. S. Millett. 1981. A thallium-205 NMR investigation of the thallium (I)-gramicidin complex. *J. Magn. Reson.* 45:42–47.

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

Hladky, S. B., and D. A. Haydon. 1970. Discreteness of conductance change in bimolecular lipid membranes in the presence of certain antibiotics. *Nature* (*Lond.*). 225:451–453.

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

Jack, J. J. B., D. Noble, and R. W. Tsien. 1975. Electric current flow in excitable cells. Oxford University Press, Clarendon Press, Oxford. 229–240.

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

Koeppe, R. E. II, J. M. Berg, K. O. Hodgson, and L. Stryer. 1979. Gramicidin A crystals contain two cation binding sites per channel. *Nature* (*Lond.*). 279:723–725.

Koeppe, R. E. II, K. O. Hodgson, and L. Stryer. 1978. Helical channels in crystals of gramicidin A and of cesium-gramicidin A complex: an x-ray diffraction study. *J. Mol. Biol.* 121:41–54.

Koeppe, R. E. II, and M. Kimura. 1983. Computer building of β-helical polypeptide models. *Biopolymers.* In press.

Koeppe, R. E. II, and L. B. Weiss. 1981. Resolution of linear gramicidins by preparative reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 208:414–418.

Kohler, H. -H., and K. Heckman. 1980. The relation between binding affinities and selectivity of a pore. *J. Membr. Sci.* 6:45–69.

Krasne, S., G. Eisenman, and G. Szabo. 1971. Freezing and melting of lipid bilayers and the mode of action of nonactin, valinomycin and gramicidin. *Science* (*Wash., DC*). 174:412–415.

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.

Levitt, D. G., S. R. Elias, and J. M. Hautman. 1978. Number of water molecules coupled to the transport of sodium, potassium, and hydrogen ions via gramicidin, nonactin or valinomycin. *Biochim. Biophys. Acta.* 512:436–451.

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

Mazet, J. -L, R. E. Koeppe II, and O. S. Andersen. 1983. Single-channel studies on modified gramicidins. *Biophys. J.* 41(2,pt.2): 382 *a*. (Abstr.)

McBride, D., and G. Szabo. 1978. Blocking of gramicidin channel conductance by Ag^+. *Biophys. J.* 21(2,pt.2):25 *a*. (Abstr.)

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

Myers, V. B., and D. A. Haydon. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. *Biochim. Biophys. Acta.* 274:313–322.

Neher, E. 1975. Ionic specificity of the gramicidin channel and the

thallous ion. *Biochim. Biophys. Acta.* 401:540–544. Errata ibid, 1977. 469:359.

Nelson, D. J., and F. Sachs. 1979. Single ionic channels observed in tissue-cultures muscle. *Nature (Lond.*). 282:861–863.

Noda, M., H. Takahashi, T. Tanabe, M. Toyosata, S. Kikyotani, V. Furutani, T. Hirose, H. Takashima, S. Inayama, T. Miyata, and S. Numa. 1983. Structural homology of *Torpedo californica* acetylcholine receptor subunits. *Nature (Lond.*). 302:528–531.

Parsegian, V. A. 1969. Energy of an ion crossing a low dielectric membrane: solutions to four relevant electrostatic problems. *Nature (Lond.*). 221:844–846.

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.)

Renugopalakrishnan, V., and D. W. Urry. 1978. A theoretical study of Na^+ and Mg^{++} binding to the carbonyl oxygen of *N*-methyl acetamide. *Biophys. J.* 24:729–738.

Rosenberg, P. A., and A Finkelstein. 1978 *a*. Interactions of ions and water in gramicidin A channels. Streaming potential across lipid bilayer membranes. *J. Gen. Physiol.* 72:327–340.

Rosenberg, P. A., and A. Finkelstein. 1978 *b*. Water permeability of gramicidin A-treated lipid bilayer membranes. *J. Gen. Physiol.* 72:341–350.

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

Sarges, R., and B. Witkop. 1965 *b*. Gramicidin. VII. The structure of valine- and isoleucine-gramicidin B. *J. Am. Chem. Soc.* 87:2027–2030.

Sarges, R., and B. Witkop. 1965 *c*. Gramicidin. VIII. The structure of valine- and isoleucine-gramicidin C. *Biochemistry.* 4:2491–2494.

Schagina, L. V., A. E. Grinfeldt, and A. A. Lev. 1978. Interaction of cation fluxes in gramicidin A channels in lipid bilayer membranes. *Nature (Lond.*). 273:243–245.

Sigworth, F. 1982. Fluctuations in the current through open ACH-receptor channels. *Biophys. J.* 37(2,pt.2):309 *a*. (Abstr.)

Szabo, G., G. Eisenman, and S. Ciani. 1969. The effects of the macroelectralide actin antibiotics on the electrical propertiers of phospholipid bilayer membranes. *J. Membr. Biol.* 1:346–382.

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

Turner, G. I., J. F. Hinton, R. E. Koeppe II, J. A. Parli, and F. S. Millett. 1983. Difference in association of Tl(I) with gramicidin A and gramicidin B in trifluoro-ethanol determined by Tl-205 NMR. *Biochim. Biophys. Acta.* 756:133–137.

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

Urry, D. W., K. U. Prasad, and T. L. Trapane. 1982 *a*. Location of monovalent cation binding sites in the gramicidin channel. *Proc. Natl. Acad. Sci. USA.* 79:390–394.

Urry, D. W., J. T. Walker, and T. L. Trapane. 1982 *b*. 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.* 13:5249–5265.

Veatch, W. R., R. Mathies, M. Eisenberg, and L. Stryer. 1975. Simultaneous fluorescence and conductance studies of planar bilayer membranes containing a highly active and fluorescent analogue of gramicidin A. *J. Mol. Biol.* 99:219–235.

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.

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.

DISCUSSION

Session Chairman: V. Adrian Parsegian *Scribes*: John T. Durkin and Stephen Slatin

EISENMAN: In discussing the tyrosine compounds, you suggest that a single side chain substitution could produce highly asymmetrical alterations in the current-voltage characteristics for the hybrid channels. Have you observed such behavior?

MAZET: Yes. In most cases of substitution at position 1 we see two hybrid peaks. Our explanation is that it is due to the existence of two opposing orientations of the hybrid channel. We create an asymmetry across the membrane by applying a 200 mV potential. Our observation of two peaks implies that the I-V curve of a hybrid channel is asymmetric.

ANDERSEN: When we compare analogues with more or less isosteric polar and nonpolar side chains, where there is a change in conductance there is a change in the shape of the I-V.

EISENMAN: Our recent comparison of energy profiles (Eisenman and Sandblom, this meeting) indicates there may be significant species differences in the locations of the ion binding sites. In particular, the site for Cs is near the mouth of the channel, whereas that for Na lies considerably deeper. This offers an alternate interpretation for your finding that tyrosine replacements at position #1 dramatically change the afffinity for Na.

This also implies that the comparison of selectivity based on conductances in Cs and Na (Table IV) will need to be extended to a full comparison of the energy profiles for Cs vs. Na, peak by peak and well by well, before the true selectivity differences will be appreciated. Even at low occupancy, the rate determining barrier for Cs is at the mouth of the channel, whereas for Na the barriers at the mouth and the middle of the channel are of equal importance.

MAZET: I agree that more detailed investigations of the selectivity characteristics are necessary.

BUSATH: Do you think the change induced by your side-chain modifica-