

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-

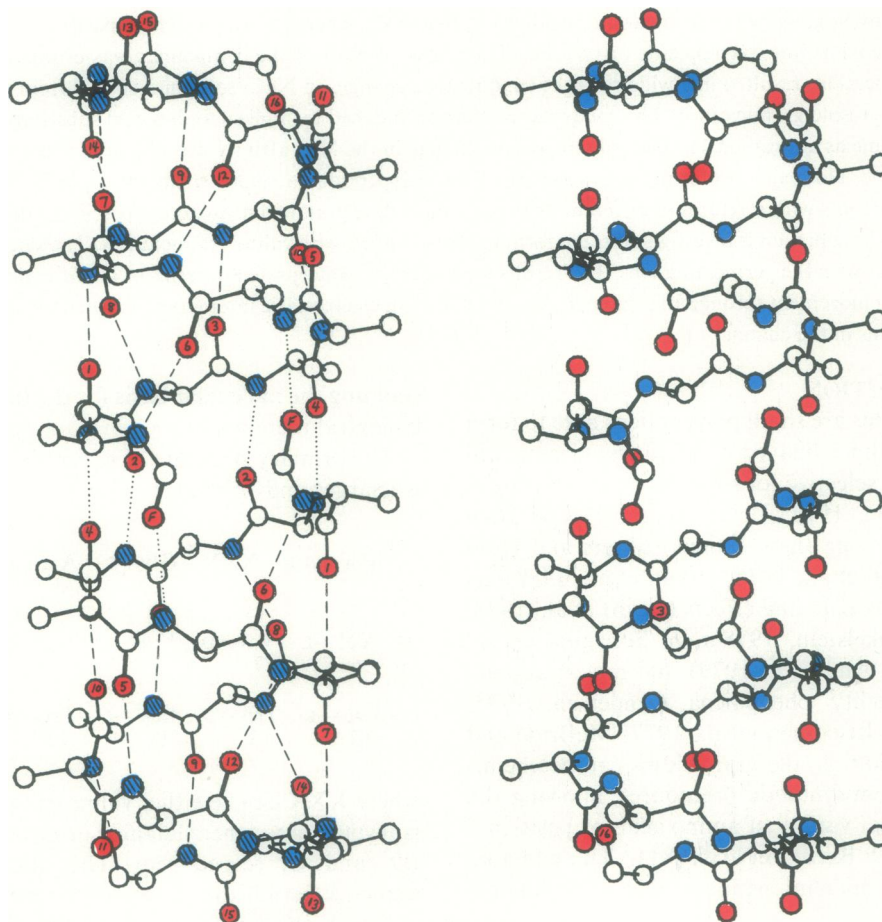


FIGURE 1 Stereoscopic Oak Ridge Thermal Ellipsoid Program (ORTEP) representation of the peptide backbone in the antiparallel β -helical gramicidin dimer. The backbone coordinates are taken from Table I of Koeppe and Kimura (1983). Intramolecular (---) and intermolecular (···) hydrogen bonds are indicated. 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.

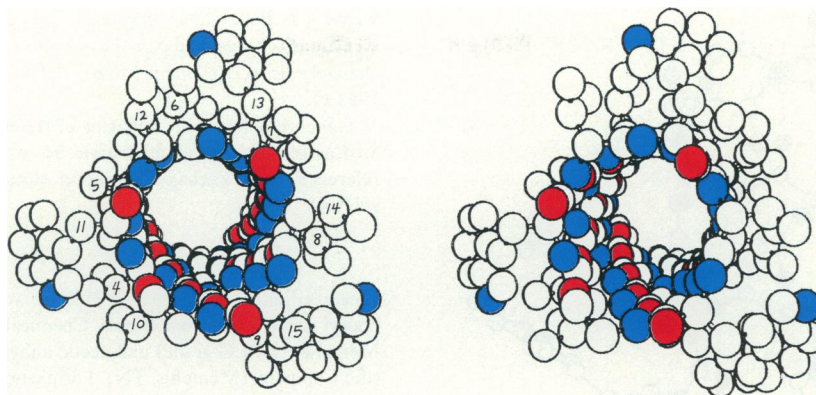


FIGURE 2 ORTEP drawing, without hydrogens, of the cylindrical channel lumen viewed from the end. This figure also illustrates the channel entrance (minus the surrounding lipid molecules). The coordinates of the backbone atoms are from Table I of Koeppe and Kimura (1983). The side chains of the upper 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 $\sim 0.01\%$ 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 $25 \pm 1^\circ\text{C}$ by the pipette technique of Szabo et al. (1969) across a hole (area $\sim 1.6 \text{ mm}^2$) 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 (1983a). 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

¹Russell, E. W. B., L. B. Weiss, F. I. Navetta, R. E. Koeppe II, and O. S. Andersen. Single-channel studies on linear gramicidins with altered amino acid side chains. Effects of altering the polarity of the NH_2 -terminal amino acid in gramicidin A. Submitted for publication.

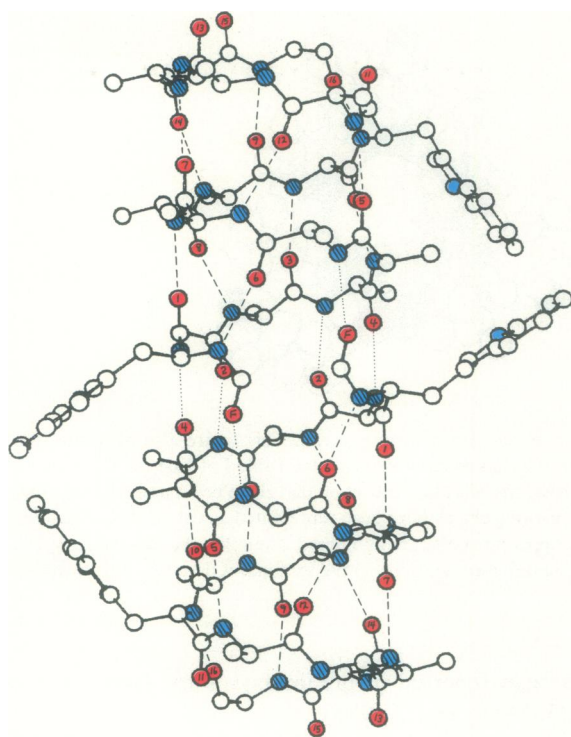


FIGURE 3 ORTEP drawing of the peptide backbone with tryptophanes drawn at positions 1 and 11. Note that the indole group at position 1 in one monomer can come quite close to the indole at position 11 in the other monomer. In this illustration the orientations of all of the indole rings with respect to 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).

\pm 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

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

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*)

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

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 \cdot s), Andersen, 1983 *b*).

The chromatographic behavior of the different analogues was evaluated as the relative retention time, R_t , 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 "Sequal" 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

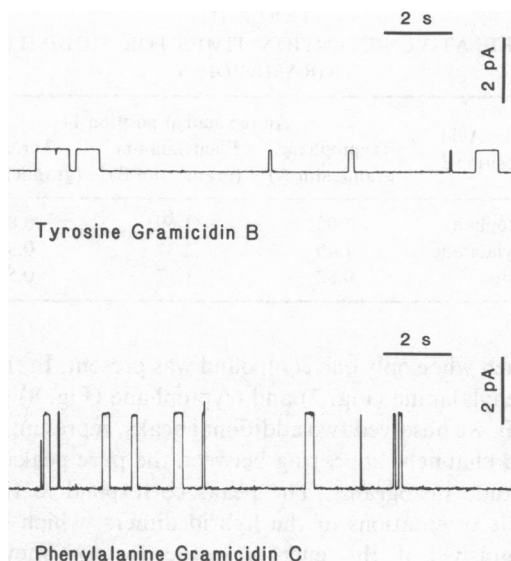


FIGURE 4 Comparison of single-channel events observed with tyrosine gramicidin B and phenylalanine gramicidin C. 1.0 M NaCl, 200 mV, 25 °C. Note that both traces were obtained at the same amplification and chart speed.

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

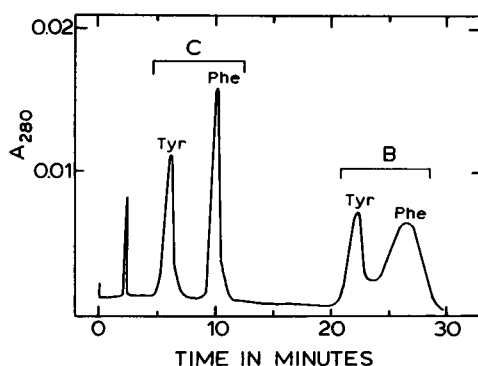


FIGURE 5 Comparisons of the HPLC chromatographic properties of tyrosine and phenylalanine gramicidins B and C. Previously purified samples were rechromatographed in separate runs on the same day and then overlaid to give the comparison indicated. The elution solvent was 83% methanol and 17% water at 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)
	<i>M</i>			
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 $\sim 3\%$ (see Legend to Fig. 6).

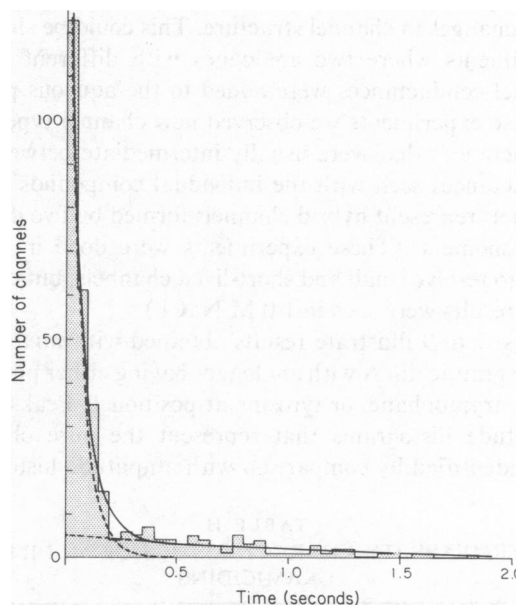


FIGURE 6 Lifetime histogram of single-channel events seen with tryptophane gramicidin A. A total of 312 channels were collected on a stripchart recorder, and the histogram was assembled from these. Three channels, with durations >2.0 s, are not illustrated in the histogram. Multiple channel openings 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 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

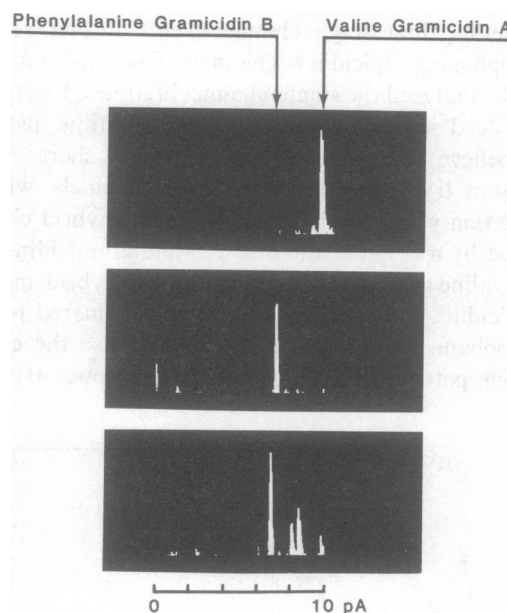


FIGURE 7 Amplitude histogram of single-channel current steps observed with a mixture of phenylalanine gramicidin A and valine gramicidin A. *Top*, histogram of pure gramicidin A. *Middle*, histogram for phenylalanine gramicidin B alone. *Bottom*, histogram for the mixture. The pure peaks were identified 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 \pm 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 \pm SD) in four independent measurements. 1.0 M CsCl, 200 mV; channels lasting longer than 23 ms were used in the analysis.

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

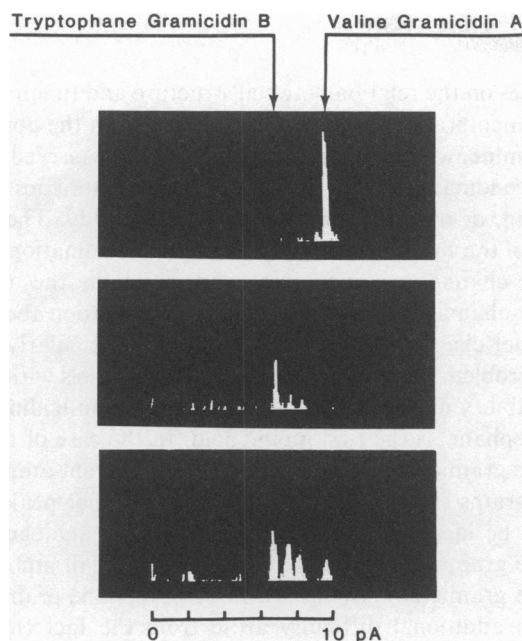


FIGURE 8 Amplitude histogram of single-channel current steps observed with a mixture of tryptophane gramicidin B and valine gramicidin A. *Top*, histogram of pure gramicidin A. *Middle*, histogram for tryptophane gramicidin B alone. Note that the histogram is not quite clean, as there are two peaks to 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 \pm 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 \pm 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.

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 Appen-

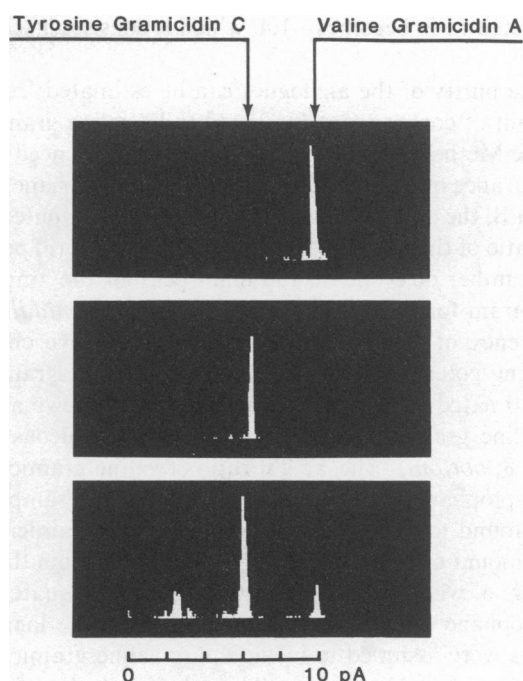


FIGURE 9 Amplitude histogram of single-channel current steps observed with a mixture of tyrosine gramicidin C and valine gramicidin A. *Top*, histogram of pure gramicidin A. *Middle*, histogram for tyrosine gramicidin C alone. *Bottom*, Histogram for the mixture. The pure peaks were identified by comparison 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 \pm 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 \pm SD) in 10 independent measurements. 1.0 M CsCl, 200 mV; channels lasting longer than 4.4 ms were used in the analysis.

dix):

$$n_H \geq 2 (n_a n_b)^{0.5} \quad (3)$$

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 $\sim 0.02\%$. 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 $\sim 0.06\%$ 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 $\sim 99.99\%$ 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^+}	$g_{\text{Cs}^+}/g_{\text{Na}^+}$
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 \pm 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, $\sim 0.06\%$, 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)

$$f_{\text{H}} \geq 2 (f_{\text{a}} f_{\text{b}})^{0.5}. \quad (4)$$

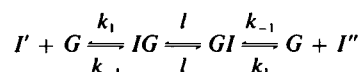
A characteristic prediction of this equation is that if the total frequency of hybrid channels, f_{H} , is not equal to the predictions of the binomial distribution, then the frequencies of each of the two orientations of the hybrid must be different (see Appendix).

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:



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

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

where

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

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., Luger, 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 transmem-

brane 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

$$f_a = f'_a f''_a \quad (\text{A1})$$

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

$$h_{ab} = f'_a f''_b \quad (\text{A2})$$

and

$$h_{ba} = f'_b f''_a. \quad (\text{A3})$$

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

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

It follows that

$$f_H \geq 2 (f_a f_b)^{0.5}. \quad (\text{A5})$$

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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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 affinity 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-

tions is a change in the dipole of the side chain, or in its electron withdrawing power? What evidence can be brought to bear for your preference?

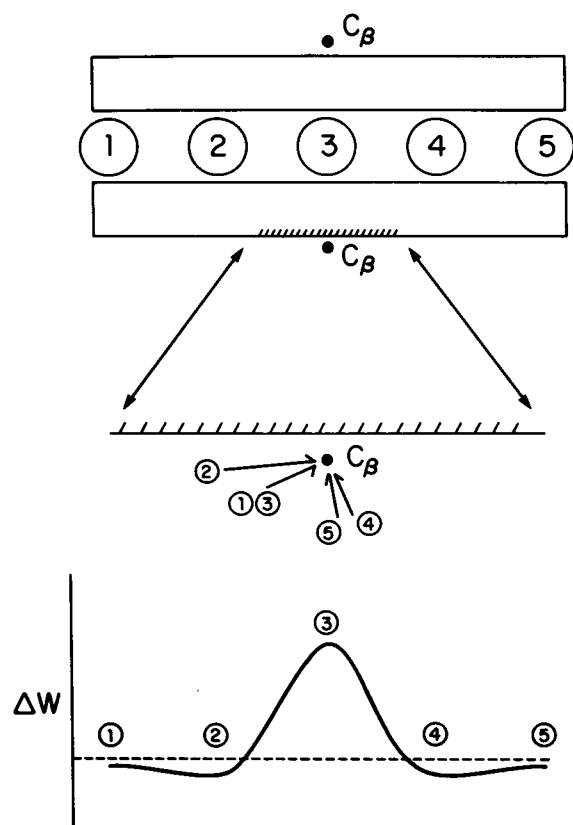


FIGURE A *Top*: schematic representation of a head-to-head hydrogen-bonded gramicidin dimer. C_β represents the position of the β -carbon of amino acid 1, the numbered circles denote five different positions of an ion moving through the channel. *Middle*: enlargement of the channel wall around one of the β -carbons. The arrows denote, for each of the ion positions, the average position and moment of a dipole directed along the β - γ bond and with its positive end pointing towards the β -carbon. (The position and average moment of the dipole is computed by assuming that the ion moves along the channel axis, and that the side chains rotate freely around the α - β bond using the Boltzmann distribution to weigh the different orientations. Dipole-dipole interactions between the side chains in each monomer are disregarded.) *Bottom*: the change in free energy for ion movement through the channel which results from the introduction of a dipolar side chain at position 1.

MAZET: Let me present our dipole interpretation. From D. W. Urry's 1971 structure, the replacements that we made correspond to a modification of a side-chain dipole at the center of the channel (See Fig. A). If we consider that a charge in the channel tends to reorient the dipole so as to minimize the interaction energy, this model predicts both the decrease in the translocation rate constants and the increase in affinity of the channel for the ion.

ROUX: Have you done opening and closing time analysis? Is there increased noise in the open state?

ANDERSEN: We do not see substates in the single-channel current transitions. There is no increased noise in the open level. Lifetime histogram have not been investigated in detail for all the analogues. But lifetimes of compounds where amino acid #1 is linear through the β

carbon tend to be less than the lifetimes of compounds where amino acid #1 has a branch at the β carbon (valine and isoleucine). We also find that the average lifetimes tend to be shorter for compounds with a polar side chain at position #1 than those with a nonpolar side chain there.

WALLACE: At this point in the meeting we've seen a number of models for gramicidin, and have heard comments about the purported x-ray structure. But two points need to be made. First, the crystals that have been looked at have been formed from an organic solvent system, where the molecule has been shown to have a very different structure from that in lipid vesicles. Second, even that crystal structure has not yet been solved. We have to remember that these are still models.

ANDERSEN: The point is that, given a molecularity of two, nobody has yet been able to fold the peptides up in such a way that the permeation pathway is formed by the side chains.

FINKELSTEIN: I think Bonnie Wallace's comment is well taken. There is no direct structural evidence for the structure of the gramicidin channel in lipid bilayers. On the other hand, it would be unfortunate if the impression were given that the Urry model of the channel was in the same class as the present status of, say, the models of the acetylcholine receptor channel or of the alamethicin channel that were presented. There is a lot of data on permeability properties of analogues. The Veatch model is clearly not compatible with these data. There are no data that I'm aware of that are inconsistent with Urry's model, and there are a lot of data that are consistent.

WALLACE: I agree there is a variety of evidence on the general folding motif which points to Urry-type models. But specific details are being discussed here, particularly binding sites based on crystal structures probably not relevant to the membrane-bound form, and details about particular interactions with ions. We have to be cautious in what we say about details of the models.

MAZET: The question we try to address here is not about the structure of gramicidin itself. With this molecule which has been studied by itself for a rather long time, it is now possible to tackle problems of biological relevance without knowing the exact structure of the channel. That's the important part of the report.

MAKOWSKI: In some cases, physiological measurements can act as a structural method. The Urry model, for instance, is in part based on such measurements. Another case of interest to me is the size of molecules which can go through the gap junction. The permeability studies have allowed us to make estimates of the diameter of the narrowest portion of the channel. Different experimental methods, including physiological methods, give us different sets of information. These sets of information can be complementary.

WILSON: Not only are we as a group making unproven assumptions about structure, we are also making assumptions about theory which are equally unproven. All of us tend to understand rates of ion passage through channels in terms of a free energy vs. distance picture. However, it is entirely possible that knowing the free energy all the way through the channel will not tell us the rate. The Eyring activated complex picture, while extremely useful in chemistry, is only an approximation. There are well known cases in liquid state reactions where it doesn't work. Remember that in Eyring's derivation there is something he called κ , a transmission coefficient. In ordinary chemical reactions we're talking about breaking and making bonds, a motion of only a few ångströms. In passing through a channel, we're talking about much longer distances, such that free energy barriers may be quite broad and passage over them may not occur in a simple or quick manner. For the Eyring picture to make sense as a predictive scheme, κ needs to be near unity. In the extreme, one could imagine a channel in which the free energy was perfectly flat throughout the channel, but κ , which could also depend upon the structure, changes as

a function of distance along the channel. In this way conductance changes could be related to structure without any change in free energy. I'm not saying this is necessarily true, and I'm not saying the Eyring model is wrong. But it is an unproven supposition that knowing the free energy tells us the rate of passage.

EISENMAN: I have a question for you, Kent. Can you produce a binding constant from transmission coefficient considerations alone instead of Eyring transition states, without violating local reversibility?

WILSON: The free energy as a function of position does tell us the equilibrium distribution of ions. That connection is perfectly clear — it's just equilibrium thermodynamics. But the problem and the uncertainty is the connection of equilibrium thermodynamics to kinetics. The free energy difference can give us the ratio of forward and reverse rate constants, but it can't tell us either one alone.

EISENMAN: So you still need to know the energy profile to characterize the equilibrium distribution of ions, but you might want to describe jumping from well to well in terms of transmission coefficients instead of Eyring transition states.

WILSON: It may not be as simple as a jump. It may be diffusional motion, a lot of wiggling back and forth. There are cases in the liquid state where one knows that as the reacting system comes to the top of the barrier, it crosses back and forth many times before it makes it over permanently. Thus the real rate and the Eyring rate may differ by an order of magnitude.

KOEPPE: Urry has independent evidence on the binding sites in membranes based on ^{13}C NMR that indicates the binding sites are near carbonyls #11 and #13. In his model, those sites are 2.5 Å from the ends of the channel. This evidence agrees with our original x-ray assignments, but is quite independent of any x-ray assumptions.

BLANK: One of the problems of having only one discipline represented at a meeting is that there tends to be a single point of view. An electrochemist looking at the problem of channel conductance will see it very differently. He knows that proteins can conduct electrons (not all proteins, but some proteins). It's quite possible that electrons are involved in these processes, yet this has not been mentioned here. Also, when proteins are close to each other, as in an oligomeric channel, a transfer of electrons from one protein to another by quantum mechanical tunneling is possible.

Another important point of view that has not been represented is that of the surface chemist. At an electrode or membrane surface, there are complex reactions of electrons and ions, inhomogeneities of electrical double layers, and discontinuities in physical properties. In dealing with ion transport through channels, these factors should be considered.

KOEPPE: Despite any problems that may be discussed with Eyring or any other rate theory, from a molecular point of view we have a well-defined system. We're trying to make highly specific modifications in highly specific places which will help bring the models from a phenomenological point of view to a molecular point of view. In some cases we are changing just one atom.

EHRENSTEIN: Your Fig. A, which shows an energy barrier based on rotation of side groups, has no numbers on the ordinate. Calculations you make based on that may be a good way of testing the validity of the Eyring ideas we've discussed.

MAZET: That model is too simplistic to quantitatively describe the data. But within an order of magnitude we estimate that for the dipole of fluorophenylalanine or of tyrosine substituted at position #1, and for a mean dielectric constant of 2 to 3, the height of the central barrier would change by ~ 2 kT.

EHRENSTEIN: Would that be enough to account for your measured changes in conductance?

MAZET: Qualitatively speaking, it is enough.

LATORRE: The maximum conductance difference you find is a factor of seven. In terms of energy, that means <2 kcal. Maybe we're never going to be able to pick that up from a structural point of view.

BUSATH: Your answer to my initial question was that probably the dipole orientation is a factor in the reduction of channel conductance. What about the possibility that the electron withdrawing power of the side chain affects the electronegativity of the carbonyl, and thus its ability to either coordinate the ion or hydrogen bond with water molecules in the plug? To what extent might that be affecting conductance in the channel?

ANDERSEN: It's very difficult to distinguish between a dipolar effect and an inductive, electron-withdrawing effect. We tried for a long time to decide between these possibilities: To what extent are the conductance changes the result of the local solvation of the permeating ion? To what extent are they the result of ion-dipole interactions, which occur over a larger distance in the molecule? Jean-Luc Mazet has just indicated that our data are consistent with the dipolar model. I should say that we have evidence, using a model-dependent interpretation of our electrical measurements, that the ion binding sites in valine gramicidin A are very close to those deduced from Urry's NMR measurements, or from Koeppe's x-ray crystallographic data. To the extent that we have done the same measurements with these compounds, the ion binding sites do not move very much. That is, the ions seem to bind quite some distance from the place in the molecule where we make a position #1 substitution. The change in affinity with the tyrosine compounds may therefore indeed indicate that there is some ion-dipole interaction. To what extent there is, in addition, an electron withdrawing from the carbonyl oxygens in the center of the channel, I can't say. It's something we've been worried about, and it's certainly something one should think about.

MAZET: The electrostatic model is supported by preliminary experiments, the interpretation of which has been suggested by Dr. Kosower. We have substituted fluorophenylalanine at position #1. The effects on conductance of fluorine at the meta and the para position are similar, although one is electron donating and the other is electron withdrawing.