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Amino Acid Sequence Modulation of Gramicidin Channel Function: Effects of Tryptophan-to-Phenylalanine Substitutions on the Single-Channel Conductance and Duration†

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ABSTRACT: Linear gramicidins with one, two, or three Trp → Phe substitutions in the gramicidin A sequence form $\beta^{6.3}$-helical channels that have widely varying conductances and average durations. The variations in single-channel conductance and average duration are uncoupled. The single-channel conductance decreases as a monotonic function of the number of Trp → Phe substitutions, and the relative conductance decrease induced by a given Trp → Phe substitution is only weakly affected by substitutions at other positions. These results suggest that each Trp influences the conductance independently, most likely through electrostatic interactions between the Trp dipole(s) and the permeant ion (as was deduced previously for aromatic side-chain substitutions at position one [Koeppe, R. E., Mazet, J.-L., & Andersen, O. S. (1990) *Biochemistry 29* (2), 512–520]). Trp → Phe substitutions exert a complex, nonadditive influence on average duration as well as the energetics of heterodimer formation. These changes are presumably due to sequence-specific differences in the channel's surface chemistry—which may be related to ability of the Trp indole NH moieties to form hydrogen bonds with the lipid backbone oxygens and/or interfacial H_2O.

Two central questions in molecular biophysics are how the function of a membrane-spanning ion channel is determined by the channel's conformation and how both conformation and function are governed by the primary sequence. In order to address these questions, it is necessary not only to know a channel's conformation but also to understand the relationship between the physicochemical characteristics of individual amino acid side chains and the energetics of channel formation and ion permeation. We will in this article examine these general questions using the linear gramicidins as a family of prototypic channel formers.

The linear gramicidins are well suited for elucidating the relations that exist between structure and function because the general channel structure is known (see below); the channels are selective for monovalent cations, single-channel experiments thus allow for precise measurements of channel function; individual side chains can be altered readily by using peptide chemical methods (Mazet et al. 1984); and the structural and functional consequences of a sequence substitution can be evaluated (Russell et al., 1986; Durkin et al., 1990; Koeppe et al., 1990).

The amino acid sequence for [Val^1]gramicidin A (gA) is (Sarges & Witkop 1965)

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formyl-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-ethanolamine

The alternating D- and L-amino acids allow the gramicidins to fold into single-stranded $\beta^{6.3}$-helices, as originally proposed by Urry (1971). Many lines of experimental evidence support the general features of the "Urry" structure [e.g., Durkin et al. (1990) and Andersen et al. (1991)] in which two $\beta^{6.3}$-helices form a symmetrical, antiparallel, formyl-NH-terminal-to-formyl-terminal dimer (Arsen'ev et al., 1985, 1986; Nicholson & Cross, 1989; Cifu et al., 1991; Koeppe et al., 1991). The peptide backbone forms the wall of a pore that provides the polar environment necessary for efficient ion translocation across the lipid bilayer. The amino acid side chains project outward to form the channel's exterior surface and do not contact the permeating ions. Nonetheless, the amino acid side chains are important modulators of three measures of channel function: conductance, selectivity, and average duration (Bamberg et al., 1976; Morrow et al., 1979; Heitz et al., 1982; Prasad et al., 1983; Mazet et al., 1984; Russell et al., 1986; Koeppe et al., 1990; Sawyer et al., 1990). In this article we examine the role of the Trp residues at positions 9, 11, 13, and 15.

Total Trp → Phe substitution causes a 20-fold decrease in single-channel conductance, alters the voltage dependence of Cs^+ conductance, and lowers the average single-channel duration (Heitz et al. 1982, 1986; Daumas et al., 1989; Fonseca et al., 1989). A Trp → Phe substitution could alter the conductance by eliminating electrostatic interactions between the dipolar side chains and the permeant ion (Tredgold et al., 1977; Morrow et al., 1979; Mazet, 1984; Etchebest & Pullman, 1985);[1] by changing the torsion angles of the peptide backbone, which would alter the geometry of ion coordination by the channel; by altering channel motion, i.e., the librations of the carbonyl oxygens (Morrow et al., 1979; Urry et al., 1981); or by a combination of these effects.

The average single-channel lifetime (the channel duration) is a measure of the gramicidin dimer dissociation rate constant. The tryptophanyl's influence on channel duration is a complicated function of both enthalpic and entropic contributions. The duration depends on poorly defined interactions between the indole side chains and interfacial H_2O, phospholipid backbone oxygens, acyl chains, and head groups, as well as adjacent amino acids.

We have examined the properties of symmetrical (homodimeric) channels formed by gramicidins with one, two, or three Trp → Phe substitutions as well as the ability of each Trp → Phe substituted gramicidin to form asymmetrical heterodimers with gA. Trp → Phe substitutions were chosen because Phe is aromatic, like Trp, but it is nonpolar, unlike Trp, which is able to be a hydrogen-bond donor through the indole NH, and has a dipole moment of ≈2 Debye along the $C_5 \rightarrow N_1$ axis (Weiler-Feilchenfeld et al., 1970). Thus, Trp → Phe substitutions should yield information as to how a neutral, dipolar amino acid affects peptide–ion interactions (conductance) as well as peptide–lipid and peptide–peptide interactions (average duration and heterodimer formation).

Some of these results have appeared in preliminary form (Becker et al., 1989, 1990).

Materials and Methods

Formyl-L-Val-Gly: L-Valylglycine was from Sigma Chemical Co. (St. Louis, MO) and it was formylated by use of formic–acetic anhydride and recrystallized as described previously (Weiss & Koeppe, 1985).

BOC-L-Trp resin, BOC-L-Phe resin, and BOC-amino acids were from Peninsula Laboratories (Belmont, CA).

Ethanolamine from Aldrich Chemical Co. (Milwaukee, WI) was purified by vacuum distillation and stored in sealed ampules at −20 °C until used. Trifluoroacetic acid from Aldrich was 99+%, spectrophotometric grade. A 4 N HCl solution in dioxane was supplied in sealed ampules by Pierce Chemical Co. (Rockford, IL). Other reagents for peptide synthesis were of the highest grade available.

Diphytanoylphosphatidylcholine was from Avanti Polar Lipids (Birmingham, AL). It was further purified by ion-exchange chromatography (Andersen 1983). *n*-Decane was 99.9% pure from Wiley Organics (Columbus, OH).

Micropipet glass was borosilicate from Corning Glass Works (Corning, NY). Trioctylsilane was from Petrarch Chemical Co. (Bristol, PA).

NaCl was GR grade from EM Science (Cherry Hill, NJ). Before use it was roasted for at least 24 h at 550–650 °C and stored in an evacuated desiccator over NaOH. The water was deionized Millipore Corp. Milli-Q water (Bedford, MA).

Synthesis of Gramicidin A Analogues. Analogues were synthesized by a combination of solid-phase (residues 3–15) and solution-phase (formyl-L-Val[1]-Gly[2]) methods, a procedure chosen to allow future substitutions at positions 1 and 2 without total resynthesis. A Du Pont Model 1000 peptide synthesizer was used to deprotect and extend BOC-L-Trp or BOC-L-Phe resins, with use of the appropriate BOC-amino acid precursors, for residue 14 back through number 3, ending with BOC-L-Ala[3]. BOC-D-Leu was introduced by a direct dicyclohexylcarbodiimide (DCCD) coupling procedure (Stewart & Young, 1984); all other BOC-amino acids were coupled as the symmetrical anhydrides (Stewart & Young, 1984), which were preformed and prefiltered to remove dicyclohexylurea.

The resulting BOC-13-residue peptides were cleaved from the resin in ethanolamine/methanol (50:50) or ethanolamine/DMF (50:50) for 12–24 h at 55 °C. Some of the hydrophobic peptides precipitated after cleavage from the resin; in those cases either DMF or dichloromethane was added to redissolve the peptide before filtering off the resin. The filtered peptides were reprecipitated by addition of 4 volumes of H_2O, dried, resuspended in methanol/DMF or methanol/dichloromethane (with heating if necessary), collected by centrifugation, and purified on a preparative phenyl-silica HPLC column (Koeppe & Weiss, 1981; Koeppe et al., 1985). The BOC group was removed with 2 N HCL in dioxane/methanol at 50 °C for 1 h under N_2; the deprotected peptides were purified on AGMP-50 ion-exchange columns (Bio-Rad) as previously described for desformyl-gramicidin (Weiss & Koeppe, 1985). Diphenyl phosphorazidate was used to couple formyl-L-Val-Gly to a 1 μmol sample of each deprotected 13-residue peptide, and the final products were twice purified by reversed-phase HPLC (Weiss & Koeppe, 1985).

Planar Bilayer Experiments. Single gramicidin channels were studied at 25 ± 1 °C in planar diphytanoylphosphatidylcholine/*n*-decane bilayers using the bilayer punch (Andersen, 1983). Single-channel current transition ampli-

[1] Tredgold et al. (1977) first proposed that dipolar aromatic side chains may modulate the single-channel conductance of gramicidin channels, but they did not observe any difference in the channels that nominally were formed by W(9,11,13,15)FgA or W13FgA and channels formed by gA. This, in retrospect, erroneous result could be explained if their samples were contaminated by gA (because the gel filtration columns used to purify the sequence substituted gramicidins retained gA from a previous purification), because gA's relatively higher channel-forming potency would cause it to predominate over Trp → Phe trisubstituted channels [see Andersen (1984)].

tudes and durations were determined in symmetrical 1.0 M NaCl solutions at applied potentials of 100 or 200 mV. The 3–20-μL aliquots of stock solutions containing the appropriate gramicidin dissolved in ethanol (to $\approx 10^{-8}$ M) were added to both aqueous solutions. The total amount of ethanol in the electrolyte solutions never exceeded 0.5% (v/v). Channel activity was titrated empirically to $\approx$1 appearance/s in order to ensure accurate counting of the number of single-channel transitions. In the heterodimer (hybrid channel) experiments, care was taken to ensure that each gramicidin type was at the same concentration at both membrane–solution interfaces. To this end, the aqueous solutions were stirred for 5 min after addition of the gramicidins, at which time the membrane was broken and reformed several times.

Data Analysis. Measurement of Single-Channel Currents. The single-channel current transitions were identified on-line (Andersen, 1983). The current signal was filtered (30–500 Hz) with an 8-pole Bessel filter before input to the A/D converter. The current was sampled at $10f_c$, where f_c is the filter's −3-dB cutoff frequency. The pre- and posttransition current levels were determined as the average of 20 sample points obtained immediately before or after the transition (except for the short-lived channels formed by W(9,13,15)FgA where only 10 sample points were used).

Calculation of Single-Channel Conductance and Number of Channels per Experiment. Current transition amplitude histograms were converted to chord conductance histograms. The mean and standard deviation of the conductance and the number of channels in each experiment were calculated after the limits around the peaks were assigned, usually using an iterative algorithm (Durkin et al., 1990). In heterodimer experiments with W(9,13,15)FgA and gA the limits were assigned manually because of their close proximity. The final mean and standard deviation of the conductance at a single potential is the average of at least four experiments weighted by the number of channels in each experiment.

Measurement of Average Channel Durations. The continuous probability distribution of gramicidin single-channel durations is given by

$$N(t)/N(0) = \exp(-t/\tau) \quad (1)$$

where $N(t)/N(0)$ is the probability density at time t and τ is the average channel duration (Hladky & Haydon, 1972; Andersen, 1978). The algorithm used for the measurement of the individual gramicidin channel durations is described by Durkin et al. (1990). Briefly, the peaks delimited in the conductance analysis define the group of channels used for the duration analysis. Channel appearances and subsequent disappearances were matched, with the intervening time being the channel duration. If more than one channel of the selected type was conducting at a given time, a random number generator was used to assign appearances to disappearances. Duration histograms from several experiments were pooled and transformed into survivor histograms. Both duration and survivor histograms are exponential decays with the same (single) time constant (the average single-channel duration) (Cox & Lewis, 1966), which was estimated by using a maximum likelihood algorithm (Hall & Sellinger, 1981).

Results

Eight Trp → Phe sequence substituted gramicidin analogues were examined: four with one substitution (W9FgA, W11FgA, W13FgA, and W15FgA); one with two substitutions [W(9,15)FgA]; and three with three substitutions [W(11,13,15)FgA, W(9,13,15)FgA, and W(9,11,13)FgA].[2] The numbers designate the position(s) of the Trp → Phe sequence substitutions.

Table I: Properties of Trp → Phe Substituted Gramicidin Channels[a]

gramicidin	conductance (ps)	av duration (ms)
W9FgA	6.0 ± 0.1	1000
W11FgA	8.7 ± 0.3	2300
W13FgA	11.2 ± 0.2	800
W15FgA	10.9 ± 0.2	790
W(9,15)FgA	4.1 ± 0.1	750
W(11,13,15)FgA	3.4 ± 0.1	2100
W(9,13,15)FgA	3.0 ± 0.5	5
W(9,11,13)FgA	2.1 ± 0.1	2300
gramicidin A	15.0 ± 0.2	840

[a] Single-channel conductances and average durations of gA and Trp → Phe sequence substituted gramicidins are given under conditions of 1.0 M NaCl and 200 mV. Mean ± standard deviation is given for conductance values.

Single-Channel Characteristics. All of the analogues form channels that are qualitatively similar to gA channels (Figure 1A,B), but with rather different overall "channel-forming potencies", which we determine on the basis of the nominal gramicidin concentration in the (5-mL) aqueous solutions necessary to give a channel appearance rate $\approx$1/s.[3] This concentration varied from 4×10^{-10} M (n = 34, where n denotes the number of independent experiments) for the trisubstituted gramicidins to 6×10^{-11} M (n = 8) for the monosubstituted gramicidins, which should be compared to 2×10^{-11} M (n = 10) for gA. Somewhat surprisingly, the channel-forming potency decreases as the number of Trp → Phe substitutions increases.

Like gA, Trp → Phe substituted gramicidins form "simple" channels, where channel formation is represented by an abrupt transition from the base-line current to a new, stable conducting current level. Channel dissociation is represented by a transition from the conducting current level back to the base line. The current transition amplitude histograms of channels formed by gA and the Trp → Phe sequence substituted gramicidins are narrow, symmetrical peaks (Figure 1A,B). The single-channel survivor plots are likewise described by single-exponential decays (Figure 1A,B). Nevertheless, the different Trp → Phe substituted gramicidins form channels that exhibit large variations in conductance and average duration from one channel type to another.

In 1.0 M NaCl (200 mV), Trp → Phe substitutions lower the conductance of symmetrical (homodimeric) channels from 15.0 ± 0.2 pS in gA channels to between 11.2 ± 0.2 and 2.1 ± 0.1 pS (Table I). The conductances correlate with the number of Trp residues in the channels: channels formed by gA and the singly substituted W9FgA, W11FgA, W13FgA, and W15FgA form a "high" conductance group, with conductances greater than $\approx$6 pS; channels formed by trisubstituted W(11,13,15)FgA, W(9,13,15)FgA, and W(9,11,13)FgA form a "low" conductance group with conduc-

[2] Attempts to synthesize W(9,11,15)FgA by the same procedure as was used for the other analogues were not successful because the deprotected 13-residue peptide was too insoluble under the conditions used for the formyl-L-Val-Gly coupling reaction. Thus, amino acid sequence-dependent alterations in behavior can be seen even during a gramicidin's synthesis.

[3] The channel-forming potency thus determined is comprised of three terms: the effective adsorption coefficient of gramicidin at the membrane/solution interface; the partitioning of the adsorbed gramicidin among various conformers [including the insertion into the bilayer; cf. Cifu et al. (1991)]; and the association rate constant for channel formation from channel-forming monomers. [The predominant mechanism of channel formation is the transmembrane association of monomers from *opposite* monolayers (O'Connell et al., 1990).]

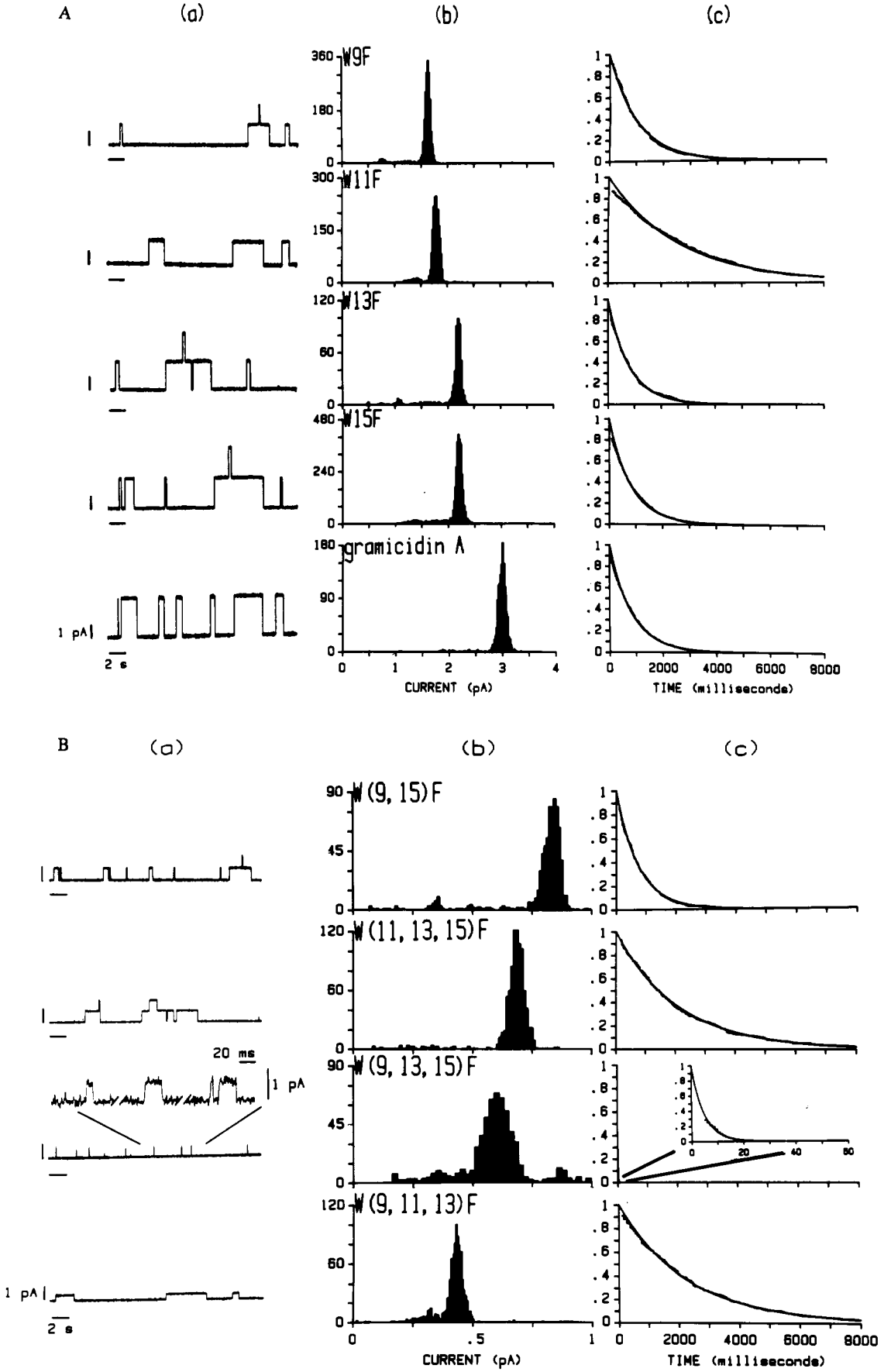

FIGURE 1: Properties of channels formed by sequence-substituted gramicidins. (A) Channels formed by Trp → Phe monosubstituted gramicidins and gA. (a) Single-channel current records, (b) current transition amplitude histograms, and (c) normalized duration survivor plots for (from top to bottom) W9FgA, W11FgA, W13FgA, W15FgA, and gA. (B) Channels formed by Trp → Phe di- and trisubstituted gramicidins. (a) Single-channel current records, (b) current transition amplitude histograms, and (c) normalized duration survivor plots for (from top to bottom) W(9,15)FgA, W(11,13,15)FgA, W(9,13,15)FgA, and W(9,11,13)FgA. Conditions: 1.0 M NaCl, 200 mV. Current traces were filtered at 50 Hz. The higher resolution current trace of W(9,13,15)FgA is filtered at 500 Hz. The results are summarized in Table I.

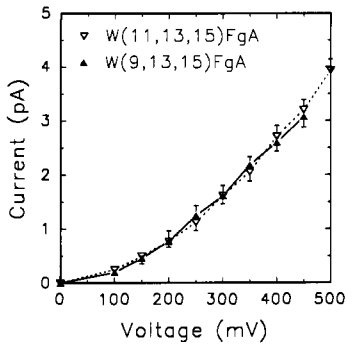

FIGURE 2: Superimposition of current–voltage relationships for channels formed by W(11,13,15)FgA and W(9,13,15)FgA in 2.0 M NaCl.

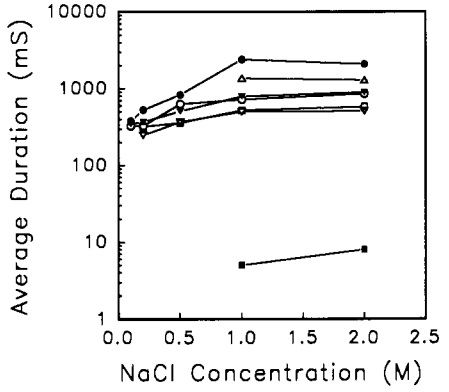

FIGURE 3: Single-channel duration as a function of permeant ion concentration for channels formed by Trp → Phe sequence substituted gramicidins. The average durations increase toward an asymptote as [NaCl] increases. Symbols: ▼, W9FgA; ●, W11FgA; ▽, W13FgA; ○, W15FgA; □, W(9,15)FgA; △, W(11,13,15)FgA; ■, W-(9,13,15)FgA.

tances less than ≈3 pS; and channels formed by the disubstituted W(9,15)FgA are intermediate, at ≈4 pS. The correlation between the conductance and number of Trp residues persists throughout an extended range of voltages. In 2.0 NaCl, for example, the W(9,13,15)FgA and W(11,13,15)FgA channels have current–voltage relations that are superimposable (Figure 2).

Channels formed by Trp → Phe substituted gramicidins display a 500-fold range in single-channel duration (τ), and the substitutions can increase or decrease τ (Table I). For all analogues, τ increases with increasing [NaCl] (0.1–1.0 M) and becomes approximately [NaCl]-independent above 1.0 M (Figure 3). The variations in channel duration persist through both the [NaCl]-dependent and the [NaCl]-independent ranges. In 1.0 M NaCl (200 mV), τ varies from ≈5 ms for W(9,13,15)FgA channels to ≈800 ms for gA channels and to ≈2500 ms for W11FgA and W(9,11,13)FgA channels. The most conspicuous common feature in these results is that the residue at position 11 is an important determinant of channel duration because gramicidins with a Phe at position 11 form the channels that have the longest durations.

Interestingly, there is no correlation between the durations and the conductances (Figure 4). In 1.0 NaCl (200 mV), W(9,13,15)FgA and W(11,13,15)FgA form channels that have similar conductances (and current–voltage relations, Figure 2) but durations that differ ≈500-fold.

Structural Equivalence. Trp → Phe substituted gramicidins can produce dramatic changes in single-channel conductance and duration *without* altering the channel's qualitative features: single-channel current transitions obtained with W-(9,13,15)FgA and with W11FgA, for example, "look" like gA

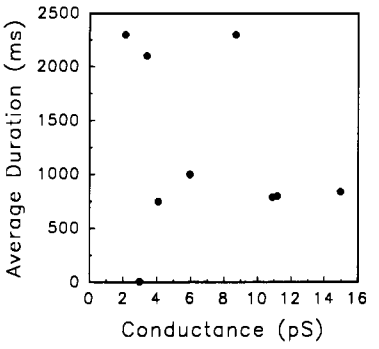

FIGURE 4: Average duration versus single-channel conductance. The conductances and durations are uncorrelated. Conditions: 1.0 M NaCl, 200 mV.

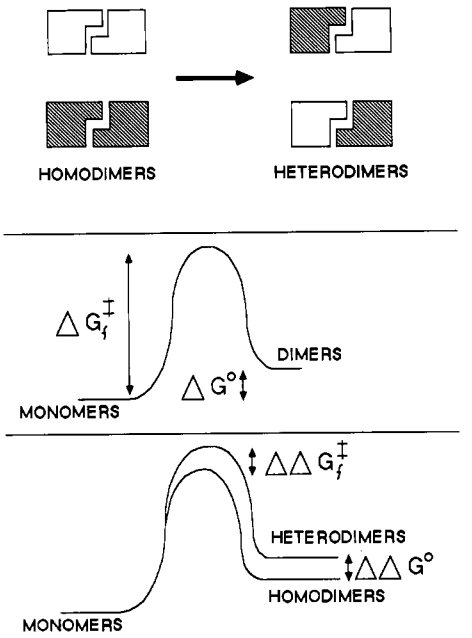

FIGURE 5: Schematic representation of the types of channels formed in a heterodimer experiment and the energetics of heterodimer formation and equilibrium. (Top) Four channel types are formed when a Trp → Phe sequence substituted gramicidin and gA are added to the same bilayer: two different homodimers and a heterodimer with two different orientations. (Middle) The dimerization reaction and a representation of its reaction coordinate diagram are shown. (Bottom) The energetics of homo- and heterodimer formation are shown.

single-channel current records—even though the average single-channel durations differ 500-fold and the conductances differ 5-fold. This general similarity suggests in itself that Trp → Phe sequence substitutions do not *substantially* alter the channel's β-helical motif. Nevertheless, the rather different behavior of the W(9,13,15)FgA channels could result from structural alterations, i.e., from a change in helix sense. The possibility of a conformational change was tested.

If amino acid substitutions alter the β-helical backbone conformation of gramicidin channels, such a change would be reflected in the statistical properties of heterodimers formed between gA and the amino acid substituted analogue, which can be quantified by two measures of the energetics of heterodimer channel formation and stability (Figure 5): $\Delta\Delta G^{\ddagger}_{f}$ and $\Delta\Delta G^{\circ}$ [cf. Durkin et al. (1990)]. $\Delta\Delta G^{\ddagger}_{f}$ is the activation energy for heterodimer formation relative to the average of the activation energies for the corresponding homodimers given by

$$\Delta\Delta G^{\ddagger}_{f} = \Delta G^{\ddagger}_{h} - (\Delta G^{\ddagger}_{gA} + \Delta G^{\ddagger}_{Sub})/2 \qquad (2)$$

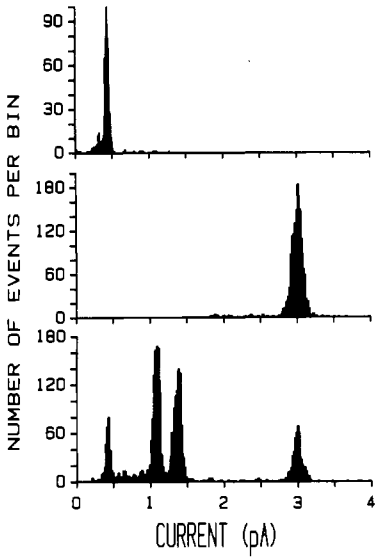

FIGURE 6: Histograms of single-channel current amplitudes for W(9,11,13)FgA (top), gA (middle), and their mixture (bottom). The top histogram contains 1163 transitions, of which 883 (76%) are in the main peak at 0.4 pA. The middle histogram contains 1430 transitions, of which 1376 (96%) are in the main peak at 3.0 pA. The bottom histogram contains 2950 transitions, of which 326 (11%) are in the peak at 0.4 pA, 983 (33%) are in the peak at 1.1 pA, 894 (31%) are in the peak at 1.4 pA, and 477 (16%) are in the peak at 3.0 pA. The relative heterodimer appearance rate is 2.4 (see Table II).

where the subscripts h, gA, and Sub refer to the heterodimers and the two homodimers, respectively. $\Delta\Delta G^\circ$ is the standard free energy difference for the heterodimer–monomer equilibrium relative to that of the homodimer–monomer equilibria and is given by

$$\Delta\Delta G^\circ = \Delta G^\circ_h - (\Delta G^\circ_{gA} + \Delta G^\circ_{Sub})/2 \quad (3)$$

$\Delta\Delta G^\circ = \Delta\Delta G^\ddagger_f - \Delta\Delta G^\ddagger_d$, where $\Delta\Delta G^\ddagger_d$ is the activation energy for heterodimer dissociation relative to that for homodimer dissociation. When each monomer type is at the same concentration in each half of the lipid bilayer (Durkin et al., 1990) then

$$\Delta\Delta G^\circ = -RT \ln [n_h\tau_h/(2(n_{gA}\tau_{gA}n_{Sub}\tau_{Sub})^{0.5})] \quad (4)$$

$$\Delta\Delta G^\ddagger_f = -RT \ln [n_h/(2(n_{gA}n_{Sub})^{0.5})] \quad (5)$$

where n_{gA}, τ_{gA}, n_{Sub}, τ_{Sub}, n_h, and τ_h denote the number and average duration of the homodimers and heterodimers in a given experiment, respectively. (All channel types are observed over the same period of time, and the channel appearance rates are proportional to the number of each channel type observed in an experiment.)

All Trp → Phe substituted gramicidins can form heterodimers with gA [see also Sawyer et al. (1990)]. The sum of the amplitude histograms from several experiments in which W(9,11,13)FgA and gA were added symmetrically to both aqueous phases is shown in Figure 6. The current amplitude histograms from these experiments show four channel types (peaks): two peaks whose positions match the symmetrical W(9,11,13)FgA and gA channels and two intermediate peaks representing asymmetrical heterodimers formed by the combination of a W(9,11,13)FgA monomer and a gA monomer in two orientations. (At this potential the two orientations of the heterodimers can be distinguished by the virtue of the intrinsic asymmetry of the potential energy profile of these channels).

The results we obtained are summarized in Table II. From these results $\Delta\Delta G^\circ$ and $\Delta\Delta G^\ddagger_f$ were estimated (Table III).

Table II: Appearance Rates and Average Durations in Gramicidin Heterodimer Experiments[a]

gramicidin analogue	relative appearance rate	durations: Trp → Phe homodimer (ms)	durations: heterodimer (ms)	durations: gramicidin A homodimer (ms)
W9FgA	1.06 ± 0.03	750	830	910
W11FgA	0.96 ± 0.18	2400	1500	810
W13FgA	1.01 ± 0.11	490	590	660
W15FgA	1.04 ± 0.26	790	930	960
W(9,15)FgA	1.58 ± 0.45	520	630	920
W(11,13,15)FgA	2.12 ± 0.24	1360	880	710
W(9,13,15)FgA	1.40 ± 0.41	5	20	700
W(9,11,13)FgA	3.02 ± 0.37	2300	1020	850

[a] The relative appearance rate is defined as $n_h/[2(n_{gA}n_{Sub})^{0.5}]$, where n_{gA}, n_{Sub}, and n_h are the number of gA, Trp → Phe substituted gramicidin, and heterodimers in an experiment (given as mean ± standard deviation). A relative appearance rate of 1 indicates no energetic cost for heterodimer formation, i.e., $\Delta\Delta G^\ddagger_f = 0$. Notice the day-to-day variation in the gA channel's average duration. Conditions: 1.0 M NaCl, 200 mV.

Table III: Energetics of Heterodimer Association and Equilibrium[a]

gramicidin	$\Delta\Delta G^\ddagger_f$ (kJ/mol)	$\Delta\Delta G^\circ$ (kJ/mol)
W9FgA	−0.2	−0.2
W11FgA	0.0	−0.2
W13FgA	0.1	0.0
W15FgA	−0.1	−0.3
W(9,15)FgA	−1.1	−0.9
W(11,13,15)FgA	−1.8	−1.6
W(9,13,15)FgA	−0.8	1.9
W(9,11,13)FgA	−2.6	−2.0

[a] Energetics of heterodimer formation and equilibrium is given, calculated from appearance rates and durations in Table II and eq 4 and 5. Conditions: 1.0 M NaCl, 200 mV.

For heterodimers formed by gA and all gramicidins with a single Trp → Phe substitution, $\Delta\Delta G^\circ$ and $\Delta\Delta G^\ddagger_f$ are both within $RT/4$ of zero (Table III). (RT = 2.5 kJ/mol at 25 °C.) Interestingly, $\Delta\Delta G^\circ$ and $\Delta\Delta G^\ddagger_f$ both become less than zero as the number of Trp → Phe substitution increases to 2 or 3, except for the W(9,13,15)FgA/gA heterodimers where $\Delta\Delta G^\ddagger_f$ is less than zero but $\Delta\Delta G^\circ$ is greater than zero. Thus, for the di- and trisubstituted analogues, heterodimer formation is energetically favored relative to homodimer formation. Since there is no energetic cost associated with heterodimer formation, we conclude that Trp → Phe substituted gramicidin channels are structurally equivalent to gA channels. This structural equivalence, however, relates to the conformation of the peptide backbone, not necessarily to the individual side-chain orientations.

Discussion

The indole group of Trp possesses two features that make it distinct from other bulky (usually hydrophobic) side chains: the indole group has a dipole moment of ≈2 Debye along the $C_5 \rightarrow N_1$ axis (Weiler-Feilchenfeld et al., 1970); and the indole NH group can hydrogen bond (Stryer, 1988) to water or to the polar phospholipid headgroup. These two features are important for gramicidin channel function and may also account for the reason why Trp residues in the membrane-spanning segments of other integral membrane proteins tend to cluster toward the membrane–solution interface (Henderson et al., 1990; Michel & Deisenhofer, 1990).

We begin by discussing the effects of Trp → Phe substitutions on channel structure (heterodimer formation) and channel-forming potency and the implications for the homo-

and heterodimer formation and dissociation rate constants. We then turn to the conductance and duration results and suggest that the indole groups influence channel function by two distinct mechanisms: by altering channel duration through direct interactions with (adjacent) lipid and H_2O, and by altering the permeant ions' energy profile without contacting the ions directly. Finally, we relate the differences in channel duration to possible differences in the channels' surface chemistry.

Structural Equivalence

The dipole moment and hydrogen-bonding ability of the indole side chains (outlined above) are important for channel function, as evidenced by the variations in the single-channel conductances and durations among the channels formed by the various Trp → Phe substituted gramicidins. Importantly, however, all of the Trp → Phe substituted gramicidins form channels that have a single, predominant β-helical backbone conformation that is equivalent to that of gA channels. This is shown by the qualitative similarity of the various channels and by the ability of each of the substituted gramicidins to combine with gA to form heterodimers.

The parameters $\Delta\Delta G^\circ$ and $\Delta\Delta G^\ddagger_f$ provide information about subunit-specific interactions among the monomers that form the different channel types (Durkin et al., 1990). If the monomers had to refold to form heterodimers, or if there were a "strain" on the heterodimer, that would be reflected in $\Delta\Delta G^\ddagger_f$ or $\Delta\Delta G^\circ$ being greater than zero. Because $\Delta\Delta G^\ddagger_f \leq 0$ for all combinations tested, all the Trp → Phe substituted gramicidins form channels that are structurally equivalent to gA channels, i.e., right-handed formyl-NH-to-formyl-NH $\beta^{6.3}$-helical dimers [see Koeppe et al. (1991)].

For the monosubstituted gramicidins, there is no energetic preference for heterodimers or homodimers ($\Delta\Delta G^\ddagger_f \approx 0$ and $\Delta\Delta G^\circ \approx 0$). For the di- and trisubstituted gramicidins, however, heterodimer formation is slightly preferred, $\Delta\Delta G^\ddagger_f < 0$. Futhermore, since $\Delta\Delta G^\circ \approx \Delta\Delta G^\ddagger_f$ in all cases, except W(9,13,15)FgA, the equilibrium preference for heterodimers (as reflected in $\Delta\Delta G^\circ$) is primarily due to a change in monomer association (i.e., in $\Delta\Delta G^\ddagger_f$) while the energetics of dissociation ($\Delta\Delta G^\ddagger_d$) are unaffected. Thus, once a heterodimer has formed there is no energetic cost (e.g., unusual strain on the backbone) that would promote dissociation. For W-(9,13,15)FgA, however, $\Delta\Delta G^\ddagger_f \neq \Delta\Delta G^\circ$; that is, heterodimer formation with gA is favored but dissociation is even more favored; i.e., in this case the heterodimers are destabilized relative to the homodimers. We do not understand the basis for these alterations, but all gramicidins with a Trp at position 11 form channels that have shorter durations than the corresponding channels formed with a Phe at that position.

A negative $\Delta\Delta G^\ddagger_f$ implies that heterodimer formation is more favorable than would be predicted if both monomers in a heterodimer contributed independently to the formation rate constant (i.e., if there were no monomer-specific interactions). The structural basis for this energetic preference of heterodimer formation is unclear because the "transition state" is unknown. But altered peptide–lipid interactions could be important because the membrane deformation surrounding the channel contributes to the energetics of channel formation and dissociation (Huang 1986; Helfrich & Jakobsson, 1990; see below). Because of specific peptide–lipid interactions, the membrane deformation surrounding the asymmetrical heterodimeric channels may be asymmetric across the bilayer (just as the amino acid sequence of the heterodimers themselves is asymmetric across the bilayer). There may thus be no simple (linear) relation between the energetics of deforming the membrane surrounding the heterodimers or adjacent to the "parent" homodimers.

Channel-Forming Potencies and Average Durations

Dimeric gramicidin channels are in thermal equilibrium with nonconducting gramicidin monomers [Hladky & Haydon, 1972; Bamberg & Läuger, 1973; Zingsheim & Neher, 1974; see also Cifu et al. (1991)] and form by the transmembrane apposition of monomers from opposing halves of the bilayer (O'Connell et al., 1990). To a first approximation, channel formation and disappearance can thus be described as shown in Scheme I, where k_1 and k_{-1} denote the overall rate constants for channel formation and dissociation, respectively, and f is the actual dimer formation rate (channel appearance rate). A gramicidin's channel-forming potency is a measure of k_1 (as well as other factors, see footnote 3), while its average duration (τ) is a measure of k_{-1} (since $k_{-1} = 1/\tau$).

Scheme I

$$\underset{\text{(nonconducting)}}{\text{2 monomers}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(conducting)}}{1\ \beta^{6.3}\text{-helical dimer}}$$

$$f = k_1[\text{monomer}]^2$$

Channel-Forming Potency: Relative Magnitudes of the Formation Rate Constants. All the Trp → Phe substituted gramicidins form channels, but with varying channel-forming potencies. The channel-forming potency, as previously defined, is inversely proportional to the square root of the overall rate constant for channel formation (Scheme I, see also footnote 3) (Bamberg & Läuger, 1973; Veatch & Stryer, 1977; Cifu et al., 1991). The (geometric) mean of the square of the gramicidin concentration needed to give an appearance rate of $\approx$1/s was 4×10^{-22} M^2 for gA, 4×10^{-21} M^2 for the monosubstituted gramicidins, and 2×10^{-19} M^2 for the trisubstituted gramicidins. Thus, compared to gA, the overall formation rate constant is decreased by $\approx$10-fold for the monosubstituted and $\approx$500-fold for the trisubstituted gramicidins, respectively.[4]

The differences in channel-forming potencies show that the overall energy barrier for homodimer formation is higher for the analogues than for gA, while the relative excess of heterodimers means that the heterodimer formation energy barrier is lower than would be expected if both monomers contributed to it independently. Thus, sequence-substituted homodimer formation seems to incur an additional energetic cost that is not completely reflected when heterodimers are formed with gA; e.g., there exists a strain during the formation of a sequence-substituted homodimer that is relieved when at least one of the two channel-forming molecules is replaced by a gA molecule. (In principle, the "excess" strain could arise in the formation of either homodimer. In light of the decreased channel-forming potency of the Trp → Phe substituted analogues, however, it is most likely to be associated with these compounds.)

Channel Duration: Relative Magnitudes of the Dimer Dissociation Constants. Trp → Phe substitution alters k_{-1}, which is remarkable because the substitutions are near the channel entrances, whereas dimerization is at the formyl NH terminus. There is no simple relation between k_{-1} and the identity of the residues at the four positions tested here except

[4] In lyso-PC micelles, the *transition* from the solvent conformation to the "channel" conformation depends on the gramicidin's amino acid sequence (Sawyer et al., 1990). So, Trp → Phe substitution may affect the heterodimer formation rate constant by altering the conformation of the nonconducting monomers and/or the conformation of the transition state.

that a Phe at position 11 always seems to decrease k_{-1} (increase τ): the durations of channels formed by of W11FgA, W-(11,13,15)FgA, and W(9,11,13)FgA are greater than that of gA channels, while the durations of channels formed by W9FgA, W13FgA, W15FgA, W(9,15)FgA, and W-(9,13,15)FgA are less than or approximately equal to that of gA channels.

Monosubstitution at positions 9, 13, and 15 or the (9,15) disubstitution does not alter τ significantly. Likewise, when Trp-9 and Trp-13 are substituted by Phe in W11FgA to give W(9,11,13)FgA or when Trp-13 and Trp-15 are substituted by Phe in W11FgA to give W(11,13,15)FgA, τ is unaffected. By contrast, when Trp-13 and Trp-15 are substituted by Phe in W9FgA to give W(9,13,15)FgA, τ decreases dramatically. The amino acids at positions 9, 11, 13, and 15 acids therefore exert a nonadditive influence on τ.

Ion Permeation

All the Trp → Phe substituted gramicidins form channels with conductances that are less than that of gA channels, an effect that also was seen with a Trp → Phe substitution at position 1 (Mazet et al., 1984). The indole groups at positions 9, 11, 13, and 15 in gA thus enhance cation permeation through the channel. The magnitude of the conductance decrease is roughly proportional to the number of Trp → Phe substitutions. A similar effect was found on the Cs^+ permeability of gramicidins in which two or more Trps were replaced by napthylalanines (Daumas et al., 1989).

A simple kinetic description of Na^+ movement through gramicidin channels is provided by the symmetrical, three barrier–two site–one ion model (Läuger, 1972; Hladky, 1974; Procopio & Andersen, 1979; Finkelstein & Andersen, 1981) given in Scheme II, where ion movement is divided into three steps (movement over the entry, translocation, and exit barriers) and Na_l^+, Na^+G, GNa^+, and Na_r^+ denote ions in the left aqueous phase in the left and right binding site, and the right aqueous phase, respectively.

Scheme II

$$Na_l^+ + G \leftrightarrow Na^+G \leftrightarrow GNa^+ \leftrightarrow G + Na_r^+$$

Trp → Phe substitutions alter (decrease) at least one of the rate constants in Scheme II, a change we attribute to the removal of the Trp rather than the insertion of the Phe. Mechanistically, if a Trp interacts favorably with the permeant ion as it passes across an energy barrier, i.e., stabilizing the "transition state", a Trp → Phe replacement would increase the height of that energy barrier and decrease the associated rate constants. Conversely, if a Trp were to interact unfavorably with an ion residing in an energy well, a Trp → Phe replacement would stabilize the ion–channel complex—which, again, would decrease one (or more) rate constant. In the latter case, however, the conductance decrease should be associated with an increase in the channel's ion affinity, which is not observed (Becker et al., 1989). The Trps surround the channel entrance; the entry and exit rate constants are thus likely to be altered by the substitutions. As a single substitution is moved from position 15 to 9 (toward the center of the bilayer), the translocation rate constant may be affected as well.

The effect of a Trp → Phe substitution on Na^+ permeation is largely independent of the identity of the other three aromatic residues, such that the conductances of the channels formed by the monosubstituted gramicidins can be used to predict qualitatively the conductances of the di- and trisubstituted channels (Table I). Among the monosubstituted gramicidins, the single-channel conductance decreases as the Phe is moved from position 15 to 9. In a converse manner, for the trisubstituted gramicidins the conductance *increases* as the (single) Trp is moved from position 15 to position 9. For the disubstituted gramicidin, the conductance is intermediate to the conductance of the mono- and trisubstituted gramicidins. Overall, these results suggest that electrostatic interactions between the permeant ions and the dipoles of the side chains serve to increase the single-channel conductance (Tredgold et al., 1977; Mazet et al., 1984; Etchebest & Pullman, 1985; Koeppe et al., 1990).

If the predominant effect of a Trp → Phe substitution on ion permeation is the removal of electrostatic interactions, the magnitude and sign of the interaction between the indole dipole and the permeant ion will depend on the side chain's orientation (Mazet et al., 1984; Etchebest & Pullman, 1985). Our results thus suggest that the four Trp residues are substantially equivalent and thus probably have similar orientations with respect to the channel axis.

Differential Influence of Trp → Phe Substitutions on Conductance and Duration

A simple explanation for the nonadditivity of the effect of di- and trisubstitution on the channel durations would be that the substitutions induce alterations in the peptide backbone, but this cannot explain our results. First, the channels formed by the substituted gramicidins are structurally equivalent to gA channels (see above). Second, the conductance should be sensitive to an altered backbone conformation since the permeant ions are solvated by the carbonyl oxygens of the backbone. Thus, if the difference in duration (and heterodimer energetics) resulted from backbone effects, there should be a correlation between conductance and duration, which is not found (Figure 4). Third, τ for W(11,13,15)FgA channels is ≈500-fold longer than for W(9,13,15)FgA channels, but the current voltage relationships for channels formed by these two gramicidins in 2.0 M NaCl are nearly superimposable (Figure 2). Taken together, these results imply that the variations in τ and heterodimer energetics result from nonadditive interactions at the channel's *external* surface (which contacts the lipid molecules and the aqueous solution directly), while the conductance variations result from throughspace interactions between the side chains and the permeant ions (which do not directly contact the side chains). Both effects are superimposed on an invariant peptide backbone conformation.

Channel Surface Interactions I: Membrane Deformation

Model-building studies (Venkatachalam & Urry, 1983; Koeppe & Kimura, 1984; Roux & Karplus, 1988) and two-dimensional nuclear magnetic resonance results (Arsen'ev et al., 1986) place a 2.6-nm limit on the length of the pore in gA channels. The length of the channel's hydrophobic exterior is slightly less, while the equilibrium (average) thickness of solvent-containing diphytanoylphosphatidylcholine (DPhPC) bilayers is about twice that (Benz & Janko, 1976). Accordingly, gramicidin channels can form only if the surrounding bilayer is deformed, and the deformation energy will modulate the channel duration (Rudnev et al., 1981; Elliott et al., 1983; Huang, 1986; Helfrich & Jakobsson, 1990). In our experiments, the depth of the deformation around a channel should be constant because all channels are 15 amino acid $\beta^{6.3}$-helical dimers. At a constant deformation depth, at least two mechanisms can contribute to the alterations in the single-channel duration and heterodimer energetics: the shape of the deformation may be amino acid sequence-dependent (i.e., a change in side-chain bulk could change the splay term of the deformation energy) and the magnitude of the thermal

fluctuations of the deformation needed to dissociate the channel into nonconducting monomers could be sequence-dependent. The latter possibility is supported by the finding that the much smaller τ of W(9,13,15)FgA channels relative to gA channels is also observed in "solvent-free" DPhPC/squalane bilayers, which are much thinner than DPhPC/decane bilayers (Benz & Janko, 1976) and should require almost no deformation for channel formation (M. D. Becker and O. S. Andersen, unpublished results). How then do the different chemical characteristics of the Phe and Trp side chains influence the interaction of the channel's external surface with its surroundings?

Channel Surface Interactions II: Indole Hydrogen Bonding

Hydrophobicity is an important factor in gA's insertion into lipid bilayers (Segrest & Feldmann, 1974; Jähnig, 1983). Trp → Phe substitutions will alter the hydrophobic interaction between the channel and the lipid molecules because Trp and Phe residues have different hydrophobicities. On hydropathy scales Phe side chains are uniformly hydrophobic (Eisenberg, 1984; Engelman et al., 1986; Lesser & Rose, 1990). Trp side chains, in contrast, are classified quite differently from one scale to another because they are amphiphilic [cf. Jacobs and White (1989) and O'Connell et al. (1990)]. It is thus likely that one or more of the Trp side chains in the gA channel may project into the bilayer's interfacial region to hydrogen bond to H_2O or to a lipid's ester carbonyl oxygen—in accordance with Trp's ability to interact with aqueous phase paramagnetic ions (Feigenson et al., 1977).

Given the amphiphilic nature of Trp, a Trp → Phe substitution could either increase or decrease τ for a gramicidin channel. If the substituted Trp were in a hydrophobic environment, where it could not assume a conformation that would allow it to form a hydrogen bond, a Trp → Phe substitution would increase the channel duration. Conversely, a Trp → Phe substituted gramicidin would have a shorter duration than gA if a Trp in an amphiphilic "pocket" were substituted by a Phe that was not entirely compatible with the pocket. No change would be observed if at a given position either a Trp or Phe could find an environment of similar minimum energy.

A Trp → Phe substitution at positions 13 or 15, near the interfacial region, has only a small effect on τ (W13FgA and W15FgA vs gA). At these positions, a Trp should be able to hydrogen bond to H_2O, but most of its nonpolar surface would be buried. Phe at these positions could also be largely buried. Position 11 is deeper within the bilayer, and it could be difficult for the Trp to hydrogen bond to H_2O or the lipid backbone, while a Phe at this position is likely to be buried in the bilayer's hydrocarbon core. This could explain why a Trp → Phe substitution at position 11 always increases the average duration [W11FgA, W(9,11,13)FgA, and W(11,13,15)FgA vs W9FgA, W13FgA, W15FgA, W(9,15)FgA, and W-(9,13,15)FgA].

Position 9 is even deeper into the bilayer than position 11, but a Trp → Phe substitution at position 9 has almost no effect on channel duration [W9FgA vs gA and W(9,15)FgA vs W15Fga]. This apparent puzzle may reflect multiple aspects of the position 9 side chain's contribution to channel duration, which depend not only on the hydrophobicity of the environment but also on interactions with the aromatic side chain at position 15 (Weinstein et al., 1980; Urry et al., 1981; Scarlata, 1988). The increased stability that would be gained by placing a more hydrophobic residue at position 9 may thus be negated by a Phe-9/Trp-15 or a Phe-9/Phe-15 interaction that is less favorable than the Trp-9/Trp-15 interaction. It is also possible that the interaction between interfacial H_2O and the position 9 residue could be modulated by the bulky position 15 residue. Thus, the position 9 side chain may sit in an amphiphilic pocket that can accommodate Trp as well as a Phe.

This model for Trp/Phe hydrophobicity/aromatic interactions can also provide a rationale for the permeation results: all four Trps seem to have an equivalent orientation, which is determined by the anisotropy of the interfacial region. To account for the fact that the Trp side chains do not contribute independently to the τ, we propose that subtle changes in side-chain chemistry translate into (relatively) large changes in the shape of the membrane deformation or in the transmission of the deformation energy to the dimerization site.[5]

SUMMARY

In this article we have begun to elucidate the functional and structural role of each of the four Trp residues in gA. The results show that single-channel studies can yield information about the likely orientations of the Trp residues and that the amphiphilic character of these residues must be considered explicitly when discussing their structural and functional significance. These results thus provide insights into why Trp (and Tyr) residues in integral membrane proteins tend to cluster at the membrane–solution interface.

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[5] In making these arguments we have used gA as the reference, i.e., emphasized the effects of removing the Trps one by one. The functional roles of the individual Trps could appear to be different if we "added" Trps, i.e., compared single Trp gramidicins to zero Trp gramicidins (gramicidin M$^-$; Heitz et al., 1982), because the individual side chains contribute in a nonlinear fashion to some aspects of function.

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