On the supramolecular organization of gramicidin channels

The elementary conducting unit is a dimer

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ABSTRACT The question, whether the conducting channels formed by the linear gramicidins are dimers (as is generally believed) or tetramers (as has been recently proposed [Stark G., M. Strässle, and Z. Takacz. 1986. *J. Membr. Biol.* 89:23–37; Strässle, M., G. Stark, M. Wilhelm, P. Daumas, F. Heitz, and R. Lazaro. 1989. *Biochim. Biophys. Acta.* 980:305–314]) has been addressed in single-channel experiments. The experimental approach was based on the ability of electrophysiological (single-channel) experiments to resolve the number of hybrid channel types that could form between gramicidin A or C and O-pyromellityl-gramicidin A or C (in which a pyromellitic acid residue has been esterified to the ethanolamine -OH group [Apell, H.-J., E. Bamberg, H. Alpes, and P. Läuger. 1977. *J. Membr. Biol.* 31:171–188]). The presence of the bulky, negatively charged pyromellityl group at the channel entrances endows the hybrid channels with characteristically different features and thus facilitates the resolution of the different hybrid channel types. Only two hybrid channel types were detected, indicating that the conducting channels are membrane-spanning dimers. There was likewise no evidence for lateral association between conducting channels and nonconducting monomers. These results can be reconciled with those of Stark et al. (op. cit.) if gramicidin channel formation involves a (slow) folding into $\beta^{6.3}$-helical monomers followed by the dimerization step.

INTRODUCTION

Because of their relatively simple structure and the existence of a single predominant conducting state, gramicidin channels are used extensively to characterize protein-lipid interactions and the elementary events involved in ion permeation (e.g., Pullman et al., 1988). If gramicidin channels are to be useful as prototypical membrane-spanning proteins, however, one must have rather detailed information about the basic channel structure.

There is general agreement that the elementary membrane-spanning unit is a ~2.5-nm long formyl-NH-to-formyl-NH-terminal dimer of $\beta^{6.3}$-helices as suggested by Urry (1971, 1972), in which the peptide backbone forms the wall of a pore with a diameter ~0.4 nm and the hydrophobic side chains line the exterior surface (see Andersen et al., 1988; and Koeppe et al., 1992 for recent summaries). This arrangement permits the helix to intercalate among the acyl chains of the bilayer, whereas the carbonyl oxygens can solvate small monovalent cations and H_2O.

It is widely believed that the elementary conducting unit, the channel, is a dimer, identical to the elementary membrane, spanning unit. This is supported by five independent lines of evidence: voltage-induced conductance relaxation measurements (Bamberg and Läuger, 1973, Neher and Zingsheim, 1974); simultaneous fluorescence and conductance measurements (Veatch et al., 1975); fluorescence energy transfer measurements (Veatch and Stryer, 1977); measurements of the relative appearance rates of symmetrical (homo-oligomeric) and hybrid (hetero-oligomeric) channels in gramicidin mixtures (Veatch and Stryer, 1977; see also Andersen et al., 1988); and measurements of the gramicidin-induced conductance as a function of the gramicidin concentration in the aqueous or membrane phase (Tosteson et al., 1968; Goodall, 1970; Veatch et al., 1975; Appel et al., 1978). Recently it has been suggested, however, that the channel may not be a dimer, but some higher oligomer, most likely a tetramer (Stark et al., 1986; Strässle et al., 1989). This was based primarily on two findings: that the voltage-induced conductance relaxations cannot be described as single exponential transients (see also Hladky, 1974), suggesting that the overall kinetics of channel formation is more complex than a simple monomer-dimer reaction; and that the covalently dimerized malonyl-bis-(desformyl gramicidin) (MBg) appears to induce conductance relaxations similar to those induced by [Val^1]gramicidin A (gA), which could suggest that the channels were formed by lateral association of nonconducting dimers. Indeed, at gramicidin/lipid ratios > 1/50, or so, membrane-bound gramicidins can associate to form higher oligomers (Spisni et al., 1983; Killian and de Kruijff, 1988); but this association may depend on the sample preparation, because Moll and Cross (1990), using ^{31}P nuclear magnetic resonance (NMR), did not

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observe evidence for significant lateral association of membrane-incorporated gramicidin. In any case, it is not clear whether significant lateral association could occur at the much lower gramicidin/lipid ratios where single-channel measurements and other functional studies are done ($\sim 10^{-7} - 10^{-4}$).

The uncertainty about the channel structure raises problems for the use of gramicidin channels as prototypical membrane-spanning structures. Only if the basic channel structure is known is it possible to relate function (e.g., the rate of ion permeation) to structure. A priori predictions of gramicidin single-channel conductances have, in fact, been unsuccessful (Jordan, 1987), which could reflect that the simulations were based on an incorrect structure.

We investigated whether gramicidin channels are dimers or tetramers in single-channel measurements using either gA and O-pyromellityl gramicidin A (OPgA) or [Val^1] gramicidin C (gC) and O-pyromellityl gramicidin C (OPgC), a gramicidin with a pyromellitic acid (benzene-1,2,4,5-tetracarboxylic acid) esterified to the —OH of the ethanolamine at the gramicidin's COOH-terminus. These gramicidins were chosen for several reasons: they have similar channel formation kinetics (Stark et al., 1986); they form channels that have clearly distinguishable conductances (Appell et al., 1978); and, most importantly, because hybrid channels form when both gramicidins are present (Apell et al., 1978), the two gramicidins form channels that are structurally equivalent (cf Durkin et al., 1990). gA and OPgA (or gC and OPgC) are thus especially useful to examine the dimer/tetramer question. Conventional chemical (spectroscopic) techniques cannot be used at the low gramicidin/lipid ratios that are of interest. This limitation can be circumvented in single-channel measurements, where one, by definition, studies individual molecular events. It is thus possible to resolve whether gramicidin dimers must associate laterally to form conducting channels. This functional approach to address a structural problem relies, however, on two observations: gramicidin channels have a single dominant conducting state (e.g., Andersen et al., 1987; Busath et al., 1987); and the channels' behavior is exquisitely sensitive to perturbations of the membrane environment (Sawyer et al., 1989). Gramicidin channels therefore have only a single quaternary structure, i.e., they could be either dimers or tetramers, but not some less defined collection of higher oligomer(s).

To establish the channels' molecularity, we first examined how many distinguishable channel types form when the two gramicidins are added to the aqueous phases on either side of a membrane. If gramicidin channels were dimers, we should see at least three, most likely four distinguishable channel types (Fig. 1 *A*). The four channel types would be a gA dimer, an OPgA dimer, and two hybrid channels, each formed by one gA and one OPgA. The two hybrid channel orientations can be distinguished because they come in two flavors (with either gA or OPgA facing the positive electrolyte solution), because the bulky, negatively charged group at the COOH-terminus of OPgA endows the two hybrid channel entrances with different properties. If gramicidin channels were tetramers, we should observe at least seven, and most likely ten, distinguishable channel types when both gramicidins are added to both aqueous phases (Fig. 1 *B*). The ten channel types would be the two homotetramers plus eight hybrids, two symmetrical and three pairs of asymmetrical mixed tetramers.

As a further test of lateral association between dimers, or between dimers and monomers, we examined how addition of one gramicidin affects the conductance of channels formed by the other gramicidin, specifically whether addition of OPgA would alter the conductance of gA channels. If there were significant lateral association between conducting gA channels and OPgA, one would detect this as a change in the conductance of the gA channels. The negative charges on the OPgA would produce a negative potential that would increase the local cation concentration in the vicinity of the complex, thereby increasing the conductance of the "pure" gA channels.

Some of this material has appeared in preliminary form (Cifu et al., 1989).

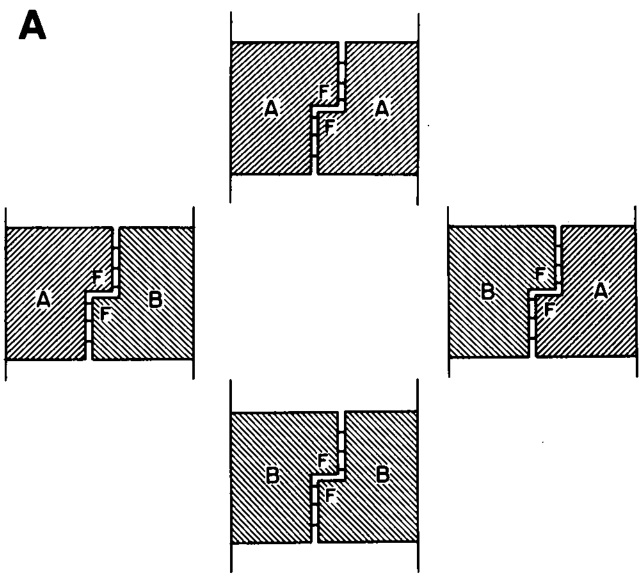
METHODS AND MATERIALS

Experimental Procedures

Single-channel currents were recorded in planar lipid bilayers formed from diphytanoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) dissolved in *n*-decane (Wiley Organics, Columbus, OH). The experimental procedures were as described previously (Andersen, 1983; Sawyer et al., 1989; Durkin et al., 1990). The experiments were done at 25 ± 1°C in symmetrical solutions of unbuffered (pH ~6) 0.05 or 1.0 = M solution of CsCl (Ultrapure grade, MCB Manufacturing Chemists, Cincinnati, OH) in deionized Millipore Milli-Q water (Millipore Corp., Bedford, MA). (In preliminary experiments, the pH was varied between 6 and 8 with no clear effect on the single-channel conductance. Consequently, because the membranes were less stable when using buffered solutions, the experiments were done using unbuffered solutions.)

The gramicidins (gA or gC) were added from ethanolic stock solutions to one or both aqueous solutions, depending on the experimental design, to give a channel appearance rate between 0.1 and 1 s^{-1} (for a 1,000 μm^2 membrane). The final gramicidin concentrations were ~10 pm for gA and ~200 pm for OPgA. The ethanol concentration never exceeded 0.3% by volume.

The amplitudes of single-channel current transitions and the times of channel appearance and disappearance events were determined on-line using the channel-recognition algorithm described by Andersen



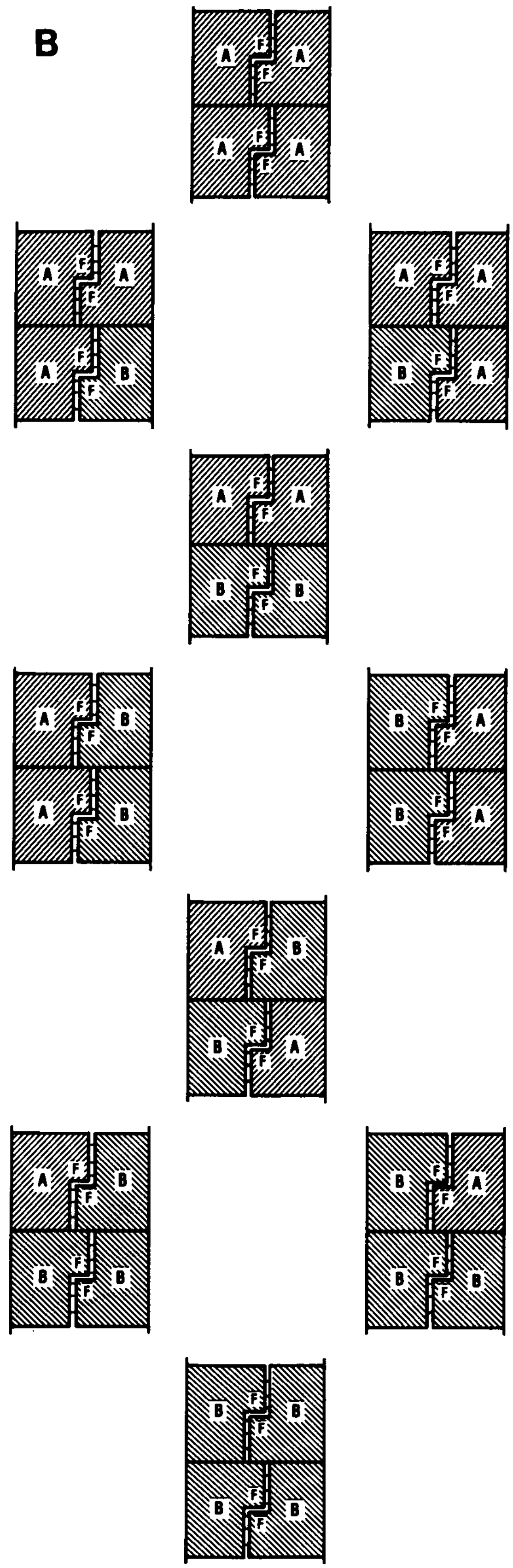


FIGURE 1 Schematic representation of the channel types expected in a hybrid experiment if the gramicidin channel is a dimer or a tetramer. (*A*) If the channel is a dimer, there will be two homo-dimeric (pure) and two hetero-dimeric (hybrid) channel types. (*B*) If the gramicidin channel is a tetramer, there are two homo-tetrameric and eight hetero-tetrameric channel types.

(1983). Current transition amplitude histograms and channel duration histograms were subsequently generated off-line.

In experiments where the gramicidins were added a symmetrically (to the *cis* or *trans* electrolyte solutions), the membrane potential difference (ΔV) were measured relative to the *trans* solution.

Analysis

Determination of single-channel currents

The peaks in the amplitude histograms were delimited by an iterative procedure, where mean and standard deviation initially were calculated based on limits that include the peak of interest. This information was used to define new limits to calculate new values for the mean and standard deviation. This cycle was repeated seven times; in each cycle the new limits were calculated with more weight given to the calculated standard deviation and less to the initial limits. As long as the initial limits included the center of the peak, the procedure converged to similar final limits: the mean ± three standard deviations.

Determination of channel appearance rates

For a given channel type, the appearance rate, f, is related to the number of events, n, in the appropriate peak in amplitude histograms by $f \simeq n/(2 \cdot T_{obs})$, where T_{obs} is the total observation time in the experiment. (The factor of 2 arises because the current transition histograms include both channel appearances and disappearances.)

Determination of average channel durations

The duration of a gramicidin channel is the time from channel appearance to channel disappearance. The distribution of gramicidin channel durations is a single exponential decay, with time constant τ.

For a given channel type (identified by its conductance), τ was determined by constructing histograms of channel durations. Single-channel events with conductances that fell outside the limits that identified the peak in question were not used. If two or more channels of the same type were conducting simultaneously, a random number generator was used to associate channel appearances with disappearances. The duration histograms were transformed into survivor histograms and estimates for τ were obtained by fitting the function $N(t) = N \cdot \exp[-t/\tau]$ (where $N(t)$ is the number of events remaining at time t and N is the expected number of events at $t = 0$) to the histogram using a maximum-likelihood algorithm (Hall and Sellinger, 1981).

Synthesis of O-pyromellityl gramicidins

O-pyromellityl gramicidins A, B, and C were synthesized by the method of Apell et al. (1977), and were purified by reversed-phase high-performance liquid chromatography (HPLC). 0.4 g of commercial gramicidin D (Sigma Chemical Co., St. Louis, MO) and 2.0 g of pyromellitic acid anhydride (Aldrich Chemical Co., Milwaukee, WI) were dissolved in 10 ml pyridine (Pierce Chemical Co., Rockford, IL) and stirred under nitrogen for 48 h at room temperature. Following neutralization with $NaHCO_3$ and dilution with NaCl (Apell et al., 1977), the product was allowed to precipate overnight at 4°C. The precipitate was collected by centrifugation, washed twice with water and dried overnight in vacuo at room temperature. A portion of the sample (200 mg) was reconstituted in 5 ml of methanol and chromatographed on a 1.5 × 100 cm Sephadex LH-20 column (Pharmacia, Inc., Piscataway, NJ), eluted with 0.07% trifluoroacetic acid (TFA) in methanol, pumped at 1.0 ml/min at room temperature (ascending). Under these conditions, the O-pyromellityl gramicidins were retarded ~30% relative to unmodified gramicidins, presumably due to specific binding between the gramicidins and the LH-20 matrix. (In the absence of TFA, the binding is more pronounced.) In contrast to an earlier suggestion (Apell et al., 1977), thd LH-20 column did not separate or enrich the modified gramicidin A, B, or C species relative to each other.

The O-pyromellityl derivatives of gramicidins A, B, and C were separated by reversed-phase HPLC in the presence of TFA. 10–100 μl aliquots of one of the LH-20 fractions were injected directly onto the reversed-phase column. (The TFA is necessary to protonate the carboxylic acid groups and allow the gramicidin derivatives to be retained and resolved by the reversed-phase column. In the absence of TFA, the charged pyromellityl derivatives elute with the solvent injection peak. They will therefore not be separated from each other.) A Zorbax-C8 column (Dupont Instruments, Wilmington, DE) eluted with 0.2% TFA in 78% methanol plus 22% water proved satisfactory for the resolution of O-pyromellityl gramicidin B and the Val[1] and Ile[1] variants of OPgA and OPgC (Fig. 2 *A*). Samples of [Val[1]]OPgA and -OPgC, suitable for single-channel analysis, were collected by hand (Fig. 2 *A*), rechromatographed without drying on a second identical Zorbax-C8 column and once again collected by hand. The samples were then dried in a vacuum centrifuge to remove the TFA, and were reconstituted at 2–5 μg/ml in 80% methanol. Such samples were diluted 10-fold with ethanol and used for single-channel analysis (Fig. 2, *B–D*).

RESULTS

Experiment at high salt concentrations

The basic experiment is illustrated in Fig. 3, which shows the results of an experiment in 1.0 M CsCl, where gC and OPgC were added symmetrically to both electrolyte solutions, such that gC and OPgC were present in both halves of the membrane. Four different channel types are observed: two symmetrical channel types, and two hybrid channel types. The peak at the highest conductance represents the symmetrical gC channels, the peak at the lowest conductance represents the symmetrical OPgC channels, and the two middle peaks represent the two hybrid channel types. The presence of only four peaks is consistent with the channels being dimers.

The orientations of the two hybrid channel types were identified in experiments (Fig. 4), in which gC was added symmetrically while OPgC was added to only one aqueous phase (the *trans* solution).[1] At positive ΔV (Fig. 4, *Top*), the OPgC half of the heterodimer will face toward the negative solution; at negative ΔV (Fig. 4, *Bottom*), the OPgC half will face the positive

[1]At this salt concentration, there appears to be a small flux of OPgA across the membrane, and perfect asymmetry was not achieved. Thus, in current transition histograms there is a small "contamination" of symmetrical OPgA channels (and of the wrong hybrid channel type), which result from the presence of OPgA on the side to which it was not added. Nevertheless, the asymmetric addition of OPgA produces a predominance of hybrid channels of one orientation.

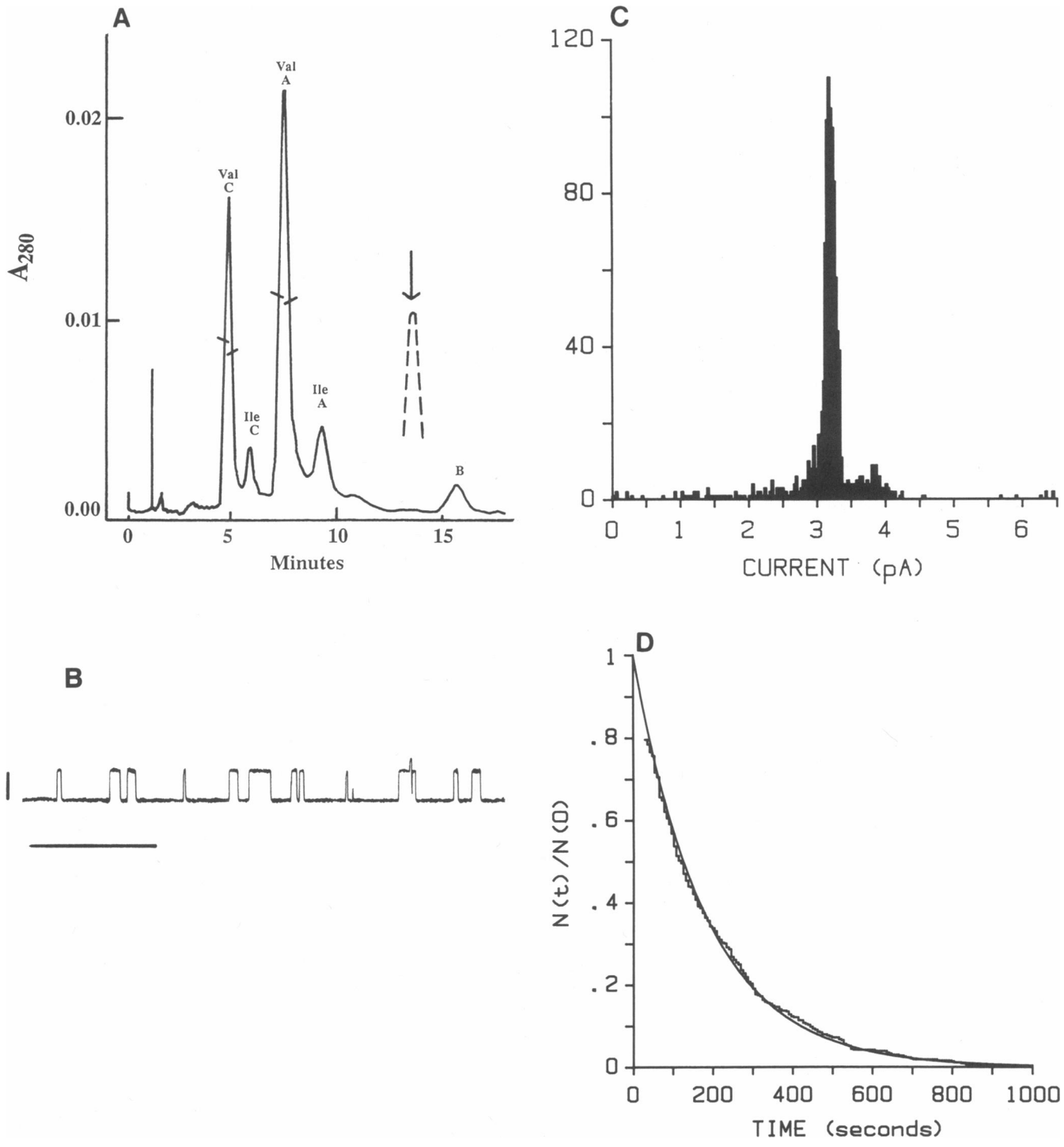


FIGURE 2 Characterization of O-pyromellityl gramicidin A. (*A*) HPLC trace. The peptide has a sharp, characteristic elution peak. The tick marks delimit the material collected for use in single-channel experiments. The hatched peak denotes where [Val^1]gA would have come off, if it were present. (*B*) Current trace of OPgA channels. There is one predominant conducting state. The calibration bars denote: 2.5 pA (vertically); and 2.5 s (horizontally). (*C*) Current amplitude histogram of OPgA channels. Sample purity is again evidenced by the sharp, single peak in the histogram. There were 1,085 resolved transitions, of which 858 (or 80%) were in the main peak. The average single-channel current for the transitions in the main peak was 3.15 ± 0.10 pA. (*D*) Survivor plot for OPgA channels. The average channel duration was determined using a maximum-likelihood fit of $N(t) = N(0) \cdot \text{Exp}(-t/\tau)$ to the distribution. $N(t)$ denotes the number of events with duration longer than the time t, $N(0)$ is the total number of channels in the population, and τ is the average channel duration. $N(0) = 429$; $\tau = 182$ ms. 0.1 M CsCl, 100 mV.

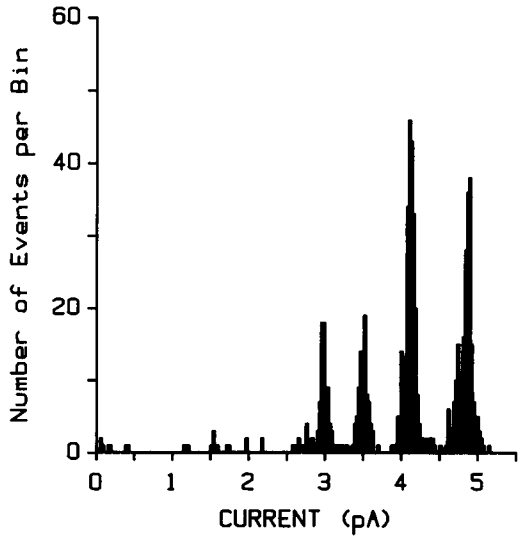


FIGURE 3 Current amplitude histogram for a hybrid channel experiment in which gC and OPgC both were added to both aqueous phases. The four peaks in the histogram represent the four channel types that are predicted by the dimer hypothesis. The average single-channel currents were, from left to right: OPgC channels, 2.98 ± 0.05 pA (n = 72); hybrid channels with OPgC toward the negative solution, 3.49 ± 0.06 pA (n = 88); hybrid channel with OPgC facing the positive solution, 4.10 ± 0.06 pA (n = 241); and gC channels, 4.83 ± 0.09 pA (n = 220). 1.0 M CsCl, 100 mV.

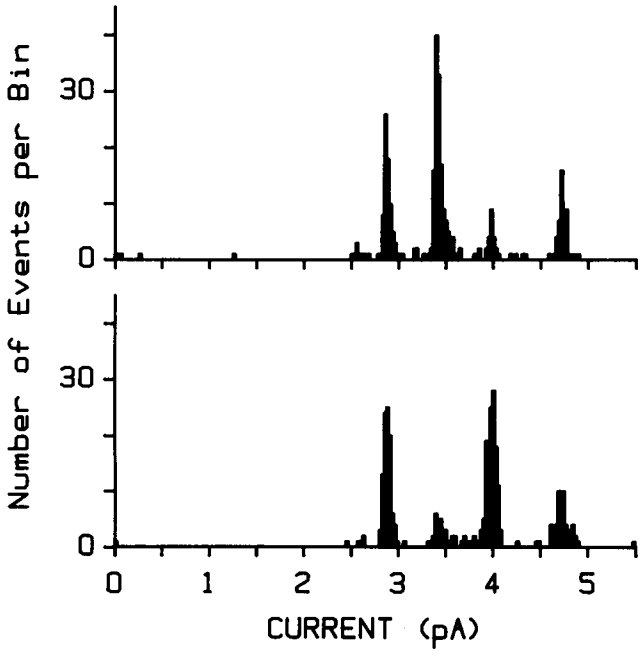


FIGURE 4 Current amplitude histograms for a hybrid channel experiment where gC was added to both aqueous phases whereas OPgC was added to only one solution. The solution containing OPgC was the electrical reference. (*Top*) Results at +100 mV. The four peaks denote the same channel types as in Fig. 3. The single-channel current amplitudes were, from left to right: 2.87 ± 0.04 pA (n = 75); 3.41 ± 0.04 pA (n = 132); 3.95 ± 0.06 pA (n = 25); 4.71 ± 0.03 pA (n = 46). (*Bottom*) Results at −100 mV. The single-channel current amplitudes were, from left to right: 2.87 ± 0.04 pA (n = 96); 3.45 ± 0.07 pA (n = 29); 3.97 ± 0.05 pA (n = 129); 4.70 ± 0.05 pA (n = 49). 1.0 M CsCl, 100 mV.

solution. In Fig. 4, *Top*, $\Delta V = +100$ mV and the main hybrid channel peak is at 3.3 pA. In Fig. 4, *Bottom*, $\Delta V = -100$ mV and the main hybrid channel peak is at 3.8 pA. In both histograms there are, additionally, a pronounced peak corresponding to the symmetrical gC channels at 4.5 pA, a peak corresponding to the symmetrical OPgC channels at 2.8 pA, and a small peak representing the other hybrid orientation. The latter two "contaminating" peaks are the result of the presence of OPgC on the *cis* side of the membrane (see also footnote[1]).

These experiments provide strong support for the dimer hypothesis, as there are four distinguishable and identifiable channel types. It has been argued, however, that single-channel measurements cannot be used in such a hybrid experiment to provide support for the dimer hypothesis because the resolution may be insufficient to resolve all ten channel types predicted by the tetramer hypothesis (Stark et al., 1986). To test whether several channel types could underlie any of the peaks in the current amplitude histograms, we examined the duration distributions for the events underlying each peak (Fig. 5). Each of the duration distributions for the channels underlying each peak is well described by a single exponential decay, which suggests that only a single channel type underlies each of the four peaks. Similar results have been observed for all other gramicidin combinations that we have tested (cf Russell et al., 1986; Andersen, et al., 1988; Durkin et al., 1990). We thus conclude that each peak in the current amplitude histogram represents a unique channel type. These results confirm and extend our previous results, that at most two hybrid channel types (heterodimers) are formed when two gramicidins are added together (Andersen et al., 1988). Importantly, the average durations (the stabilities) of the hybrid channels are intermediate to (and approximately the geometric mean of) those of the two (homodimeric) symmetrical channels. This indicates that there is little, if any, electrostatic repulsion between the negatively charged pyromellityl residues in the symmetrical OPgC homodimer (see Discussion).

Experiments at low salt concentrations

To address whether nonconducting monomers associate with conducting channels, we did experiments at low

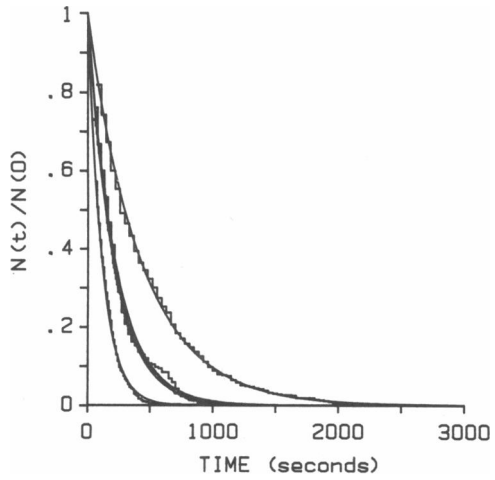


FIGURE 5 Survivor distributions for the four channel types that can be distinguished in hybrid channel experiments (Figs. 3 and 4). The results are presented as normalized survivor plots, i.e., as $N(t)/N(0)$ vs t. Single exponential decays were fitted to the results (see legend to Fig. 2). The theoretical distributions are denoted by the curves (from right to left): gC channels, τ = 440 ms ($N(0)$ = 446); low-conductance hybrid channels, τ = 220 ms ($N(0)$ = 241); high-conductance hybrid channels, τ = 210 ms ($N(0)$ = 350); OPgC channels, τ = 115 ms ($N(0)$ = 425). 1.0 M CsCl, 100 mV.

ionic strength, where the electrical screening distance in the electrolyte solution, the Debye length (L_D),[2] is comparable to a channel's diameter ($\sim$0.8 nm). Thus, if a (nonconducting) OPgA monomer were laterally associated with a (conducting) gA dimer, the conductance of the gA channel would be expected to increase because the presence of the negatively charged OPgA molecule would tend to concentrate cations at the entrance to the gA channel. These experiments were done at 0.05 M salt (L_D = 1.4 nm) to balance the need for electrostatic interactions between any adjacent channels with the need for reasonable resolving power. Fig. 6 shows results of such experiments. The top histogram show results obtained when only gA was added to both electrolyte solutions. In this experiment we see only a single predominant channel type. The bottom histogram was obtained when OPgA and gA were added to both solutions. In this case we see two peaks (this result will be further discussed below). Importantly, the leftmost (low conductance) of these peaks is at the same position

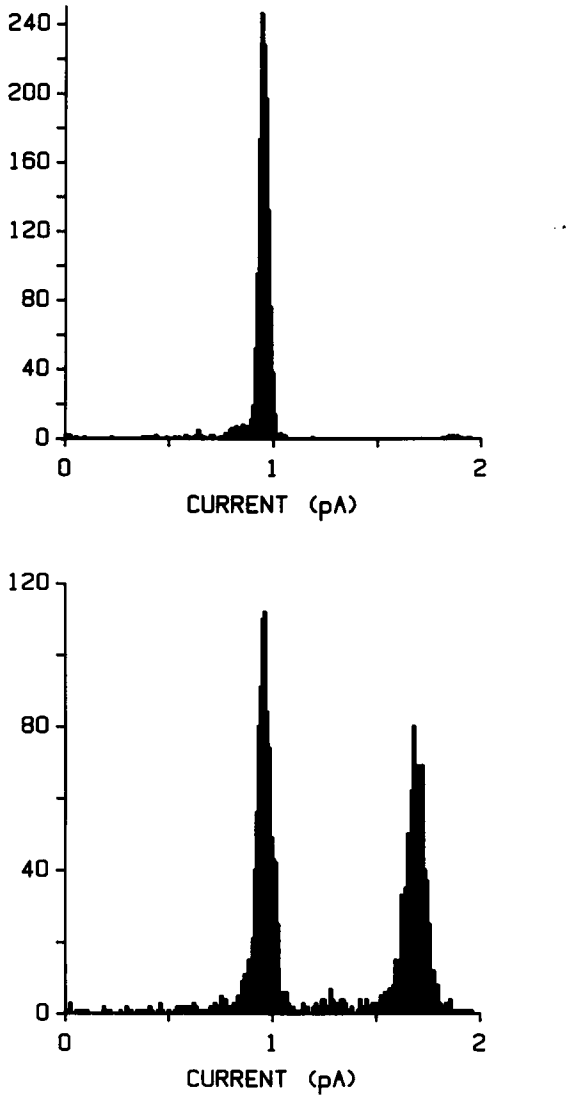


FIGURE 6 Current amplitude histogram for a hybrid experiment at low ionic strength, to test for lateral association between conducting dimers and nonconducting monomers. (*Top*) Amplitude histogram obtained with gA alone. The average single-channel current was 0.95 ± 0.03 pA (n = 1313). (*Bottom*) Amplitude histogram obtained with gA and OPgA added to both aqueous phases. There are only two peaks in the histogram. The peak to the left represents gA channels and one of the hybrid channel types, the peak to the right represents OPgA channels and the other hybrid channel type. The average single-channel currents were, from left to right: 0.95 ± 0.04 pA (n = 930); and 1.68 ± 0.05 pA (n = 822). 0.05 M CsCl, 100 mV.

as the gA peak in the upper histogram, which indicates that OPgA monomers (or nonconducting dimers) had not associated with conducting gA channels. These results thus show that gramicidin channels are unlikely to function as tetramers or higher oligomers.

We would have expected to see four peaks in Fig. 6 (cf Fig. 1), and more if gramicidin channels were formed by

[2]For a uni-univalent electrolyte, the Debye length is defined as (e.g., Aveyard and Haydon, 1973):

$$L_D = (2 \cdot F^2 \cdot C/(\epsilon_0 \cdot \epsilon_r \cdot RT))^{0.5},$$

where F is Faraday's constant, C is the bulk salt concentration, ϵ_o the permittivity of free space, ϵ_r the relative dielectric constant (78.3 and 25°C), R the gas constant, and T temperature in Kelvin.

higher oligomers, but only two peaks are seen in the bottom histogram. The seeming discrepancy arises because we, to clarify the issue, selected experimental conditions at which one hybrid channel type had the same conductance as OPgA channels, whereas the other type had the same conductance as gA channels. This is seen in the experiments where one of the two gramicidins added is added asymmetrically (Figs. 7 and 8). In the experiment summarized in Fig. 7, gA was present on both sides of the membrane but OPgA on only the *trans* side. We should thus observe gA channels but only one hybrid channel type. The hybrid channels will, however, have different conductances depending on the polarity of the applied potential. When the OPgA side is positive (Fig. 7, *Top*), two peaks are seen. These peaks represent gA channels (the leftmost, or low-conductance peak) and hybrid channels (the rightmost, or high-conductance peak). The hybrid peak appears at the same position at which the OPgA peak appeared in Fig. 6. When the potential is reversed (Fig. 7, *Bottom*), the rightmost peak disappears, revealing that it represented a hybrid channel type. The peak that was at the position of the gA channels remains, but there is no other clearly defined peak. The single remaining peak therefore represents two channel types with similar conductances: symmetrical gA channels, and hybrid channels with the pyromellityl group on the negative side. In the experiment summarized in Fig. 8, OPgA was added to both sides but gA was added only to the *cis* side. When the gA side is negative (Fig. 8, *Top*), only a single peak is seen, which is the position of the high-conductance channels in Fig. 6. This peak represents a mixture of OPgA channels and the hybrid channel type that was seen in Fig. 7, *Top*. When the potential is reversed (Fig. 8, *Bottom*), a second peak appears (at the same position at which the gA peak appeared in Fig. 6). This new peak represents the other hybrid channel type. A comparison of the histograms in Figs. 7 and 8 with those in Fig. 6 thus shows that the two peaks in Fig. 6 indeed represent the four expected channel types.

As was the case at high salt concentration (Fig. 5), the average durations of the two hybrid channels are intermediate to those of the symmetrical channel types (Table 1). Once again, therefore, it appears that there is little electrostatic repulsion between the pyromellityl residues in OPgA channels.

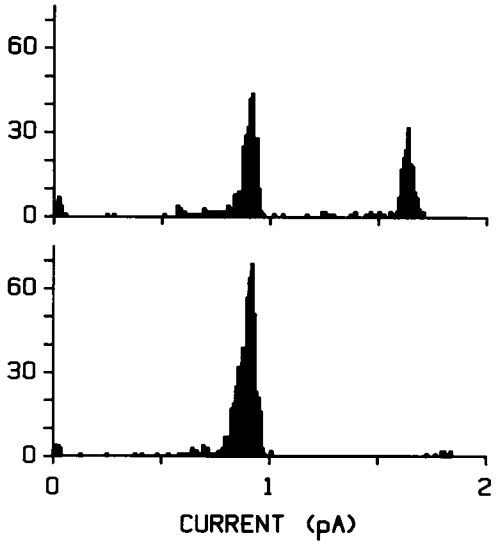


FIGURE 7 Experiment at low ionic strength where gA was added symmetrically while OPgA was added to only one solution. When the OPgA-containing solution was positive (*Top*), there were two peaks. The single-channel currents were, from left to right: 0.90 ± 0.03 pA (n = 267); and 1.63 ± 0.02 pA (n = 164). When the OPgA-containing solution was negative (*Bottom*), there was only one well-resolved peak. The single-channel current was 0.89 ± 0.04 pA (n = 518). 0.05 M CsCl, 100 mV.

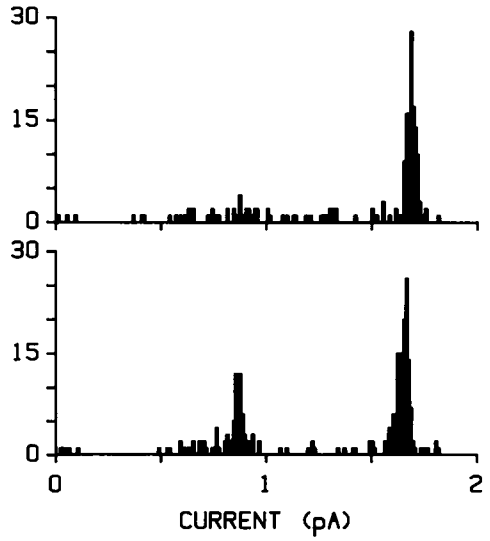


FIGURE 8 Experiment at low ionic strength where OPgA was added to both aqueous phases, while gA was added to only one solution. When the gA-containing solution was negative (*Top*), there was one well-resolved peak. The single-channel current was 1.68 ± 0.02 pA (n = 109). When the gA-containing solution was positive (*Bottom*), there were two peaks. The single-channel currents were, from left to right: 0.86 ± 0.02 pA (n = 61); and 1.64 ± 0.03 pA (n = 128). 0.05 M CsCl, 100 mV.

DISCUSSION

We have examined the question, whether gramicidin channels are dimers or tetramers, through experiments that relied on our ability to produce extremely pure

TABLE 1 **Energetics of O-pyromellityl-gramicidin/gramicidin heterodimers**

[CsCl]	$f_h/(2(f_a f_b)^{0.5})$*	$\tau_h/(\tau_a\tau_b)^{0.5})$**	$\Delta\Delta G_f^{\mp}$	$\Delta\Delta G_b^{\mp}$	$\Delta\Delta G^0$
(M)			kJ/mole	kJ/mole	kJ/mole
1.0	1.2 ± 0.2	0.95	−0.5	−0.1	−0.3
0.05		1.33		0.7	

*Normalized heterodimer appearance rate: f_h, f_a, and f_b denote the appearance rates for the heterodimers and two symmetrical channels, respectively. **Normalized heterodimer duration: τ_h, τ_a, and τ_b denote the average durations for the heterodimers and symmetrical channels respectively. $\Delta\Delta G_f^{\mp}$, $\Delta\Delta G_b^{\mp}$, and $\Delta\Delta G^0$ are defined in Eqs. 1–4 (see Discussion).

gramicidin samples and on the use of single-channel measurements. The results support the conclusion that the elementary conducting unit, the gramicidin channel, is a dimer. This was shown in two related ways. First, only two hybrid channel types are formed when two gramicidins are present in the membrane, even with the highly asymmetric hybrids formed between OPgA and gA (or OPgC and gC). Second, there is no association of conducting gramicidin channels with other gramicidins in the membrane at low gramicidin/lipid ratios.

We first discuss the evidence that single gramicidin channels are dimers; next, the kinetics and energetics of channel formation and gramicidin aggregation at high gramicidin/lipid ratios; then the asymmetry of the hybrid channels and the electrostatic interactions between the charged ends in the OPgA homodimer.

Only two hybrid channel types are observed. The channel is a dimer

Gramicidin channels have a single predominant conducting state (Andersen et al., 1987; Busath et al., 1987; Sawyer et al., 1989). This observation makes it possible to study the molecularity of gramicidin channels, because any channel type has conductances that fall within a narrow range. Thus, if the channels are dimers one should observe at least one, but no more than two hybrid peaks in amplitude histograms. If a gramicidin channel is made up of more than two molecules, then many more channel types should be observed (e.g., Fig. 1). But only four channel types are observed when gA and OPgA are added together. Similar results were obtained with CsCl or NaCl, at concentrations ranging from 0.025 M to 1 M, and at potentials ranging from 25 to 300 mV (results not shown).

The argument that only two hybrid channel types can be identified, has been used previously to support the dimer hypothesis (Veatch and Stryer, 1977; Andersen et al., 1988). The particular properties of OPgA, and the experimental resolution it allows, make the argument for the dimer hypothesis definitive. The bulky, negatively charged carboxyl end of OPgA creates a situation in which the OPgA-gA heterodimer is extraordinarily asymmetrical. A more detailed discussion of this asymmetry will be undertaken below. For now it is sufficient to note that the structural asymmetry produces a functional asymmetry that is observed as a clear separation of the two hybrid peaks even at relatively low potentials (Figs. 3,4,6–8). We furthermore find that a single exponential decay can describe the duration distribution for the channels underlying each peak (Fig. 5), which enables us to conclude that there are no undetected hybrid peaks cf Stark et al. (1986).

For all gramicidin combinations tested, we observe usually one and never more than two hybrid channel types (Mazet et al., 1984; Durkin et al., 1986, 1990; Russell et al., 1986; Koeppe et al., 1990, 1992; Sawyer et al., 1990). These observations provide strong support for the prevailing notion that the elementary conducting unit is a dimer (cf Fig. 1*A*), a conclusion that is consolidated by the present results, because there is no evidence for the lateral association of OPgA monomers or dimers with membrane-spanning gA dimers (Figs. 6–8).

Kinetics of gramicidin channel formation

Our conclusion, that the elementary conducting unit is a dimer, conflicts with that reached by Stark et al. (1986; see also Strässle et al., 1989). Using temperature- and voltage-jump measurements, these investigators concluded that the elementary conducting unit is a tetramer (or possibly some higher oligomer) formed by the lateral association of nonconducting dimers. Their model was based on the finding that channels formed by gA, OPgA, and MBg had similar current relaxation behavior. For all three channel types, the current transients could not be described by single exponential transients. For a quantitative description, it was necessary to use at least two exponentials: a fast component (similar to that observed by many investigators), and a slower component that usually has received little attention, and often been attributed to be an artifact resulting from voltage-induced membrane changes (e.g., Bamberg and Janko, 1977) because it is less pronounced in solvent-depleted membranes (Bamberg and Benz, 1976). Importantly, Stark et al. (1986) showed that the second, slower exponential transient is an inherent property of gramicidin channels.

But the presence of two exponential components in the gA- or OPgA-induced current relaxations is consistent not only with the notion that gramicidin channels

are tetramers, i.e.,

$$4\,M \rightleftharpoons 2\,D' \rightleftharpoons T, \qquad (1)$$

where M, D', and T denote (nonconducting) monomers and dimers, and (conducting) tetramers, respectively; but also with notion that the channels are dimers, e.g.,

$$2\,M' \rightleftharpoons 2\,M \rightleftharpoons D, \qquad (2)$$

or

$$2\,M \rightleftharpoons D' \rightleftharpoons D, \qquad (3)$$

where M' and M denote non-$\beta^{6.3}$-helical and $\beta^{6.3}$-helical monomers, and D' and D denote nonconducting and conducting dimers. For either of these schemes, the conductance relaxation will be composed of two exponential components (see the Appendix). For Scheme 2 (but generally not 1 or 3), the relaxations will at low gramicidin densities (membrane conductances) approach a single exponential transient, with a time constant (τ) that is equal to the conducting channels' average duration. The presence of two exponential components in the conductance relaxations provide strong evidence that channel formation involves a multistep process (even though the monomer $\leftrightarrow$ dimer transition is the kinetically dominant one, cf Bamberg and Läuger, 1973, and Zingsheim and Neher, 1974). One likely mechanism that satisfies all experimental observations on gA is, that channel formation involves transitions between nonconducting monomers and conducting dimers coupled to the folding of "surface-denatured" monomers into β-helices (Scheme 2).

Indeed, other lines of evidence suggest that monomer refolding may be a slow step in gramicidin channel formation: at high gramicidin/lipid ratios (e.g., 1/15) the structure of membrane-adsorbed (or incorporated) gramicidin shows a marked "solvent history" dependence (Killian et al., 1988; LoGrasso et al., 1988; Baño et al., 1989), which presumably results from a slow unwinding of double-stranded (intertwined) dimers; but the single-channel properties (determined at gramicidin/lipid ratios $\sim 10^{-7}$) show no sign of a "solvent history" dependence (Sawyer et al., 1990), although the channel appearance rate increases steadily for at least 30 min after gramicidin addition to the aqueous solution bathing a bilayer (Haydon and Hladky, 1972; Kemp and Wenner, 1976; O'Connell et al., 1990). These findings together suggest that the refolding into $\beta^{6.3}$-helical monomers could be slow (and rate limiting for channel formation).

In any case, the close agreement between the results of Stark et al. (1986) for the chemically dimerized MBg and the monomeric gA or OPgA is problematical, because the average single-channel durations for gA and MBg channels differ ~100-fold (Bamberg and Janko, 1977). The voltage- or temperature-induced conductance relaxations should thus contain terms with time constants that differ by the same factor when the membrane conductance approaches 0 (see Appendix), in contrast to what was observed.[3] But the purity of the MBg sample was not documented by HPLC traces, single-channel current traces, or current transition histograms. This is important because a (small) gA contamination in the MBg could significantly affect the results (the relative importance of any gA channels in a mixture of gA and MBg would increase as the total gramicidin concentration [or membrane conductance] squared). The relaxation behavior observed with a contaminated MBg sample and a gA sample could thus be indistinguishable because the membrane conductance resulted primarily from the gA channels.

Energetics of gramicidin association

At high gramicidin/lipid ratios ($\gtrsim 1/50$), there may be lateral association among gramicidins in (hydrocarbon-free) phospholipid bilayers (e.g., Killian and de Kruijff, 1988; but see Moll and Cross, 1990). The aggregation constant has not been quantified; but one can estimate it, and the energetics of the aggregation process, by approximating it as a dimer $\leftrightarrow$ tetramer equilibrium. The lateral association constant (K_L) can then be expressed (in mole-fraction units) as $K_L = x_T/(x_D)^2$, where x_D and x_T denote the mole fractions of membrane-spanning dimers and tetramers in the membrane, respectively. At a total gramicidin/lipid mole-fraction of 0.02, if $x_D \simeq x_T$ ($\simeq 0.003$) then K_L is ~300 and the standard free energy difference for lateral association (ΔG_L°) is ~ -14 kJ/mol. There is thus a modest energetic preference for the aggregated state, which presumably arises from attractive interactions among the tryptophans in adjacent dimers (Cavatorta et al., 1982; Spisni et al., 1983; Killian et al., 1987). At the gramicidin/lipid mole fractions used in single-channel experiments ($\sim 10^{-6}$). If there were significant lateral interactions, if $x_D \simeq x_T$ ($\simeq 1.6 \cdot 10^{-7}$), then K_L is $\sim 6 \cdot 10^6$ and ΔG_L° is ~ -40 kJ/mol. To have any significant lateral association at the single-channel level, the dimer–dimer interactions must by very substantial.

In hydrocarbon-containing bilayers there is an additional energetic contribution that favors lateral aggrega-

[3]For Scheme 1, if the monomer(-)dimer ($M(\text{-})D'$) equilibrium is shifted so strongly to the right that one can disregard the monomers (cf Stark et al., 1986), the conductance relaxations will be single exponential transients and the limiting relaxation time constant (as the total gramicidin concentration approaches 0) will be equal to the average duration of the conducting species.

tion among gramicidin channels: the channels are shorter than the membrane thickness, such that the formation of a membrane-spanning channel would deform the host bilayer. The associated deformation energy contributes to the overall energetics of channel formation (Huang, 1986; Helfrich and Jakobsson, 1990), which could make it advantageous for channels to coalesce to share the membrane-induced strain. The energetic preference is modest, however, (10–15 kJ/mol in solvent-containing phospholipid bilayers [Helfrich and Jakobsson, 1990]). Thus, at the very low gramicidin/lipid ratios used in single-channel measurements, where the time averaged channel activity usually is on the order of one conducting channel in a 1,000 μ^2 membrane, there should be only insignificant lateral association among membrane-spanning dimers (see above).

Interactions between the charged ends of OPgA channels. Energetics of channel formation

Following Durkin et al. (1990), hybrid-specific interactions between the chemically dissimilar subunits in the hybrid channels can be evaluated by calculating the standard free energy of the hybrid (heterodimeric) channels relative to the homodimeric channels ($\Delta\Delta G°$), or the activation energies for channel formation ($G_f^{\mp}$) and dissociation ($\Delta\Delta G_d^{\mp}$). For $\Delta\Delta G°$,

$$\Delta\Delta G° = \Delta G_h° - (\Delta G_a^0 + \Delta G_b°)/2, \quad (4)$$

where the $\Delta G°$'s denote the standard free energies for the heterodimeric and two homodimeric channel types, respectively. Similar expressions pertain to the $\Delta\Delta G^{\mp}$'s. In terms of experimental parameters,

$$\Delta\Delta G° = -RT \cdot \ln\{f_h \cdot \tau_h/(2 \cdot (f_a \cdot f_b \cdot \tau_a \cdot \tau_b)^{0.5})\}, \quad (5)$$

where the f's and τ's denote channel appearance rates and average durations, while the subscripts h, a, and b denote the hybrid and two symmetrical channel types, respectively. Correspondingly,

$$\Delta\Delta G_f^{\mp} = -RT \cdot \ln\{f_h/(2 \cdot (f_a \cdot f_b)^{0.5})\}, \quad (6)$$

and

$$\Delta\Delta G_d^{\mp} = RT \cdot \ln\{\tau_h/(\tau_b)^{0.5}\}. \quad (7)$$

In the symmetrical OPgA channels, the presence of a net charge at either end of the channel would be expected to produce an electrostatic repulsion between the two monomers, which would destabilize the OPgA homodimers relative to the gA homodimers or OPgA/gA heterodimers. The electrostatic repulsion between the charged ends of the symmetrical OPgA channels thus would be expressed as a hybrid-specific (lack of) interaction between the monomers in a heterodimer, i.e., $\Delta\Delta G°$ being <0 (either $\Delta\Delta G_f^{\mp} < 0$ or $\Delta\Delta G_d^{\mp} > 0$). A priori, one would expect the greatest changes to be in $\Delta\Delta G_d^{\mp}$, because the charges on the two OPgA's would be closest together in the $\beta^{6.3}$-helical dimer.

In 1.0 M CsCl, no such interactions were observed. The $\Delta\Delta G$'s were within $RT/2$ of 0 (Table 1), which provides quantitative evidence that gA and OPgA channels are structurally equivalent (and that electrostatic interactions are minimal). In 0.05 M CsCl, only $\Delta\Delta G_d^{\mp}$ could be evaluated in experiments with asymmetrical gramicidin addition (the hybrid channels could not be identified unambiguously because of overlap with the symmetrical channels [Figs. 6–8]); it was likewise within $\frac{1}{2}$ RT of 0. We thus conclude that there is little detectable electrostatic repulsion between the charges at the entrances of symmetrical OPgA channels.

This absence of significant electrostatic interactions is at first sight surprising because the electrostatic interaction between two trivalent ions separated by 2.6 nm of a homogeneous medium of dielectric constant 80 is ~ 2.5 RT. But the charges at the entrances may not be trivalent, because one or more of the pyromellityl moiety's pKs may be shifted in the alkaline direction.[4] In any case, the screening imposed by the aqueous electrolyte could diminish electrostatic interactions between the channel entrances, which also would imply that electrostatic repulsion between two monovalent cations within the channel, but close to the channel entrances, could be modest. (Mathias et al. [1991] found that the screening imposed by the aqueous electrolyte effectively abolished electrostatic interactions across a membrane of dielectric constant 2. Whether that conclusion also holds for channels, with their more complex dielectric structure, remains to be evaluated.)

Asymmetry of the hybrid channel

The remarkable asymmetry of the hybrid channels (Figs. 3, 4, 6, and 7) results from two competing effects caused by the presence of the bulky, negatively charged pyromellityl moiety at a channel entrance. The negative charge will act to establish a local negative potential at the entrance (relative to the bulk solution), which will attract cations to the entrance (and alter the electrostatic potential profile along the channel [Franken-

[4]The pK_a's of benzene 1,2,4,5-tetracarboxylic acid are approximately 2, 3, 4.5, and 5.5, whereas those of benzene-1,2,4-tricarboxylic acid are approximately 2.5, 4, and 6 (e.g., Kortüm et al., 1961). It thus appears that the highest pK remains relatively invariant with respect to the total number of (free) carboxyl groups on the benzene ring. Thus, for the pyromellityl group not to be trivalent, one (or more) of the pK's would have to be shifted 3–4 pH units in the alkaline direction.

haeuser, 1960; Muller and Finkelstein, 1972; Green et al., 1987; MacKinnon et al., 1989]), whereas the bulk associated with the pyromellityl group will act to decrease the intrinsic rate constant for ion association/dissociation (Fig. 9).[5]

At low salt concentrations, the electrostatic effect dominates. Symmetrical OPgA (or OPgC) channels and hybrid channels with the OPgA toward the positive solution have the higher conductances (Figs. 7 and 8) because the local negative potential at the entrance increases the local cation concentration and thereby increase the overall association rate constant (an effect that may in part be blunted by the associated alteration in the electrostatic field along the channel interior). Hybrid channels with the gA towards the positive solution have nothing to attract ions to the channel and behave similarly to gA channels (Fig. 7); the bulky pyromellityl group at other end of the channel may, in fact, decrease the dissociation rate constant (an effect that again may be blunted by the alteration in the electrostatic field along the channel).

As the salt concentration (ionic strength) increases, the advantage afforded by the charge at the channel entrance (in terms of acting to decrease the overall association barrier for cations) becomes less, because the potential difference between the channel entrance and bulk solution approaches 0. The bulky group at the entrance, and the associated increase of the energy barrier for the association/dissociation steps, thus becomes dominant. Again, hybrid channels with the OPgA towards the positive solution have a higher conductance than hybrid channels having the opposite orientation (although both have lower conductance than symmetrical gA channels).

The change in the relative importance of the electrostatic and the "bulk" effects explains the different order of the homodimer and heterodimer peaks at 0.05 M (Figs. 6–8) and 1.0 M CsCl (Figs. 3 and 4), and why the symmetrical OPgA channels, with steric hindrances of both entrances have lower conductances than the hybrid channels (or the symmetrical gA channels) at high ionic strength.

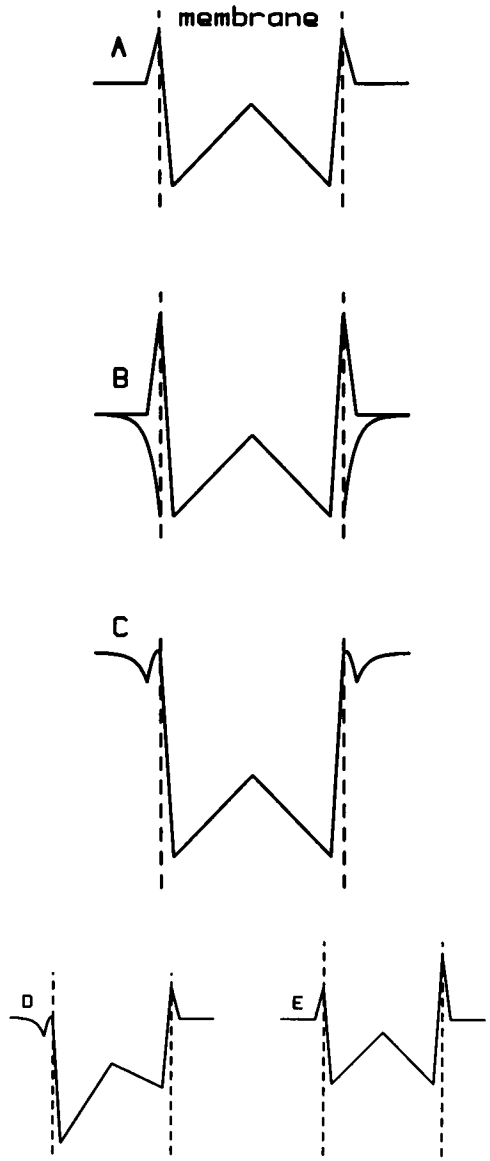


FIGURE 9 Schematic representation of the potential energy profiles in gramicidin channels at 0 mV. (*A*) Symmetrical gA channel. (*B*) The two components of the energy profile in symmetrical OPgA channels. There is an increased intrinsic barrier for ion entry/exit, and an electrostatic attraction of cation to the channel entrances. (*C*) Overall potential profile for symmetrical OPgA channels at low ionic strength. (At high ionic strength, the electrostatic attraction disappears, and the energy profile will resemble the intrinsic profile in *B*). (*D*) Energy profile for an OPgA/gA heterodimer at low ionic strength. The major energy barrier is for ion exit at the OPgA side of the channel (*left*). (*E*) Energy profile for a gA/OPgA heterodimer at high ionic strength. The major energy barrier is, again, for ion exit at the OPgA side of the channel (*right*).

[5]With des(ethanolamine)-taurine[16]-gA, the single-channel conductance in 1.0 M salt is higher than that of gA channels (Roeske et al., 1989). With des(ethanolamine)gA, a net charge at the channel entrance is likewise associated with an increased single-channel conductance (Reinhardt et al., 1986). It is thus unlikely that the lesser conductance of the symmetrical OPgA (or OPgC) channels at high salt concentrations results because of a more favorable electrostatic interaction between the permeating ion and the channel, which also would decrease the dissociation rate constant.

Conclusions

We have used a negatively charged analogue of gA (or gC) to establish the molecularity of gramicidin channels. The results can be reconciled with those of Stark et al. (1986) by invoking a multistep mechanism for channel formation, in which there is a slow interconversion between gramicidin monomer conformations followed by a comparatively rapid monomer(-)dimer step (cf Bamberg and Läuger, 1972; Zingsheim and Neher, 1974).

Somewhat surprisingly, there was little detectable electrostatic repulsion between the charged groups at the entrances of the symmetrical OPgA (or OPgC) channels, a finding that may have implication for understanding ion–ion repulsion in multi-ion channels. Relative to the native gA channels, the permeability properties of the OPgA channels is modified by the electrostatic interactions between the pyromellityl residues and the permeating ions as well as the steric constraints associated with the bulk of the residues, which makes these channels useful for investigating ion-channel interactions, particularly the ion entry step.

APPENDIX

Kinetics of gramicidin channel formation

Three simple models that can account for the bi-exponential gramicidin conductance transients are given by Schemes A1, A2, and A3,

$$4\,M \underset{k_m^{d'}}{\overset{k_{d'}^{m}}{\rightleftharpoons}} 2\,D' \underset{k_{d'}^{t}}{\overset{k_t^{d'}}{\rightleftharpoons}} T, \tag{A1}$$

$$2\,M' \underset{k_{m'}^{m}}{\overset{k_m^{m'}}{\rightleftharpoons}} 2\,M \underset{k_m^{d}}{\overset{k_d^{m}}{\rightleftharpoons}} D, \tag{A2}$$

or

$$2\,M \underset{k_m^{d'}}{\overset{k_{d'}^{m}}{\rightleftharpoons}} D' \underset{k_d^{d}}{\overset{k_{d}^{d'}}{\rightleftharpoons}} D, \tag{A3}$$

where M' and M denote non-$\beta^{6.3}$-helical and $\beta^{6.3}$-helical monomers, D' and D denote nonconducting and conducting dimers, and T conducting tetramers, respectively, while the k's denote the rate constants (the super- and subscripts denote source and destination, respectively).

The kinetics are described by the following sets of equations: for Scheme A1,

$$d[M]/dt = -2 \cdot k_{d'}^{m} \cdot [M]^2 + 2 \cdot k_m^{d'} \cdot [D'], \tag{A1a}$$

$$d[D']/dt = k_{d'}^{m} \cdot [M]^2 - k_m^{d'} \cdot [D'] - 2 \cdot k_t^{d'} \cdot [D']^2 + 2 \cdot k_{d'}^{t} \cdot [T], \tag{A1b}$$

$$d[T]/dt = k_t^{d'} \cdot [D']^2 - k_{d'}^{t} \cdot [T]; \tag{A1c}$$

for Scheme II,

$$d[M']/dt = -k_{m'}^{m} \cdot [M'] + k_{m'}^{m} \cdot [M], \tag{A2a}$$

$$d[M]/dt = k_{m'}^{m} \cdot [M'] - k_{m'}^{m} \cdot [M] - 2 \cdot k_d^{m} \cdot [M]^2 + 2 \cdot k_m^{d} \cdot [D], \tag{A2b}$$

$$d[D]/dt = k_d^{m} \cdot [M]^2 - k_m^{d} \cdot [D]; \tag{A2c}$$

and for Scheme II,

$$d[M]/dt = -2 \cdot k_{d'}^{m} \cdot [M]^2 + 2 \cdot k_m^{d'} \cdot [D'], \tag{A3a}$$

$$d[D']/dt = k_{d'}^{m} \cdot [M]^2 - k_m^{d'} [D'] - k_d^{d'} \cdot [D'] + k_{d'}^{d} \cdot [D], \tag{A3b}$$

$$d[D]/dt = k_d^{d'} \cdot [D'] + k_{d'}^{d} \cdot [D]. \tag{A3c}$$

In each case, we assume that the total amount of gramicidin in the membrane is constant:

$$[M] + 2 \cdot [D'] + 4 \cdot [T] = [G]\ (= \text{const}), \tag{A1d}$$

$$[M] + [M'] + 2 \cdot [D] = [G]\ (= \text{const}), \tag{A2d}$$

$$[M] + 2 \cdot [D'] + 2 \cdot [D] = [G]\ (= \text{const}), \tag{A3d}$$

such that only two out of the three differential equations describing each scheme (Eqs. A1,*a–c*, etc.) are independent.

The time courses of the conductance relaxations were obtained under the additional assumptions: that only the rightmost state in each scheme is conducting; and that the displacement from equilibrium is small, such that the kinetics could be reduced to a (linear) relaxation problem (e.g., Eigen and de Maeyer, 1963). The deviations from equilibrium are denoted by

$$\mu = M - M_{eq},\ \mu' = M' - M'_{eq},\ \delta = D - D_{eq},\ \delta' = D' - D'_{eq},$$

$$\text{and}\quad \tau = T - T_{eq},$$

and the time courses will be described by: for Scheme A1,

$$d^2\tau/dt^2 + A \cdot d\tau/dt + B \cdot \tau = 0, \tag{A4a}$$

$$A = k_m^{d'} + 4 \cdot k_{d'}^{m} \cdot [M]_{eq} + k_d^{t} + 4 \cdot k_t^{d'} \cdot [D']_{eq}, \tag{A4b}$$

$$B = k_{d'}^{t} \cdot (k_m^{d'} + 4 \cdot k_{d'}^{m} \cdot [M]_{eq}) + 2 \cdot k_t^{d'} \cdot [D]_{eq} \cdot (8 \cdot k_{d'}^{m} \cdot [M]_{eq} - k_{d'}^{t}); \tag{A4c}$$

for Scheme A2,

$$d^2\delta/dt^2 + A \cdot d\delta/dt + B \cdot \delta = 0, \quad \text{(A5}a\text{)}$$

$$A = k_m^d + 4 \cdot k_d^m \cdot [M]_{eq} + k_m^{m'} + k_{m'}^m, \quad \text{(A5}b\text{)}$$

$$B = k_m^{m'} \cdot (k_m^d + 2 \cdot k_d^m \cdot [M]_{eq}) + k_{m'}^m \cdot k_d^m \cdot [M]_{eq}; \quad \text{(A5}c\text{)}$$

and for Scheme A3,

$$d^2\delta/dt^2 + A \cdot d\delta/dt + B \cdot \delta = 0, \quad \text{(A6}a\text{)}$$

$$A = 2 \cdot k_{d'}^m \cdot [M]_{eq} + k_d^{d'} + k_d^{d'} + k_{d'}^d, \quad \text{(A6}b\text{)}$$

$$B = 2 \cdot k_{d'}^d \cdot [M]_{eq} \cdot (k_d^{d'} + k_{d'}^d) + k_m^{d'} \cdot k_{d'}^d. \quad \text{(A6}c\text{)}$$

For either of the schemes, the time-course of the conductance relaxations, $G(t)$, is given by

$$G(t) = \alpha_1 \cdot \exp\{\lambda_1 \cdot t\} + \alpha_2 \cdot \exp\{\lambda_2 \cdot t\}, \quad \text{(A7)}$$

where

$$\lambda_{1,2} = (-A \pm \{A^2 - 4 \cdot B\}^{0.5})/2$$

where the + is for λ_1 and the − for λ_2; and subject to the initial conditions: for Scheme A1,

$$\alpha_1 + \alpha_2 = \tau(0), \quad \text{(A8}a\text{)}$$

$$\lambda_1 \cdot \alpha_1 + \lambda_2 \cdot \alpha_2 = d\tau/dt|_{t=0}$$

$$= -k_{d'}^t \cdot \tau(0) + 2 \cdot k_t^{d'} \cdot [D]_{eq} \cdot \delta(0), \quad \text{(A8}b\text{)}$$

such that, as $[G] \to 0$ then $\lambda_1 \to -k_d^t$, and $\lambda_2 \to -k_d^{d'}$ (and both $\alpha_1/\tau(0)$ and $\alpha_2/\tau(0)$ will generally converge towards finite limiting values); for Scheme A2,

$$\alpha_1 + \alpha_2 = \delta(0), \quad \text{(A9}a\text{)}$$

$$\lambda_1 \cdot \alpha_1 + \lambda_2 \cdot \alpha_2 = d\delta/dt|_{t=0}$$

$$= -k_m^d \cdot \delta(0) + 2 \cdot k_d^m \cdot [M]_{eq} \cdot \mu(0), \quad \text{(A9}b\text{)}$$

such that, as $[G] \to 0$ then $\lambda_1 \to -k_m^d$ and $\lambda_2 \to -(k_m^{m'} + k_{m'}^m)$ (and $\alpha_1/\delta(0) \to 1$ whereas $\alpha_2/\delta(0) \to 0$); for Scheme A3,

$$\alpha_1 + \alpha_2 = \delta(0), \quad \text{(A10}a\text{)}$$

$$\lambda_1 \cdot \alpha_1 + \lambda_2 \cdot \alpha_2 = d\delta/dt|_{t=0}$$

$$= -k_{d'}^d \cdot \delta(0) + k_d^{d'} \cdot \delta'(0), \quad \text{(A10}b\text{)}$$

such that, as $[G] \to 0$ both $\alpha_1/\delta(0)$ and $\alpha_2/\delta(0)$ generally will converge towards finite limiting values).

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REFERENCES

Andersen, O. S., J. T. Durkin, and R. E. Koeppe II. 1988. Do amino acid substitutions alter the structure of gramicidin channels? Chemistry at the single molecule level. *In* Transport Through Membranes: Carriers, Channels and Pumps. A. Pullman, J. Jortner, and B. Pullman, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 115–132.

Andersen, O. S., R. E. Koeppe II, J. T. Durkin, and J.-L. Mazet. 1987. Structure-function studies on linear gramicidins: site-specific modifications in a membrane channel. *In* Ion Transport Through Membranes. K. Yagi and B. Pullman, editors. Academic Press, Tokyo. 295–314.

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

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

Aveyard, R., and D. A. Haydon. 1973. An Introduction to the Principles of Surface Chemistry. Cambridge University Press, London. 232 pp.

Bamberg, E., and R. Benz. 1976. Voltage-induced thickness changes of lipid bilayer membranes and the effect of an electric field on gramcidin A channel formation. *Biochim. Biophys. Acta.* 426:570–580.

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

Bamberg, E., and P. Läuger. 1973. Channel formation kinetics of gramicidin A in lipid bilayer membranes. *J. Membr. Biol.* 11:177–194.

Busath, D. D., O. S. Andersen, and R. E. Koeppe II. 1987. On the conductance heterogeneity in membrane channels formed by gramicidin A: a cooperative study. *Biophys. J.* 51:79–88.

Cavatorta, P., A. Spisni, E. Casali, L. Lindner, L. Masotti, and D. W. Urry. 1982. Intermolecular interactions of gramicidin A′ transmembrane channels incorporated into lysophosphatidylcholine lipid systems. *Biochim. Biophys. Acta.* 689:113–120.

Cifu, A. S., R. E. Koeppe II, and O. S. Andersen. 1989. On the supramolecular organization of gramicidin channels. *Biophys. J.* 55:502*a*. (Abstr.)

Durkin, J. T., O. S. Andersen, E. R. Blout, F. Heitz, R. E. Koeppe II, and Y. Trudelle. 1986. Structural information from functional measurements. Single-channel studies on gramicidin analogs. *Biophys. J.* 49:118–121.

Durkin, J. T., R. E. Koeppe II, and O. S. Andersen. 1990. Energetics of gramicidin hybrid channel formation as a test for structural equivalence. Side-chain substitutions in the native sequence. *J. Mol. Biol.* 211:221–234.

Frankenhaeuser, B. 1960. Sodium permeability in toad nerve and in squid nerve. *J. Physiol.* 152:159–166.

Goodall, M. C. 1970. Structural effects in the action of antibiotics on the ion permeability of lipid bilayers. III. Gramicidins "A" and "S", and lipid specificity. *Biochim. Biophys. Acta.* 219:471–478.

Green, W. N., L. B. Weiss, and O. S. Andersen. 1987. Batrachotoxin-modified sodium channels in planar lipid bilayers. Ion permeation and block. *J. Gen. Physiol.* 89:841–872.

Helfrich, P., and E. Jakobsson. 1990. Calculation of deformation energies and conformations in lipid membranes containing gramicidin channels. *Biophys. J.* 57:1075–1084.

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

Huang, H. W. 1986. Deformation free energy of bilayer membrane and its effect on gramicidin channel lifetime. *Biophys. J.* 50:1061–1070.

Jordan, P. C. 1987. Microscopic approaches to ion transport through transmembrane channels. The model system gramicidin. *J. Phys. Chem.* 91:6582–6591.

Kemp, G., and C. Wenner. 1976. Solution, interfacial, and membrane properties of gramicidin A. *Arch. Biochem. Biophys.* 176:547–555.

Killian, J. A., K. N. J. Burger, and B. de Kruijff. 1987. Phase separation and hexagonal H_{II} phase formation by gramicidin A, B, and C in dioleoylphosphatidylcholine model membranes. A study on the role of the tryptophan residues. *Biochim. Biophys. Acta.* 897:269–284.

Killian, J. A., and B. de Kruijff. 1988. Proposed mechanism for H_{II} phase induction by gramicidin in model membranes and its relation to channel formation. *Biophys. J.* 53:111–117.

Killian, J. A., K. U. Prasad, D. Hains, and D. W. Urry. 1988. The membrane as an environment of minimal interconversion. A circular dichroism study on the solvent dependence of the conformational behavior of gramicidin in diacylphosphatidylcholine model membranes. *Biochemistry.* 27:4848–4855.

Koeppe, R. E. II, J.-L. Mazet, and O. S. Andersen. 1990. Distinction between dipolar and inductive effects in modulating the conductance of gramicidin channels. *Biochemistry.* 29:512–520.

Koeppe, R. E. II, L. L. Providence, D. V. Greathouse, F. Heitz, Y. Trudelle, N. Purdie, and O. S. Andersen. 1992. On the helix sense of gramicidin A single channels. *Proteins.* 12:49–62.

LoGrasso, P. V., F. Moll III, and T. A. Cross. 1988. Solvent history dependence of gramicidin A conformations in hydrated lipid bilayers. *Biophys. J.* 54:259–267.

MacKinnon, R., R. Latorre, and C. Miller. 1989. Role of surface electrostatics in the operation of a high-conductance Ca^{2+}-activated K^+ channel. *Biochemistry.* 28:8092–8099.

Mathias, R. T., G. J. Baldo, K. Manivanan, and S. McLaughlin. 1991. Discrete charges on biological membranes. *In* Electrified Interfaces in Physics, Chemistry, and Biology. R. Guidelli, editor. Kluwer Academic Publishers. Dordrecht, The Netherlands. In press.

Maxwell, W. R., and J. R. Partington. 1937. The dissociation constants of some small polybasic acids. Part III. *Trans. Faraday Soc.* 33:670–678.

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

Moll, F., III, and T. A. Cross. 1990. Optimizing and characterizing alignment of oriented lipid bilayers containing gramicidin D. *Biophys. J.* 57:351–362.

Muller, R. U., and A. Finkelstein. 1972. The effect of surface charge on the voltage-dependent conductance induced in thin lipid membranes by monazomycin. *J. Gen. Physiol.* 60:285–306.

O'Connell, A. M., R. E. Koeppe II, and O. S. Andersen. 1990. Kinetics of gramicidin channel formation in lipid bilayers: transmembrane monomer association. *Science (Wash. DC).* 250:1256–1259.

Pullman, A., J. Jortner, and B. Pullman, editors. 1988. Transport Through Membranes: Carriers, Channels and Pumps. Kluwer Academic Publishers, Dordrecht, The Netherlands. 570 pp.

Reinhardt, R., and E. Bamberg. 1986. Single channel conductance changes of the desethanolamine-gramicidin through pH variations. *In* Electrical Double Layers in Biology. M. Blank, editor. Plenum Publishing Corp., New York. 91–102.

Roeske, R. W., T. P. Hrinyo-Pavlina, R. S. Pottorf, T. Bridal, X.-Z. Jin, and D. Busath. 1989. Synthesis and channel properties of [Tau^{16}]gramicidin A. *Biochim. Biophys. Acta.* 982:223–227.

Russell, E. W. B., L. B. Weiss, F. I. Navetta, R. E. Koeppe II, and O. S. Andersen. 1986. Single-channel studies on linear gramicidins with altered amino acid side chains. Effects of altering the polarity of the side chain at position 1 in gramicin A. *Biophys. J.* 49:673–686.

Sawyer, D. B., R. E. Koeppe II, and O. S. Andersen. 1989. Induction of conduction heterogeneity in gramicidin channels. *Biochemistry.* 28:6571–6583.

Sawyer, D. B., R. E. Koeppe II, and O. S. Andersen. 1990. Gramicidin single-channel properties show no solvent-history dependence. *Biophys. J.* 57:515–523.

Sawyer, D. B., L. P. Williams, W. L. Whaley, R. E. Koeppe II, and O. S. Andersen. 1990. Gramicidins A, B, and C form structurally equivalent ion channels. *Biophys. J.* 58:1207–1212.

Spisni, A., I. Pasquali-Ronchetti, E. Casali, L. Lindner, P. Cavatorta, L. Masotti, and D. W. Urry. 1983. Supramolecular organization of lysophosphatidylcholine-packaged gramicidin A'/ *Biochim. Biophys. Acta.* 732:58–68.

Stark, G., M. Strässle, and Z. Takacz. 1986. Temperature-jump and voltage-jump experiments at planar lipid membranes support an aggregational (micellar) model of the gramicidin A ion channel. *J. Membr. Biol.* 89:23–37.

Strässle, M., G. Stark, M. Wilhelm, P. Daumas, F. Heitz, and R. Lazaro. 1989. Radiolysis and photolysis of ion channels formed by analogs of gramicidin A with a varying number of tryptophan residues. *Biochim. Biophys. Acta.* 980:305–314.

Tosteson, D. C., T. E. Andreoli, M. Tieffenberg, and P. Cook. 1968. The effects of macrocyclic compounds on cation transport in sheep red cells and thin and thick lipid membranes. *J. Gen. Physiol.* 51:373s–384s.

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

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

Veatch, W. R., R. Mathies, M. Eisenberg, and L. Stryer. 1975. Simultaneous fluorescence and conductance studies of planar bilayer membranes containing a highly active and fluorescent analog of gramicidin A. *J. Mol. Biol.* 99:75–92.

Veatch, W. R., and L. Stryer. 1977. The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. *J. Mol. Biol.* 113:75–92.

Zingsheim, H. P., and E. Neher. 1974. The equivalence of fluctuation analysis and chemical relaxation measurements: a kinetic study of ion pore formation in thin lipid membranes. *Biophys. Chem.* 2:197–202.