LOW CONDUCTANCE GRAMICIDIN A CHANNELS ARE HEAD-TO-HEAD DIMERS OF $\beta^{6.3}$-HELICES

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ABSTRACT Weakly conductive, atypical channels were observed to form from highly purified Val[1]-gramicidin A in planar lipid bilayer membranes. The structure of these low-conductance channels (minis) was investigated by a detailed study of their channel forming characteristics. The possibility that minis originate from primary structural analogs or degradation products of gramicidin was considered and ruled out. In particular, spontaneous conductance changes in single channels demonstrated that minis can derive directly and reversibly from "standard" channels having the most common conductance level. The fraction of channels which are minis does not vary with changes in membrane gramicidin concentration, indicating that mini and standard channels have the same molecularity, that is, both are dimers. The mean lifetime of mini channels is only slightly shorter than that of standard channels, indicating that the six hydrogen bonds that stabilize the head-to-head dimer are minimally affected in minis. The fraction of channels which are minis is unaffected by the ionic strength, ionic composition, or pH of the bathing solution; it is also unaffected by the lipid composition of the bilayer. These findings are consistent with the hypothesis that minis arise from minor changes in the conformation of the Val[1]-gramicidin A molecule near the channel entrance or exit.

INTRODUCTION

Val[1]-gramicidin A can be isolated from related peptides in gramicidin D and highly purified (>99%) using HPLC (Koeppe and Weiss, 1981; Prasad et al., 1982*a*; Mazet et al., 1984). The cation conductive channels formed by purified gramicidin A in lipid bilayer membranes exhibit, however, a broad spectrum of discrete conductance states (Hladky and Haydon, 1972). Although the majority of channels have conductances falling within a narrow range, a considerable fraction (between 10%, Mazet et al., 1984 and 35%, Busath and Szabo, 1981) have lower conductances distributed rather uniformly below the standard conductance (minis). The same behavior is displayed by synthetic Val[1]-gramicidin A (Prasad et al., 1982*a*), synthetic or purified Val[1]-gramicidin B (Bamberg et al., 1976; Prasad et al., 1982*b*), synthetic or purified Val[1]-gramicidin C (Bamberg et al., 1976), and Phe[9]-Val[1]-gramicidin A (Prasad et al., 1983).

Extensive evidence (for a recent review, see Andersen, 1984) indicates that the predominant standard gramicidin channels are dimers of $\beta^{6.3}$ helices formed by gramicidin A in the membrane. The structure of the low-conductance channels is less clear. Bamberg et al. (1976) suggested that channels with lower conductances had either variant conformations or unusual lipid environments. They noted however, that the latter possibility is rather unlikely since these channels had a constant conductance during their 0.1 to 1.0 s lifetime which, given normal gramicidin diffusivity, is too long for the lipid environment to remain unaltered. Ovchinnikov and coworkers (Shepel et al., 1976; Ovchinnikov, 1979; Ovchinnikov and Ivanov, 1983) suggested that the low conductance channels may be a result of double helical conformations of the type proposed by Veatch et al. (1974). Urry et al. (1981) suggested that the position of the side chains (which radiate away from the backbone-lined channel) could be the cause of decreased channel conductance. In support of this hypothesis they found a decrease in the frequency of low conductance channels for Leu[5]-gramicidin A, where the bulky replacement side chain was expected to reduce the freedom of other side chains (Urry et al., 1984).

Busath et al. (1987) examined a persistent difference between two different labs in observed gramicidin mini frequency. The difference was not due to impurity of the peptide sequence, as both labs used the same source of peptide. Doping the gramicidin sample with formaldehyde or benzene did not increase the mini frequency. They were unable to identify the precise source of the difference but felt their results suggested some other chemically active containment in the lipid, saline, or gramicidin solvent. However, their results would not rule out peptide conformational differences as a source of mini channels.

In an early paper we noted that low conductance channels may convert reversibly to standard channels and that their current–voltage relations are asymmetric (Busath and Szabo, 1981). Here we examine in detail the

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properties of low-conductance channels. The data suggests that these atypical channels are head-to-head dimers of $\beta^{6.3}$ helices having minor conformational alterations near the channel openings.

METHODS

The experimental procedures were similar to those described previously (Busath and Szabo 1981). The chamber consisted of a Teflon block having a 3 ml reservoir and a polyethylene pipette inserted from the side (Fig. 1). The pipettes had an aperture at the tip, from 25 to 650 μm in diameter. With a bilayer present, the input capacitance of the 25-μm pipette was ~10 pF, and seal resistances ranged from 20 to 50 GΩ with only 20 to 50 KΩ series resistance. This approach permits easy membrane preparation for high-frequency single-channel measurements. Lipid bilayers were painted on the aperture using a polyethylene spatula under UV-free illumination. Lipid solutions were dispersions of glyceryl-1-monoolein (GMO, NuCheck, 50 mg) in hexadecane (Hex, Aldrich Chemical Co., Inc., Milwaukee, WI gold label, 1 ml), decane (Dec, Aldrich gold label, 1 ml), or squalene (Squ, purified over alumina, 1 ml; Sigma Chemical Co., St. Louis, MO); of bacterial phosphatidylethanolamine (PE, Applied Science Laboratory Waltham, MA 15 mg) in Dec (1 mL); of phosphatidyl serine (PS, Supelco, 15 mg) in decane (1 ml) and of diphytanoyl phosphatidyl choline (DPPC, Calbiochem, 15 mg) in decane (1 ml). All lipid solutions were made from components as supplied, without further purification. Salt solutions were composed of filtered, deionized water (18 MOhm, Milli-Q System from the Millipore Co.) and analytic grade salts which were first roasted ~3 h at ~600°C. Final solutions were filtered with a 0.22 μm Millipore filter (type TF, low extractables) to remove particulates. In control experiments, the final filtration did not affect the frequency of low conductance channels though it did reduce fluctuations of the lipid bilayer conductance. Membrane potential was applied by way of Ag–AgCl electrodes. Membrane current was measured using a low noise current-to-voltage converter, digitized, and stored on magnetic disk for subsequent analysis. All experiments were done at room temperature (23 ± 1°C).

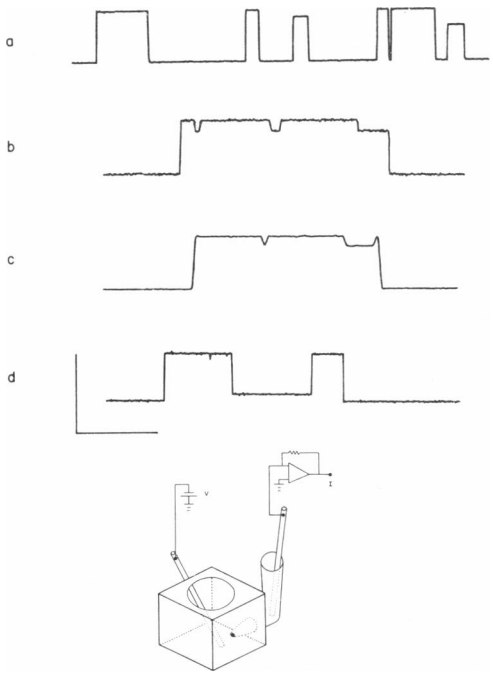

FIGURE 1 Membrane current traces in various experimental conditions. Upward deflections are conductance increases. The vertical calibration bar represents 75 pS for all but the second trace for which it is 30 pS. The horizontal bar represents 7.5, 1.25, 1.25, and 19.5 s for traces 1–4 respectively. GMO/hexadecane bilayers were used for all but the last trace for which squalene was the lipid solvent. Membranes were bathed in 1 M KCl except for the second trace which was in saturated sodium acetate. The three lower traces are all examples of standard channels undergoing reversible conductance decreases. *Insert:* The lipid bilayer apparatus comprised of a polyethylene pipette inserted in a teflon block.

For each experiment, data was collected continuously, usually for 10–30 min, after a bilayer was formed. Current transitions reflecting channel openings and closings were detected with an interactive computer program. Points between transitions were averaged and transition amplitudes calculated as differences between adjacent segments. These were binned according to the resolution of the A/D converter. The main peak was analyzed using an expedient method as follows. A range of bins which appeared to contain ~95% of the main peak was selected by eye. The mean, γ_s, and standard deviation, SD (γ_s), of transition amplitudes in this range were then used to produce a normal curve which was compared with the histogram. The fit was considered reasonable if the normal curve passed through the top of most of the bars in the standard peak. Of the total number of current transitions observed, t_T, the number falling outside (above or below) the range used to analyze the main peak, t_m, were defined as atypical "mini" channels. The mini frequency, f_m, is defined as $f_m = 100 \times t_m/t_T$. The fraction of channels which are minis (versus standards) is subject to counting error. Assuming that minis and standards occur as independent Poisson processes, we estimate the counting error in f_m to be

$$\mathrm{SE}(f_m) = 100\, C_m C_s \sqrt{(1/C_s + 1/C_m)}/(C_s + C_m)^2, \qquad (1)$$

where C_m, the number of mini channels in an experiment, is estimated as $t_m/2$ and the number of standard channels is $C_s = (t_T - t_m)/2$. For the experiments reported here, the main peak (standard channel) conductance, γ_s, fluctuated only slightly ~46 pS, the fluctuations being of the magnitude and direction expected for the measured fluctuations in room temperature. The main peak was usually quite narrow (SD[γ_s] ~ 0.5 pS) allowing precise distinction between standard and mini channels. We will summarize the results of several experiments as $f_m \pm \mathrm{SE}(f_m)$, t_T, $\gamma_s \pm$ SD (γ_s).

Bis-desformyl malonyl gramicidin was a gift from Dr. Dan W. Urry. Gramicidin A was purified from gramicidin D (ICN Pharmaceuticals Inc., Irvine, CA) using high performance liquid chromatography and stored in reagent grade ethanol at 3°C in ultraviolet light-absorbing glass vials for periods of years. Initial purity of a sample of gramicidin A obtained from Dr. Urry was established using nuclear magnetic resonance spectroscopy, amino acid analysis, circular dichroism spectroscopy, and HPLC (Prasad et al., 1982*a*). The purified gramicidin A was shown to elute with the same retention time from the HPLC column as the gramicidin A fraction of the original gramicidin D sample and also as gramicidin A synthesized de novo.

In control experiments, the channel conductance histograms were unaffected by storage procedures and did not differ appreciably for samples obtained from three different sources. Amino acid hydrolysis of a fraction of the sample used for these experiments yielded no kynurenine (an oxidation product by tryptophan). Busath and Waldbillig (1983) demonstrated that extensive gramicidin photooxidation results in the production of mini channels. We found that a small exposure of our sample to UV substantially modified the gramicidin absorption spectrum without perceptibly increasing the mini frequency. Because the absorption spectrum of our original sample lacked evidence of photolysis, accidental UV exposure is not likely to be the explanation for naturally occurring minis.

RESULTS

A close relationship between standard and mini channels is suggested by the observation of conversions between them (Fig. 1). In the first trace six channels conduct at a single

level throughout their lifetime. The third and sixth are minis; the others are standards. The next three traces show expanded views of standard channels which changed state spontaneously and reversibly during their lifetime. These and similar examples were observed for a variety of membrane lipids and aqueous salt solutions, indicating that spontaneously occurring state changes are not specific to a particular membrane or aqueous environment. In GMO-Hex bilayers for which we collected the largest amount of data, we have observed changes from the standard conductance to a variety of mini levels as well as between mini levels. While we have not observed enough statistically established state changes to determine the spectrum of accessible conductance states, they appeared to be distributed throughout the range covered by the minis. Likewise, channels formed by covalently dimerized bis-desformyl-malonyl-gramicidin usually undergo state changes during their lifetime. In one experiment, two channels were observed, one lasting 72.2 min. and the other 65.6 min. Both assumed multiple long-lived states, a total of 178 state changes during the aggregate lifetime of 138 min. for the two channels, corresponding to 0.022 state changes/s. Taken together, these observations demonstrate the ability of a single primary sequence to generate channels with a variety of conductance states.

The results of five experiments with Gramicidin A which were scrutinized for state-changes are summarized in Table I. It contains for each experiment: (*a*) the number of digitized current samples, (*b*) the number of channel current transitions, and (*c*) the number of "off" transitions not matching "on" transitions (i.e,. state changes). The observed state changes are not merely a consequence of undetected simultaneous closings and/or openings of channels having different conductances (Appendix). The final column of Table I contains the probability that all unmatched off transitions in each experiment were due to coincidences, calculated according to Eq. 2 *A* of the Appendix. The total row displays the negligibly small product of the probabilities for all five experiments. The total expected number of coincidences for all five experiments was 0.71 suggesting that most if not all of the 26 unmatched transitions represented genuine state changes.

Of the 26 state changes observed in these five experiments which contained an aggregate channel conductance time of 1.48×10^4 s, 19 were between the standard state and an easily detected but low conductance state between 1 and 9 pS (e.g., trace 4, Fig. 2). To estimate the energy of activation for this particular state change, we assessed the rate constants for the reaction:

$$\underset{(46\ \text{pS})}{\text{Typical Channel}} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \underset{(1\text{–}9\ \text{pS})}{\text{Mini Channel}} \qquad (2)$$

The equilibrium constant, $k_f/k_b = 0.028$, is similar to the ratio of the occurrence of 1–9 pS channels to that of 46 pS channels, 0.043. The energies of activation for these state changes, calculated using Eyring absolute rate theory according to:

$$k_i = \frac{kT}{h} e^{-\Delta G^\dagger/RT}, \qquad (3)$$

where R,T,h, and k have their standard thermodynamic meanings and $\Delta G^\dagger$ is the free energy barrier to the state change, were 22.2 kcal/mol for the transition from standard channel to 1–9 pS mini and 20.0 kcal/mol for the transition back to standard.

We attempted to determine whether minis are dimers. Alternatives include monomeric, trimeric, or tetrameric structures. The multimers might be composed of monomers all attached end-to-end or they might consist of a conducting dimer laterally associated with other monomers. An end-to-end trimer would be expected to have higher resistance because of its increased length, while lateral association of a cation-filled monomer with a conducting dimer might reduce conductance in the dimer by coulombic repulsion from the ion bound to the adhering monomer (Bamberg et al., 1978). The molecularity of mini

TABLE I

Experiment	Samples	Transitions	Unmatched *off* transitions	P (coincidence)
DB4A	73,728	62	3	3×10^{-6}
DB4B	102,400	150	8	5×10^{-13}
DB4C	253,952	44	2	7×10^{-6}
DB4D	267,008	136	6	2×10^{-12}
DB4E	194,560	456	7	1×10^{-6}
TOTAL	716,648	848	26	2×10^{-41}

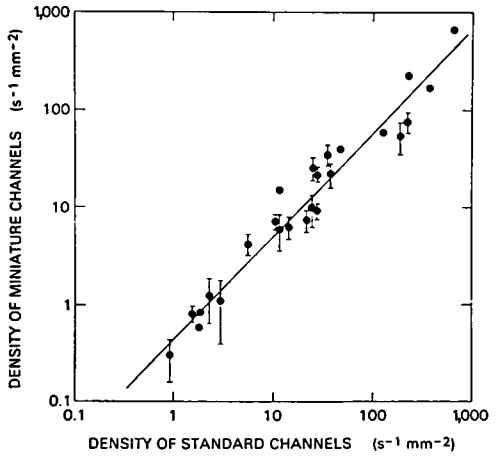

FIGURE 2 For each of 25 experiments, the rate of occurrence of variant channels per unit of membrane area is plotted logarithmically against that of standard channels. Membranes of 45, 65, 90, 400, and 640 μm diameter were used. Each experiment utilized a different bilayer and consisted of between 70 and 1,928 current transitions. Error bars represent twice the standard error due to counting of a Poisson process. The straight line is a least squares fit to the data and has a slope of 1.06. GMO/hexadecane or GMO/squalene bilayers. Standard channel peaks had means between 43.6 and 49.5 pS with standard deviations ranging from 0.10 to 0.99 pS. 1 M KCl, 0.10 V or 0.05 V.

channels was determined by comparing their frequency of occurrence with that of the standard channels. The rational for this is as follows. Taking the formation equilibrium constant of $K \cong 10^{-14}$ cm^2/mol reported for standard channels by Kolb and Bamberg (1977), it can be shown (Veatch et al., 1975) that in the range of low concentrations, ($KG \ll 1$), the membrane dimer concentration is given by KG^2, where G is the concentration of gramicidin monomers in the membrane. Note that the rate of occurrence of standard channels per unit membrane area is a measure of the dimer concentration because all dimers form observable channels (Veatch et al., 1975) and the mean channel lifetime is constant. Thus, the rate of occurrence of standard channels is proportional to the square of the gramicidin monomer concentration. Fig. 2 plots with logarithmic scales, the frequency of occurrence of mini channels versus that of standard channels, that is versus the square of the gramicidin monomer concentration. If mini channels are dimers, then a slope of 1.0 is expected for the log-log plot of Fig. 2. Other molecularities should yield slopes different from unity. The least-squares fit of a straight line to the data, shown as a solid line, yielded a slope of 1.06 ± 0.05 (S.D., $\rho = 0.975$, $N = 27$), indicating that mini channels are dimers. The membrane area varied widely, between 0.001 and 0.3 mm^2, in these experiments. The fact that the rate of occurrence of minis is proportional to that of standard channels, independent of the size of the perimeter of the lipid bilayer support, rules out the possibility that minis originate at the border of the bilayer which may present a different environment to channels.

We examined the lifetime of minis in order to determine whether or not their structure is radically different from that of the standard $\beta^{6.3}$ helical dimer. We expected, based on its junctional hydrogen bonds and channel length, for instance, that the $\beta^{4.8}$-helix (Urry et al., 1971) would have 1,000-fold shorter average lifetime than standard channels (see discussion), while double helices should have much longer lifetimes because they are held together by 28 or 30 hydrogen bonds rather than only 6 (Veatch et al., 1974). In Fig. 3, the average lifetimes for channels is plotted against conductance. Membranes utilizing three different solvents and thus having different thickness and surface tension properties, were used. For each type of membrane it can be seen that the lifetime for standard channels (extreme right data point) is similar to those of the minis in the same type of membrane. The largest difference between average lifetimes of different groups of mini channels for each type of membrane is about fourfold, indicating that the dimeric structure and length of the mini channel are quite similar to those of the standard channel.

To rule out the possibility that minis are generated by a charged or polar group bound tightly to the gramicidin pore, reducing by coulombic repulsion its cation conductance, we compared the channel conductance histograms at different ionic strengths: 0.01 M, 0.1 M, and 1.0 M KCl.

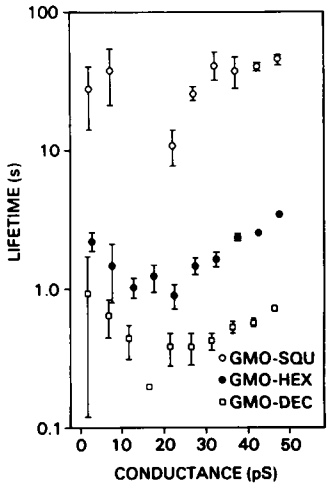

FIGURE 3 Lifetimes for channels grouped according to their conductance (±1 S.E.) are plotted against the midpoint of each groups' conductance range for channels in each of three types of bilayers, glyceryl monoolein in decane, hexadecane, or squalene. The data were composed from 1, 3, and 5 bilayers, yielding totals of 551, 586, and 445 channels, respectively for the three types of lipid solutions used. The peak conductances were 47.3 pS (decane), 44.7 – 45.0 pS (hexadecane), and 47.1 – 48.0 pS (squalene). Main peak standard deviations were all between 0.4 and 0.8 pS. Mini frequencies were 40%, 31–50%, and 25–43%, respectively. 1 M KCl, 0.1 V. The decrease in lifetimes was not due to a substantial fraction of channels with very short lifetimes because the lifetime histogram for each group had a roughly exponential distribution (data not shown).

The upper three histograms in Fig. 4 (*a*, *b*, and *c* respectively), show the results. If minis were due to charges or dipoles bound outside the channel, an increase in ionic strength would be expected to shield the bound charge and reduce the relative number of minis. The percentage of channels falling outside the main peak are 38 ± 1%, 42 ± 2%, and 55 ± 3% respectively, opposite to the trend expected from the bound charge or dipole hypothesis.

We tested the hypothesis that a titratable moiety bound near the channel mouth could produce minis. Conductance histograms (data not shown) were obtained in 1.0 M KCl at pH 3.0 and 8.0 (the latter maintained using 1 mM MOPS buffer). In two experiments at pH 3, the percentages of channels falling outside the standard channel peak were 31.3 ± 2.0% (t_T = 1097, 46.7 ± 0.4 pS) and 33.9 ± 2.3% (t_T = 867, 46.4 ± 0.3 pS). In two experiments at pH 8.0, the mini frequencies were 45.8 ± 4.3% (t_T = 277, 46.8 ± 0.5 pS) and 58.0 ± 2.1% (t_T = 1090, 47.3 ± 0.5 pS). There was no substantial difference in the histogram shapes. These values do not differ appreciably from the mini frequencies at pH ~ 5.6 (see Fig. 2), 30–50%.

The experiments described up to this point were performed with uncharged glyceryl-1-monoolein bilayers and aqueous KCl solutions. In order to test whether the bilayer lipid or aqueous salt species are critical for the formation of mini channels, we compared conductance histograms for various phospholipid membranes and salt solutions. The lower three histograms in Fig. 4 show channel conductance

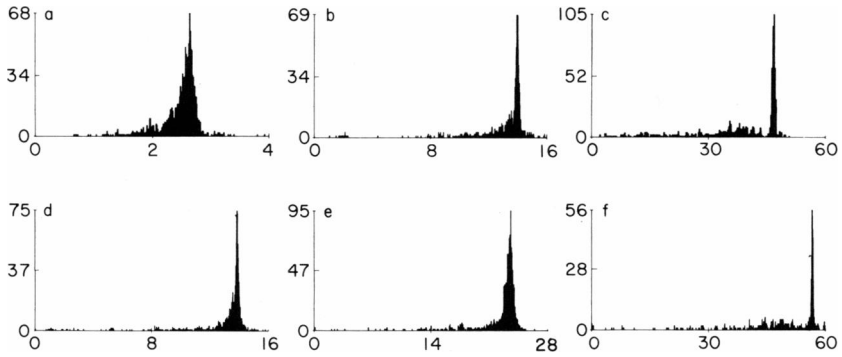

FIGURE 4 Conductance histograms for gramicidin A channels in differing environments. (*a*) 0.01 M KCl, 38 ± 1.4% minis, t_T = 2283, γ_s = 2.6 ± 0.1 pS; (*b*) 0.1 M KCl, 42 ± 2% minis, t_T = 1199, γ_s = 13.9 ± 0.1 pS; (*c*) 1 M KCl, 55 ± 3% minis, t_T = 708, γ_s = 46.4 ± 0.3 pS; *a–c*) glyceryl monoolein in hexadecane (50 mg/ml) 0.1 V; (*d*) 1 M NaCl, diphytanoyl phosphatidyl choline in decane (15 mg/ml), 51 ± 2% minis, t_T = 1,550, γ_s = 13.8 pS ± 0.1 pS; 0.2 V; (*e*) 1 M KCl, bacterial phosphatidyl ethanolamine in decane (15 mg/ml), 41 ± 2% minis, t_T = 1,384, γ_s = 23.4 ± 0.4 pS; mean standard-channel lifetime: 0.13 ± 0.01 s (S.E.), mean mini channel lifetime: 0.10 ± 0.01 s (S.E.); (*f*) 1 M CsCl and 0.1 mM EDTA, phosphatidyl serine in decane (10 mg/ml), 55 ± 3% minis, t_T = 784, γ_s = 56.6 ± 0.2 pS; *d–f*) 0.1 V.

histograms in (*d*) DPPC (1.0 M NaCl), (*e*) PE (1.0 M KCl), and (*f*) PS (1.0 M CsCl, 0.1 mM EDTA) membranes. In each case, the histogram contains a major peak of standard channels and a broad distribution of lower conductance minis. Fractions of 51% (*d*), 41% (*e*), and 55% (*f*) fell outside the standard channel peak. In another series of experiments performed using 1 M KCl (data not shown), cholesterol was added to GMO in hexadecane (10 mg total lipid per ml hexadecane) in molar ratios of 1:1 and 3:1. In seven experiments performed with 1 cholesterol: 1 GMO, the mini frequency (mean ± SD) was 23 ± 14% (t_T = 1,854, γ_s ranging from 41.2 to 43.1 pS). Using 3 cholesterol: 1 GMO, eleven experiments were performed. One experiment that was analyzed carefully yielded a mini frequency of 38.5 ± 2.2% (t_T = 985, γ_s = 31.3 ± 0.5 pS). Visual inspection of the others indicated similar results. We conclude that lipid structure and ionic species do not substantially affect the occurrence of mini channels.

We attempted to induce conformational changes directly using chemical additives, specifically amphipathic aromatics that adsorb at the lipid water interface and might induce new configurations of the hydrophobic tryptophan side chains located near the channel entrances. However, neither 200 mM benzyl alcohol nor 1 mM diphenylhydantoin added to the aqueous solution affected the mini channel frequency (f_m = 45.6 ± 3.2%, t_T = 491, γ_s = 45.3 ± 0.4 pS and f_m = 50.1 ± 2.8%, t_T = 636, γ_s = 43.3 ± 0.5 pS, respectively).

DISCUSSION

The main conclusions of this paper, namely that variant gramicidin channels are head-to-head dimers of $\beta^{6.3}$ helices, do not depend on the chemical purity of the gramicidin samples used. However, the conclusions are particularly significant if chemical purity can be assumed because it can then be asserted that variant channels result from minor conformational changes which leaves the head-to-head junction and channel length nearly unaltered. The reversible state changes illustrated in Fig. 1 are strongly suggestive of conformational changes. Several considerations lead us to believe that mini channels do not originate from contaminant peptides having sequences different from that of Val[1]-gramicidin A, although it remains possible that some other type of contaminant may yet be discovered. It is unlikely that minis are caused by variations in membrane lipid structure or polarity of the headgroup region. Variations in membrane thickness should affect primarily channel lifetimes (Hladky and Haydon, 1972) whereas variants have decreased conductance and similar lifetimes (Fig. 3). Salt composition, pH, and ionic strength are without effect on the variant channel population. These considerations lead us to believe that mini channels are caused by conformational changes within the gramicidin molecule itself.

The constant ratio of minis to standards despite a broad range in gramicidin density in the membrane, indicates that minis are not monomers or other multimeric configurations. This conclusion depends on the assumption that the standard channels are dimers and that equilibrium exists between mini channels and membrane bound monomers. The bulk of gramicidin channels and therefore at least the standard channels have been established by fluorescence methods to be dimers (Veatch et al., 1975; this result has recently been contested by Stark et al., 1986). The equilibrium assumption has not been tested. However, no substantial relaxation in the fraction of channels in the mini state could be observed over the routine course of our experiments which generally last 10–60 min.

The mini channel lifetimes indicate that variant channels are not dimers of helices having different pitches. Urry et al. (1971) suggested that β-helices having 4.8, 6.4, or 8.2 amino acids per turn would be well stabilized by hydrogen

bonds. They would be expected to differ in the number of hydrogen bonds holding the dimer together (4, 6, or 8) and also in length (3.2, 2.5, 2.0 nM). Both of these factors would affect the lifetime of the channels. Studies of the temperature dependence of channel lifetimes (Hladky and Haydon, 1972) indicate that the energy barrier to dissociation is ~19 kcal/mol, appropriate for 6 hydrogen bonds of ~3 kcal/mol each. Reduction in the number of hydrogen bonds from 6 to 4, which would be required for a narrow channel of lowered conductance, would yield lifetimes decreased by a factor of $\exp(6 \text{ kcal mol}^{-1}/RT) = 2 \times 10^4$. The lengthened channel, however, would be less subject to membrane strain. For instance, the lifetimes of standard channels in GMO Hexadecane bilayers ($\simeq$ 3.2 nM) are 6–7 times longer than in GMO-Dec bilayers ($\simeq$ 4.0 nM) in Fig. 2. Others have reported similar findings (Hladky and Haydon, 1972, Kolb and Bamberg, 1977, Rudnev et al., 1981). Thus, considering both factors, the $\beta^{4.8}$-helix would be expected to form channels having lifetimes shortened by a factor of $\simeq 10^3$ over the $\beta^{6.3}$ channel. Fig. 3 shows that although some reduction is observed, it is comparatively small. Therefore, it is unlikely that $\beta^{4.8}$-helices (or, by the same reasoning, $\beta^{8.2}$-helices) could contribute significantly to the variant channel population.

The family of double helices found to occur in organic solvents (Veatch and Blout, 1974) was proposed to give rise to groups of channels differing in conductance (Ovchinnikov, 1979). Although subsequent published conductance histograms (e.g., Bamberg et al., 1978; Busath and Szabo, 1981) have failed to substantiate the presence of discrete populations in the conductance histogram, it may be suggested that the low-conductance variants represent broadly overlapping groups formed by double helices, the main group of standard channels being due to $\beta^{6.3}$-helices. The lifetimes for double-helix channels should be very long. With 28–30 hydrogen bonds between the two monomers, the lifetimes would be expected to be governed primarily by exit of the peptide from the bilayer. For instance, covalently linked head-to-head dimers of gramicidin (malonyl bis-desformyl gramicidin), have single channel lifetimes on the order of 30–60 min which probably reflect the desorption of the peptide from the membrane (Szabo, 1981). However, Ovchinnikov and Ivanov (1983) have suggested that double helix channels could cease conducting more rapidly than expected by this rationale. They considered a dissociation mechanism proposed by Urry et al. (1975) where the two helical peptides unwind with respect to each other and they suggest that it may not be necessary for all intermolecular hydrogen bonds to break simultaneously for this to occur. It is difficult to assess what the expected channel lifetimes would be for this "zipper" mechanism. However, it seems to us that it would be very fortuitous if the zipper mechanism yielded lifetimes nearly identical to those of the $\beta^{6.3}$-helices.

The lifetimes and rate of occurence of minis vary smoothly over the conductance range. These features suggest that some continuous structural variation underlies the genesis of low conductance states. We have found (Busath and Szabo, 1984; Busath and Szabo, 1987) that fits of the current-voltage relations of variant channels suggest a continuum of increased ion-binding affinities on one side of the channel. The apparent continuum in the lifetime, appearance rate, and apparent ion affinity would be expected for a large family of conformational variants such as the side chain rotamer familty suggested by Venkatachalam and Urry (1983).

APPENDIX

State changes are events where the conductance of a channel suddenly changes to a different, nonzero value. There is a possibility, however, that the closing of one channel and the opening of a second channel could not be distinguished from a genuine state change. Therefore it is important to estimate the probability that unresolved coincidental events are mistakenly interpreted as state changes. To do this, we made the reasonable assumptions that (*a*) in the steady-state conditions of our experiments, all channels are turning on and turning off independently at stationary rates, and (*b*) there is some threshold time interval Δt for the separation of two-channel events so that events occurring closer to each other than Δt, cannot be resolved from single-state change events. We then use the Poisson distribution to determine the probability of observing n "coincidences" when only m are expected in a given set of data.

One simple definition of threshold interval is the time between digitizations when sampling at the Nyquist frequency or slower. A more rigorous approach is to consider an analog measurement and to determine the separation necessary between two coincidental events for these to deviate detectably beyond background noise from the signal produced by a single-state change. To do this, we consider that all channel events are instantaneous changes in the signal level, that the signal is then filtered with a multi-pole linear phase (Bessel), low-pass filter, and that the background noise has a uniform standard deviation σ. If, for this analog signal measurement, we count two channels as resolved when the maximum difference between the superposition of their signals and that expected from a single-state change exceeds 2σ (that is, there is >98% chance that the signal deviates from expected), then it can be shown that the threshold interval is approximately given by

$$\Delta t \simeq 2.5\, \tau\sigma/i_2, \tag{1A}$$

where τ is the risetime of the filter and i_2 is the amplitude of the second transition.

The sampling interval in our experiments closely equalled the rise-time of our filter, that is $\Delta t = 1.05\tau$. As we considered unmatched transitions separated by more

than 2σ as coincidental transitions, according to Eq. 1 *A* it is appropriate to take the threshold interval to be the sampling interval.

Next we determine the probability that two random events will occur within any of the sequential sampling intervals, Δt in an experiment. If θ_T is the rate of events and $\theta_T \Delta t \ll 1$, this is $\sim(\theta_T \Delta t)^2$. An experiment of duration T should contain $(\theta_T \Delta t)^2 N$ coincidences, where $N = T/\Delta t$ is the number of sequential intervals in the experiment. From the Poisson distribution, it is now easy to calculate the probability of observing *C* or more coincidences (apparent state changes) in an interval *T* given an expected number of coincidences, $\theta_T^2 \Delta t T$:

$$P(n \geq C) = \sum_{i=c}^{\infty} e^{-\theta_T^2 \Delta t T} (\theta_T^2 \Delta t T)^i / i! \qquad (2A)$$

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