Gramicidin Tryptophans Mediate Formamidinium-Induced Channel Stabilization

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ABSTRACT Compared with alkali metal cations, formamidinium ions stabilize the gramicidin A channel molecule in monoolein bilayers (Seoh and Busath, 1993a). A similar effect is observed with *N*-acetyl gramicidin channel molecules in spite of the modified forces at the dimeric junction (Seoh and Busath, 1993b). Here we use electrophysiological measurements with tryptophan-to-phenylalanine-substituted gramicidin analogs to show that the formamidinium-induced channel molecule stabilization is eliminated when the four gramicidin tryptophans are replaced with phenylalanines in gramicidin M^-. This suggests that the stabilization is mediated by the tryptophan side chains. Tryptophan residues 9, 13, and 15 must cooperate to produce the effect because replacement of any one of the three with phenylalanine significantly reduces stabilization; replacement of Trp-11 with phenylalanine causes negligible decrease in stabilization. In addition, formamidinium-related current-voltage supralinearity and open-channel noise are absent with gramicidin M^-. When the lipid bilayer was formed with monoolein ether rather than monoolein ester, the channel lifetimes were reduced markedly and, at low voltage and relative to those in KCl solution, were decreased by a factor of 2, whereas the open-channel noise was unaffected and the current-voltage relation was only modestly affected. These results suggest that formamidinium modifies the state of the tryptophan side chains, which, in turn, affects channel lifetime, current-voltage supralinearity, and open-channel noise through interactions with water or lipid head-group atoms including the lipid ester carbonyl.

INTRODUCTION

Tryptophans have been found to be abundant at the lipid–water interface in the photosynthetic reaction center (Deisenhofer et al., 1985; Richardson and Richardson, 1989). As pointed out by Fonseca et al. (1992) and others, they may stabilize the protein and form a major component of the protein–lipid interactions by virtue of their amphiphilic nature. This is reasonable because the indole amide can hydrogen bond readily to lipid head-group oxygens and water molecules from the bulk phase, its dipole potential can align with the interfacial potential to benefit from dipole-field stabilization, and its hydrophobic benzene ring can project into the lipid tail region of the bilayer. A few studies have been undertaken to test this hypothesis (Becker et al., 1991).

The gramicidin A (gA) channel represents an interesting model for tryptophan–lipid interaction studies because it has four tryptophan (Trp) residues at each interface. These have been shown to be stable with the indole amide projecting toward the bulk aqueous solution (Arseniev et al., 1990; Takeuchi et al., 1990; Hu and Cross, 1993). A few studies have suggested an important role of these Trp residues in gramicidin channel conductance or stability. Single-channel studies using Trp-substitution analogs demonstrate that Trp side chains stabilize channels (Fonseca et al., 1992) and increase cation conductance (Bamberg et al., 1976; Heitz et al., 1982, 1986, 1989; Sawyer et al., 1990; Becker et al., 1991, 1992; Fonseca et al., 1992). Trp photolysis has been shown to eliminate channel conductance (Busath and Waldbillig, 1983; Jones et al., 1986; Busath and Hayon, 1988; Strassle et al., 1989). Fourier-transform infrared spectroscopy and fluorescence depolarization measurements suggest Trp–lipid interactions (Takeuchi et al., 1990; Scarlatta, 1991), as does the finding that tryptophans stabilize gramicidin analogs differentially in phospholipid and monoglyceride bilayers (Fonseca et al., 1992). Molecular modeling computations suggest that phospholipid ester carbonyls may interact with Trp side chains in gramicidin (Meulendijks et al., 1989; Woolf and Roux, 1994).

Ring and Sandblom (1988) analyzed the stabilizing effects of ion occupancy on gramicidin channel lifetime using a four-site Eyring rate model to predict occupancy. They successfully related changes in channel lifetime to the H^+, K^+, and Cs^+ occupancy of the channel used to predict the current-voltage-concentration data, using the assumption that the channel termination rate constant becomes nil when the channel interior is occupied by an ion. Because channel lifetime is believed to reflect the duration of dimerization, they ascribed the stabilization of the channel to interactions of bound ions with the channel backbone that propagate to and favorably perturb the dimer junction.

Urea and guanidinium denature proteins at high concentrations, but formamidinium, $HC(NH_2)_2^+$, a related organic cation, dramatically stabilizes the gramicidin channel structure (Seoh and Busath, 1993a,b). We report here that this effect is eliminated when the gramicidin tryptophans are replaced with phenylalanines and suggest that during permeation, the ion affects the gramicidin tryptophans in ways that increase channel lifetimes and produce channel current

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noise. The gramicidin–formamidinium complex forms a simple model system in which these interactions can be studied with spectroscopic or other physical techniques to clarify the interactions of the Trp indole with surrounding molecules in the lipid–water interface.

MATERIALS AND METHODS

Planar lipid bilayers were formed on the 80-μm hole in the tip of a polyethylene pipette (Busath and Szabo, 1988a) using 1-monoolein glyceryl ester (GMO ester; NuChek Prep, Elysian, MN) or 1-monoolein glyceryl ether (GMO ether; Serdary Research Laboratories, London, Ontario, Canada) in hexadecane (Aldrich Chemical Co., Milwaukee, WI, 50 mg/ml). Formamidinium HCl (Sigma Chemical Co., St. Louis, MO) or KCl (Fisher Scientific, Pittsburgh, PA) was dissolved in distilled and purified water (Barnstead NANO Pure II, Fisher Scientific) at a concentration of 1.0 M and was used unbuffered. At the pH of unbuffered formamidinium HCl solution, ~6, formamidinium is fully ionized and gramicidin H^+ conductance is negligible.

Val^1 gA was purified from gramicidin D (ICN Nutritional Pharmaceuticals, Cleveland, OH) by analytical scale isocratic high-performance liquid chromatography (Koeppe and Weiss, 1981), using a 25-cm-long, 4.6-mm-diameter column with octylsilane beads. The mobile phase (flow rate, 1 ml/min) was 85% methanol (Caledon Laboratories, Ltd., Georgetown, Ontario, Canada) with purified water, degassed before use by filtration and stirring under vacuum. The Val^1 gramicidin C peak had a retention time (from the solvent front) of 4.7 min; that of the Val^1 gA peak, the largest peak, was 6.7 min; that of Ile^1 gA was 8.0 min. The central 30 s of the Val^1 gA peak was collected. After an injection of 2-mg gramicidin D, the eluent peak contained gA (the Val^1 species) at a concentration of ~1.2 mg/ml, which was collected and diluted ~1000-fold with methanol for single-channel experiments. The retention time for gramicidin B (W11F gA) was 3–4 min longer than that of gA. The peak was collected in the same fashion as that of gA, but the quantity of W11F gA in the original sample was ~50-fold lower than that of gA (judging by the peak heights), so the eluent was diluted only 100-fold in methanol for single-channel experiments.

Gramicidin M^- (gM) was a gift from Frederic Heitz. In this peptide the amino acid chirality sequence is inverted, and it forms left- rather than right-handed helices, which could affect the side chain positions slightly but is expected to be of little consequence for the conclusions presented here (Koeppe et al, 1992). Analogs of gA with single tryptophans replaced by phenylalanine (Phe) (W9F, W13F, and W15F gA) were gifts from Roger Koeppe and Olaf Andersen. The point mutants will be referred to as WjF, where j is the index of the mutated residue.

Single-channel currents were monitored and analyzed with techniques described previously (Seoh and Busath, 1993a). Following a method described previously (Seoh and Busath, 1993b), the zero-frequency spectral density of the open single-channel noise was approximated as the difference of the single-channel and baseline current variances, $V(i_o) - V(i_c)$, divided by the cutoff frequency of the filter, f_c. This noise statistic was divided by the mean single-channel current squared, i^2, to yield a normalized noise parameter, $S' = (V(i_o) - V(i_c))/f_c i^2$, which allows a simple comparison of noise properties of different channel types. In many models for the origin of noise, the zero-frequency spectral density is proportional to i^2. For shot noise, i is the mean channel current, which is used here for normalization. For a gated channel, i is the current in the open state. To interpret the noise in terms of a gated-channel model, it would be necessary, therefore, also to determine the probability of the open state, which is not possible with the simple analysis used here.

RESULTS

The top pair of current traces in Fig. 1 shows the behavior of standard gA channels in 1-M KCl (left) and 1-M formamidinium Cl (right) solutions. In the formamidinium solution, the conductance is lower and the typical channel lifetime longer (also see Seoh and Busath, 1993a,b). Also, the current noise is much greater than the baseline noise. The bottom panel demonstrates that these differences do not exist for gM channels. The conductances, lifetimes, and single-channel noise are approximately the same for the two solutions, as will be shown below.

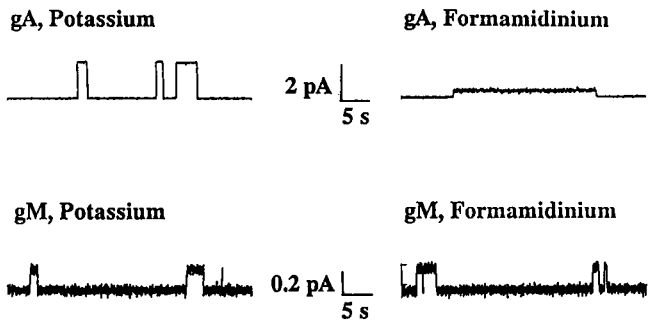

FIGURE 1 Current traces in 1-M potassium and formamidinium solutions with gA and gramicidin M channels in GMO ester/hexadecane membranes at 50 mV. Sampling frequency 100/s, cutoff frequency 30 Hz. Room temperature (22–23°C).

Using gramicidin peptides with single Trp-to-Phe mutations, we tested whether these three phenomena could be ascribed to any one of the tryptophans. From the current traces in Fig. 2 alone, it is evident that all four of the three-Trp variants have approximately the same excess single-channel noise, conductance, and prolonged channel lifetimes as gA.

To quantify the open-channel noise, we computed the statistic, S', for several standard gA channels in 1-M formamidinium solution at each of four membrane potentials as shown in Table 1. The noise measured is 3 orders of magnitude higher than the expected normalized shot noise at 50 mV. (If i is the mean channel current, the expected zero-frequency shot-noise spectral density is $2\,iq$, where q is the electronic charge and i is the mean channel current. For i = 0.43 pA (gA, 1-M formamidinium, 50 mV), the normalized shot noise, $2q/i$, is 0.75×10^{-6} s.) Furthermore, the noise decreases by ~60× at 200mV, whereas normalized shot noise should decrease by only ~4×.

As shown in Table 2, the excess noise at 50 mV is lower by an order of magnitude in gM channels than in gA channels. The single-channel noise in KCl solution is low for both gA and gM, indicating that the increased noise with formamidinium depends on the presence of the Trp side chains. No single Trp is solely responsible for the formamidinium-induced fluctuations because all four single-site mutants, WjF gA, j = 9, 11, 13, 15, display approximately the same level of noise with formamidinium as gA. It should be noted that the noise has significant components at <0.1 kHz and appears to reflect fluctuations much slower than the >10-MHz ion passage frequency. The most direct explanation for such low-frequency noise in the formamidinium currents is slow fluctuations in the structure of the channel or surrounding lipid molecules that involve the Trp side chains and are induced by the ions.

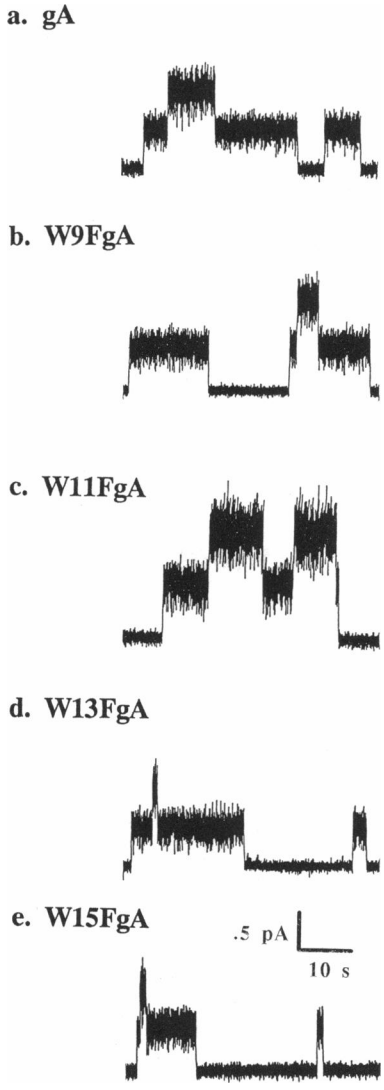

FIGURE 2 Current traces in 1-M formamidinium with gA, W9F gA, W11F gA, W13F gA, and W15F gA in GMO ester/hexadecane membranes at 50 mV. Sampling frequency 200/s, cutoff frequency 60 Hz. Room temperature (22–24°C).

TABLE 1 Voltage dependence of open-channel noise, S′, in 1-M formamidinium solution with gA channels*

V (mV)	50	100	150	200
S′	6.42 (12)	0.97 (8)	0.12 (11)	0.10 (16)

* GMO ester/hexadecane membranes. 25°C. Sampling frequency 100/s, cutoff frequency 30 Hz. The number of channels analyzed at each voltage is given in parentheses ($\times 10^{-4}$ s).

The chord conductances for gA and gM channels are plotted in Fig. 3 *a* as a function of membrane potential to compare the magnitude of the average channel conductances and the nonlinearities. In this plot, a positive slope corresponds to a supralinear current-voltage relation. For gA channels in 1-M KCl, the conductance is ~46 ps between 10 and 200 mV (Hladky and Haydon, 1972; Busath and Szabo, 1988a). In formamidinium solution, the gA channel conductance is 10-

TABLE 2 Open-channel noise, S′, in 1-M formamidinium and 1-M potassium solutions with gA, gM, and WjF gA*

Peptide	1-M Formamidinium	1-M potassium
gA	5.5 (33)	0.1 (13)
gM	0.5 (44)	0.3 (21)
W9F gA	5.3 (20)	–
W11F gA	5.8 (66)	–
W13F gA	6.8 (49)	–
W15F gA	4.8 (48)	–
gA (GMO ether)	5.8 (20)	0.05 (20)

* GMO ester/hexadecane, except last row, where GMO ether in hexadecane was used. The number of standard channels averaged together is given in parentheses. 50 mV, 22–25°C. Sampling frequency 200/s, cutoff frequency 60 Hz ($\times 10^{-4}$ s).

fold lower than in K^+ at 10 mV but is very supralinear. In contrast, the K^+ and formamidinium conductances are both low in gM channels and are only weakly supralinear. Strong supralinearity was previously noted to occur even with low (0.1-M) formamidinium concentration (Seoh and Busath, 1993a) and generally suggests that translocation through the channel is rate limiting compared with entry and exit (e.g., Hagglund et al., 1984). In the discussion we point out that in this case the disappearance of noise at high voltages should also contribute to the supralinearity.

Fig. 3 *b* displays the average channel conductances for the point-mutation W*j*F gA analogs in K^+ (*boxes*) and formamidinium (*circles*) solutions. For K^+, the W9F and W13F curves are similar to the W11F and W15F curves shown. In formamidinium, the curves for W9F, W13F, and W15F are nearly indistinguishable and are shown with small filled circles, whereas the W11F curve differs more and is shown with large open circles. The gA conductances for K^+ (upper) and formamidinium (lower) from Fig. 3 *a* are shown in Fig. 3 *b* as dashed curves for comparison. The K^+ conductance of the W*j*F analogs is significantly lower than that of gA at all voltages. The formamidinium conductance is similar to, but higher, at low voltages (and at all voltages for W11F). The W*j*F analogs display approximately the same supralinearity in the formamidinium conductance-voltage relation as gA. The Trp side chains seem to cooperate in producing supralinearity in formamidinium solutions, because complete Trp removal reduces it dramatically but removal of any one Trp does not. At low voltages the conductance is near that of the Trp-free analog gM and is noisy. At higher potentials, the excess noise disappears, and the conductance approaches that of K^+.

Dramatic changes are also observed for the single-channel lifetime. The average single gA channel lifetime in KCl rises with voltage as shown in Fig. 4 *a* (*boxes*). In contrast, with formamidinium solution (*circles*) the channel lifetimes are dramatically increased at low potentials and decrease with increased membrane potential, as reported previously (Seoh and Busath, 1993a). The voltage dependence of the stabilization suggests that the stabilization is induced by ions inside the channel. The principal alternative is that formamidinium affects lipid bilayer surface properties, such as splay constant or surface tension, which in turn affect channel lifetime

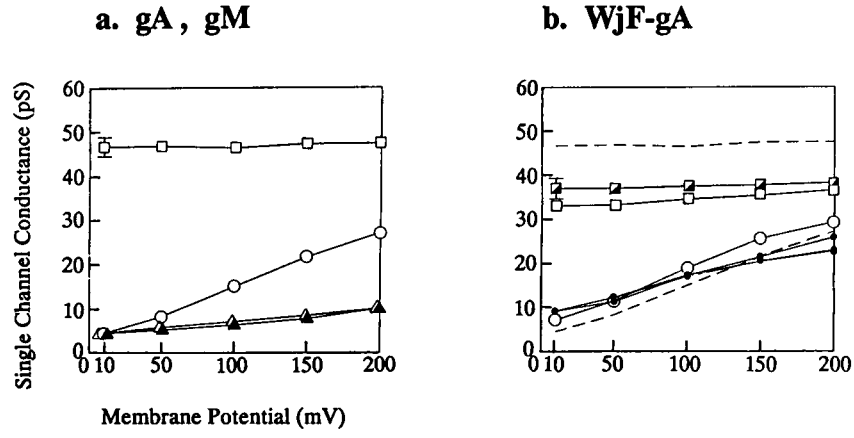

FIGURE 3 Single-channel chord conductances versus membrane potentials with (*a*) gA and gM and (*b*) W*j*F gA in 1-M formamidinium and potassium solutions. GMO ester/hexadecane. Room temperature (22–25°C). *a*: ○, formamidinium, gA; □, potassium, gA; △, formamidinium, gM; ▲, potassium, gM. *b*: ○, formamidinium, W11F gA; □, potassium, W11F gA; ◪, potassium, W15F gA; ●, formamidinium, W9F gA, W13F gA, and W15F gA. Dashed lines are for easy comparison of conductance-versus-voltage curves in gA with other curves with mutants. Standard error bars represent ±1 SE of the mean of at least 5 (up to 21) experiments for each point. Standard error bars are not shown when they would be less than symbol sizes (except SE of data with W9F gA, W13F gA, and W15F gA in the formamidinium solution whose range was ±0.04–±0.66 ps).

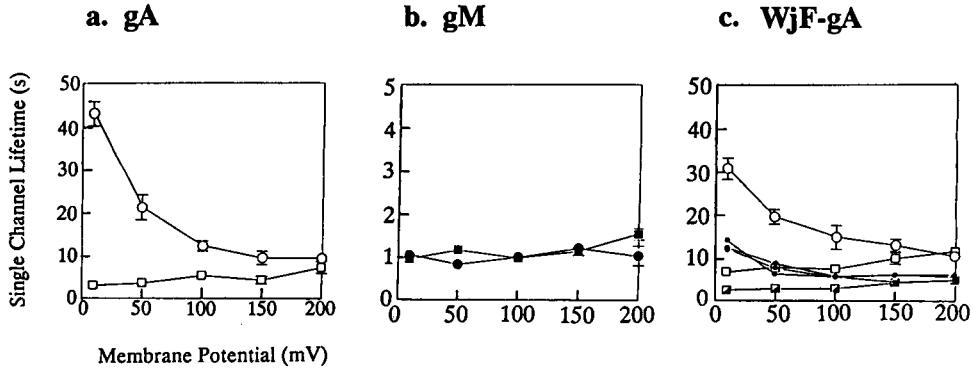

FIGURE 4 Voltage dependencies of single-channel lifetimes in 1-M formamidinium and methylammonium solutions with (*a*) gA, (*b*) gM, and (*c*) W*j*F gA. GMO ester/hexadecane. Room temperature (22–25°C). *a*: ○, formamidinium; □, potassium. *b*: ●, formamidinium; ■, potassium *c*: ○, formamidinium, W11F gA; □, potassium, W11F gA; ◪, potassium, W15F gA; ●, formamidinium, W9F gA, W13F gA, and W15F gA. Standard error bars represent %1 SE of the mean of at least 4 (up to 14) experiments for each point. Standard error bars are not shown when they would be less than symbol sizes (except SE of data with W9F gA, W13F gA, and W15F gA in the formamidinium solution whose range was ±0.33–±2.0 s).

(Huang, 1986). However, these forces should be negligible in 1-M salt and were controlled against by the experiment shown in Fig. 4 *b*. The lifetime of the gM channels in K^+ (*squares*) or formamidinium (*circles*) is ~1 s and is voltage independent. The formamidinium stabilization is absent for gM in formamidinium solution, even though any surface property changes induced by formamidinium would be present. Therefore, formamidinium must affect channel lifetime from within the channel. This finding also indicates that the gA tryptophans (absent in gM) are directly responsible for the formamidinium-induced stabilization.

To identify which of the Trp side chains contribute to the channel stabilization, we measured the lifetimes of W*j*F point mutant analogs. These are plotted in Fig. 4 *c* and listed for WISF in columns 2–4 of Table 3. The stabilization is significantly reduced when any one of Trp-9, Trp-13, or Trp-15 is mutated to Phe (*small filled circles*, Fig. 4 *c*). Mutation of Trp-11 (*large open circles*, Fig. 4 *c*) causes only a slight

TABLE 3 Single-channel lifetimes (seconds) in GMO ester (columns 2–4 and 5–7) and GMO ether (columns 8–10) lipids in 1-M KCl or 1-M formamidinium solutions with W15F gA.*

	W15F-gA, GMO Ester			gA, GMO Ester			gA, GMO Ether		
V_m (mV)	Form	K	Form/K	Form	K	Form/K	Form	K	Form/K
10	12.4	2.4	5.1	43.1	3.1	13.9	5.20	.85	6.1
50	7.8	2.8	2.8	21.4	3.7	5.8	2.12	.68	3.1
100	5.5	2.8	2.0	12.3	5.4	2.3	2.77	.76	3.7
150	4.4	4.2	1.1	9.5	4.4	2.2	1.19	.81	1.5
200	4.7	4.9	1.0	9.6	7.4	1.3	1.04	.76	1.4

* Each set contains the mean lifetimes in the two solutions (form and K) and their ratio (form/K). 22–25°C. Errors are the same as for Fig. 5 *b* (see the caption to Fig. 5).

decrease of the stabilization, indicating that it has a lesser role in stabilization. In KCl (*squares*, Fig. 4 *c*) the lifetimes are shorter and increase with voltage for W11F and W15F gA, as observed with gA; W9F and W13F gA (not shown) have lifetimes similar to those shown for W15F gA. The ratio of mean gA channel lifetimes in formamidinium to that in KCl at 10 mV is ~14 (columns 5–7 of Table 3) and decreases to ~5 in the W*j*F analogs (W15F in columns 2–4 of Table 3), except with W11F, for which the ratio decreases only to ~10. This indicates that the Trp-9, Trp-13, and Trp-15 (and, to a lesser extent, Trp-11) cooperate to stabilize channel lifetimes in formamidinium because the removal of any one Trp significantly reduces formamidinium stabilization. We note, too, that the lifetimes for the W15F analog are shorter in KCl solution than those of gA (columns 3 and 6, respectively, Table 3) and longer than those of gM (Fig. 4 *b*, *squares*), indicating that Trp-15 (and presumably Trp-9 and Trp-13 as well) stabilizes the channel somewhat even in KCl.

To test whether the supralinearity, noise, and stabilization effects depend on the lipid, we measured gA single-channel formamidinium currents (using potassium as a control) in GMO ether bilayers. The lipid differs from GMO ester in that it lacks the ester carbonyl oxygen. Fig. 5 *a* shows the single-channel potassium chord conductance (*squares*). For comparison, the data obtained with GMO ester from Fig. 3 *a* are shown as dashed curves. The supralinearity is similar in the two lipids and relatively constant with voltage, although the formamidinium conductance (*circles*) is somewhat higher in the ether. The open-channel noise with 1-M formamidinium at 50 mV is high in the ether (Table 2), similar to that observed in ester, and in 1-M KCl is low in the ether, also as in the ester. So the lipid species appears to have only modest effects on supralinearity and noise.

In contrast, the stabilization depends on the lipid species. Fig. 5 *b* shows the average channel lifetime in the ether lipid as a function of voltage in KCl (*squares*) and formamidinium (*circles*) solutions. The lifetimes in the ether lipid are much lower than in the ester lipid for both ions. This could be related to surface properties such as surface tension and interfacial potential. Because lipid surface properties could be affected by lipid species or ionic strength, we use the lifetimes in equivalent concentrations of KCl as a control. The ratios of lifetimes in the formamidinium and K^+ solutions in the columns 7 and 10 of Table 3 allow one to analyze the effects of lipid species on channel lifetime. The formamidinium-induced channel stabilization (relative to K^+), which is obvious at 10 or 50 mV (ratios of 13.9 and 5.8, respectively), decreases by one-half (to 6.1 and 3.1) when the ester membrane is replaced by the ether membrane. The ratio tends toward unity at higher potentials in both lipids. These observations, together with the elimination of stabilization in gM, suggest that interactions between ester carbonyl oxygens and tryptophans could be important in formamidinium-induced channel stabilization. The remaining channel stabilization effects might result from induced interactions of tryptophans with the other oxygens of the lipid head groups and the water when formamidinium binds in the channel.

DISCUSSION

Formamidinium-induced channel stabilization, single-channel noise, and supralinearity are eliminated when the gramicidin Trp side chains are all replaced with Phe. The stabilization and noise are also eliminated in normal gA channels at high membrane potentials. The channel stabilization and the supralinearity are reduced, but the excess open-channel noise persists when the lipid bilayer is formed from glycerylmonoolein ether instead of the ester. From these observations, we conclude 1) that the channel stabilization is mediated by the four Trp side chains, Trp-11 being the least important and that Trp-lipid interactions may be partially responsible, 2) that the high single-channel noise directly involves the Trp side chains but not interactions with the lipid 3) that the Trp-related channel fluctuations giving rise to the noise and the stabilization are inhibited at high membrane potentials, and 4) that the severe supralinearity observed in the IV (current-voltage relationship) with formamidinium is also produced by the Trp side chains.

From the point of view of traditional paradigms, these three behaviors, lifetime stabilization, channel current noise, and IV shape, would be conceptualized separately. Lifetime effects would normally be ascribed to alterations in the hydrogen bonds responsible for dimerization (Sawyer et al., 1989), which may in turn be affected by ion occupancy of

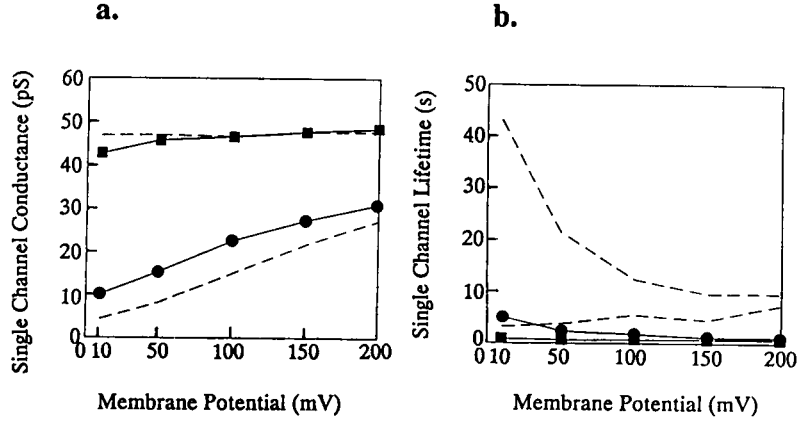

FIGURE 5 (a) Single-channel conductances versus membrane potentials with gA and (b) voltage dependencies of single-channel lifetimes in 1-M formamidinium and potassium solutions with GMO ether/hexadecane (50 mg/ml) membranes. Room temperature (21–22°C). ●, formamidinium; ■, potassium; ——, data with GMO ester/hexadecane (50 mg/ml) membranes from Figs. 3 *a* and 4 *a*. All the standard error bars were within symbol sizes. The ranges of SE for conductance data in 1-M formamidinium and potassium solutions were ±0.06–0.4 ps and ±0.02–0.6 ps, respectively, and the ranges of SE for single-channel lifetime data were ±0.06–0.37 s and ±0.03–0.08 s, respectively.

the channel through perturbations of the backbone structure (Ring and Sandblom, 1988). Current noise would be analyzed as shot noise resulting from single ions filing through the channel (Sigworth and Shenkel, 1988) or perhaps from a blocking contaminant (Heinemann and Sigworth, 1988; Hemsley and Busath, 1991). The IV shape would be analyzed in terms of the transport free-energy profile and the potential dependence of the transport steps (e.g., Sandblom et al., 1983). Here, however, these traditional approaches are inappropriate. The lifetime stabilization is eliminated by mutation of outward-pointing side chains distal to the dimer junction and unaffected by chemical modifications at the dimer junction (Seoh and Busath, 1993b). The noise is too large to be ascribed simply to transport shot noise (Sigworth and Shenkel, 1988) and cannot be due to a formamidinium contaminant because it is absent in gM channels and at higher potentials. The noise must instead represent alterations in channel structure that stop formamidinium passage for lengthy intervals, either by increased ion affinity or by steric blockage in the transport pathway. Finally, the severe supralinearity displayed by formamidinium currents in gA channels could be related to changes in the transport energy profile, which in turn depend on side chain dipole orientations, but this explanation alone does not fit the pattern of observations. Without Trp side chains, the (gM) IV for both potassium and formamidinium currents is somewhat supralinear, consistent with a "high central barrier," or, more precisely, with the rate-limiting step's being very voltage dependent. In potassium solution, the supralinearity is eliminated by the addition of tryptophans, and conductance is increased, consistent with a lowering of the central barrier by the field of the Trp dipoles (Heitz et al., 1988, Sancho and Martinez, 1991, Fonseca et al., 1992). However, in formamidinium solution the tryptophans also enhance conductance, but with a large increase rather than decrease in supralinearity. Thus, for formamidinium permeation, there must be an additional mechanism that produces supralinearity in gA.

Furthermore, the fact that these three phenomena are all eliminated with the removal of the Trp side chains and the role played by increased voltage suggests that these formamidinium effects share a common mechanism that is Trp and voltage dependent.

A unifying hypothesis

Modulation of Trp side chain motions by passing formamidinium ions could account for all these phenomena simultaneously. Formamidinium could distort the channel wall during translocation (Turano et al., 1992). At low voltages, the distortions could be coupled to slow vibrational modes in the Trp side chains. The tryptophans, which when positioned optimally do increase channel conductance over that of gM through long-range electrostatic effects of their dipoles, when they are vibrating could reduce channel conductance sterically (by applying torques to the channel wall that reduce the channel radius) or electrostatically (because of reduction in the axial component of the indole dipole) causing excess channel noise and a reduced average single channel conductance. This is an example of "fluctuating barriers" (Lauger et al., 1980). Also, the vibrations could yield a net stabilization of the dimer, for example through improved interactions with the lipid–water interface that better anchor the peptide. Perhaps one-third of the stabilizing energy derives from hydrogen bonds between indole amide hydrogens and lipid ester oxygens. A 13.9-fold increase (Table 3, 10 mV) in average gA channel duration in the presence of formamidinium (over that seen with potassium) in ester lipid bilayers corresponds to RT ln(13.9) = 1.58 kcal/mol increase in barrier to channel termination. In ether lipid, the 6.1-fold increase corresponds to a 1.08 kcal/mol barrier increase. Interactions with the ester carbonyls appear to provide 0.50 kcal/mol of the formamidinium stabilization.

At high membrane potentials, the ion passage frequency may be too high for efficient coupling with the protein lipid vibrations, just as airplane wing vibrations at low airspeeds may be reduced at higher velocity, and the structural fluctuations would be decreased. As the Trp fluctuations cease, the mechanism of enhanced dimer stabilization is lost, single-channel noise is decreased, and the average channel conductance is enhanced, resulting in the apparent supralinearity.

We have not yet attempted an analysis of the kinetics of passing ion–side chain interactions, although it would be feasible and useful to do so with phenomenological models such as Brownian dynamics or mechanical models in which the side chain, channel walls, and water/ion column are represented as solid objects, with masses being the sum of the atomic masses and force constants derived from adiabatic mapping (cf. McCammon and Harvey, 1987). However, we did perform a simple short-term dynamics computation, using the Charmm22 force field to determine whether formamidinium would induce Trp rotations (different from those induced by potassium) from within the channel through its direct interactions with the channel wall. For the computation, we used a right-handed gA monomer in the Arseniev et al. (1990) conformation with a single ion, either formamidinium (with partial charges supplied by Chemnote Molecular Simulations Inc., Burlington, MA) or potassium, placed in the channel 7–11 Å from the *N* terminus on the channel axis. Two TIP3P water molecules were placed on each side of the ion in the channel. No other waters, lipids, or ions were included in the computation. Following energy minimization, Langevin molecular dynamics (1-ps equilibration, 10-ps simulation, 300°C heat bath applied to all atoms), trajectories were computed for both the formamidinium- and K^+ containing systems. The mean dynamic structures of the two systems were compared by a least-squares fit of the peptide backbone atoms of one structure to the other, and then we estimated the angle between the planes of the indole rings to within 1°, using a protractor. The dynamics trajectory yielded average structures with significant rotations of the Trp side chain planes for residues Trp-9 (24°), Trp-13 (20°), and Trp-15 (31°). Residue Trp-11

(6°) was much less affected, consistent with the W11F lifetime results reported here.

We also note that, in potassium solutions, gA lifetimes are 4- to 10-fold greater in GMO ester than in GMO ether (Table 3) and 5- to 6-fold greater than those of gM in GMO ester (data plotted as squares in Fig. 4 *a* and *b*), indicating that the Trp side chains stabilize the channel somewhat even with the alkali metal cations. This more generalized stabilization in ester lipids could be mediated through hydrogen bond interactions with the ester carbonyls or alternatively by interactions between the indole dipole and the interfacial potential, which is 30–200 mV lower in ether lipids than in ester lipids (Paltauf et al., 1971). The greater dimer stabilization by formamidinium must be due to a higher propensity of formamidinium to induce these putative Trp-lipid headgroup oxygen interactions.

Related studies

The only solute yet identified that produces such a large effect on gramicidin channel lifetime is H^+. Ring and Sandblom (1988) found that channel lifetimes increased from ~1 s to >40 s as H^+ activity was increased from 0.01 to 1.0 M. The lifetimes increased from ~1 to ~4 s over the same range of K^+ concentrations. Interestingly, like that for formamidinium, the H^+ stabilization is reduced as membrane potential is increased toward 100 mV, suggesting a similar mechanism. However, unlike H^+, formamidinium produces a very supralinear IV even at low concentrations, which would argue against a similar mechanism, as pointed out above.

As we mentioned in the introduction, Ring and Sandblom related lifetime to occupancy, using a four-site Eyring rate theory model. We applied a similar analysis to formamidinium-induced stabilization, using a two-site model (Seoh and Busath, 1993a), but, as Ring and Sandblom (1988) predicted, this model is too simple, and we were unable to identify such a correlation. That is, our model predicted that formamidinium occupancy would be high at all concentrations studied. Four-site modeling is likely to provide the desired correlation, but we consider four-site modeling unwarranted at this time because of significant uncertainty about the permeation steps for the organic cations, which are less well understood than the alkali metal cations, and because of a high level of noise in the formamidinium current, which renders the precise conductance of the open channel ill-defined.

Dimer stabilization has also been observed in gA channels with Ag^+ and Tl^+ (McBride, 1981; Seoh, 1993), which, like formamidinium, bind tightly to the channel. These two ions also cause some excess single-channel noise in gramicidin A channels, but the noise did not disappear in gramicidin M channels (Seoh, 1993), indicating that the main source of noise is not due to the interactions between these two ions and tryptophans. Stabilization and noise might result from binding elsewhere in the channel, for instance, at the dimeric junction that was suggested (McBride, 1981) as a third binding site for these ions. Also, their dimer stabilization does not disappear at high membrane potentials as does formamidinium-induced dimer stabilization (Seoh, 1993).

Noise can also be affected by other solutes or solvent in the aqueous bath. Flicker blocks in the gA channel K^+ currents can be induced by addition to the bathing solution of guanidinium, which is chemically similar to formamidinium (Hemsley and Busath, 1991). The addition of the neutral solvent, formamide, also of similar structure, induces a high-frequency, low-amplitude noise in gA channel K^+ currents (Heinemann and Sigworth, 1988). Many examples of channel mutations that affect channel stability or noise have also been observed (e.g., Szabo and Urry, 1979; Rudnev et al., 1981; Koeppe et al., 1985; Sigworth and Shenkel, 1988; Stankovic et al., 1991; Oiki et al., 1992), but these involve structural modification of the channel and are therefore not closely related to the phenomena reported here.

Changes in the current-voltage relationship shape were observed for a series of Trp replacement analogs (Daumas et al., 1991, Fonseca et al., 1992). The replacement of Trp with a nonpolar aromatic side chain invariably led to a reduction in conductance and an increase in supralinearity, but the supralinearity was not of the degree observed with formamidinium in gA.

The usual position of the Trp side chains has been shown by two-dimensional NMR (Arseniev et al., 1990), Fourier-transform infrared spectroscopy (Takeuchi et al., 1990), and solid-state NMR (Hu and Cross, 1993) to have the indole amide dipole perpendicular to the plane of the membrane with the hydrogen projecting toward the water bath. In this position the indole would be likely to form hydrogen bonds with the water, as was suggested by O'Connell et al. (1990) to explain the low permeability of the gramicidin monomeric peptide through the bilayer. The orientation of the indole is then appropriate to enhance ion entry into and passage through the channel (Becker et al., 1991, Sancho and Martinez, 1991, Fonseca et al., 1992) by reduction of the image barrier. This may seem counterintuitive because the positively charged indole amide proton of the indole dipole is nearer the channel entry and might thus be expected to repel incoming cations. However, the bath shields the amide proton, leaving the field of the negatively charged amide nitrogen to attract cations and to reduce the absolute height of the central barrier (Sancho and Martinez, 1991).

SUMMARY

It appears that formamidinium affects Trp side chain motions as it passes through gA channels and may enhance the probability of indole interactions with lipid head-group oxygens. The relationship of channel current noise and ion passage rate could be studied with spectral analysis of currents measured with increased frequency response. If the average Trp side-chain positions are affected appreciably by formamidinium, the change may be apparent in two-dimensional NMR, Fourier transform infrared spectroscopy, solid-state NMR, and crystallographic measurements done with formamidinium in the aqueous phase. Such studies should provide additional

insight into the mobility of interface tryptophans and their interactions with lipid head groups and inform the analysis of other membrane proteins.

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