Influence of ion occupancy and membrane deformation on gramicidin A channel stability in lipid membranes

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ABSTRACT The average lifetime of gramicidin A channels in monoolein/decane bilayer membranes was measured. The results support the hypothesis of channel stabilization by ion occupancy. The effects of electric field and salt concentration are consistent with the expected effects on both occupancy and membrane compression. The lifetime in asymmetric solutions with divalent cation blockers on one side of the membrane shows a voltage dependence such that the lifetime decreases for positive voltages applied from the blocking side and increases for negative voltages. This result strongly supports the occupancy hypothesis. The lifetime increases with permeant ion concentration, and at the one molar level it also increases with voltage. The voltage dependence of lifetime for a low concentration of permeant ion depends on the total salt level. The results for these conditions are consistent with the assumption that membrane compression also influences the lifetime, even for the "soft" solvent-containing membrane considered here. It is proposed that the channel nearest neighbor lipids need not be fixed in a plane at the channel end. Using a liquid crystal model it may then be shown that surface tension is the major component of the membrane deformation free energy, which may explain the significant effects of the membrane compression on the lifetime.

INTRODUCTION

Gramicidin A is a pentadecapeptide that forms channels in lipid bilayer membranes (Hladky and Haydon, 1970, 1972) by head to head dimerization (Urry et al., 1971; Bamberg et al., 1976; Wallace et al., 1981). The channel is selective to small univalent cations and is blocked by high concentrations of divalent cations (Bamberg and Läuger, 1977).

The affinity and permeation properties have been extensively investigated using spectroscopic techniques and recently using analysis of current noise and fluctuations (Heinemann and Sigworth, 1990) and by altering the structure of the peptide (Veatch and Stryer, 1977; Heitz et al., 1982; Urry et al., 1982, 1987; Mazet et al., 1984; Koeppe et al., 1990).

The channel permeation has been modeled using molecular dynamics (Fischer et al., 1981; Mackay et al., 1984; Åqvist and Warshel, 1989; Chiu et al., 1989), by electrostatic modelling (Levitt, 1978; Andersen, 1983; Jordan, 1984; Levitt and Decker, 1988), quantum mechanical calculations (Etchebest and Pullman, 1988), and by fitting kinetic schemes to data (Neher et al., 1978; Urban and Hladky, 1979; Urry et al., 1980; Ring and Sandblom, 1983, 1988b).

The dimerization-dissociation characteristics have been investigated for their dependence on temperature, salt concentration, electric field, and bilayer properties (Hladky and Haydon, 1972; Neher and Eibl, 1977; Kolb and Bamberg, 1977; Rudnev et al., 1981; Elliott et al., 1983, 1985; Ring, 1986; Ring and Sandblom, 1983, 1988; Easton et al., 1990), but less is known about the physical mechanisms involved.

The disappearance rate of the channels has been correlated with bilayer deformation (Elliott et al., 1983, 1985, the so called ENDH model). The model assumes that a "dimple" (Hladky and Haydon, 1972) is required to accommodate the channel and that the surface tension is the driving force for dimer dissociation.

The elastic modulus for membrane deformation has been calculated and it was concluded (Hladky and Gruen, 1982) that lipid-channel interactions are more long range than previously considered. The theory for liquid crystals was applied to membranes (de Gennes, 1974; Helfrich, 1973) and, using a membrane smectic crystal (henceforth MSC) model, it was concluded that for a solventless membrane the compression and splay energies are more dominant than the surface tension (Huang, 1986).

For solvent-containing membranes it was similarly concluded (Helfrich and Jakobsson, 1990) that the major contribution was due to the compressional term, but this time surface tension also gave a significant contribution to the deformation energy.

When varying the salt concentrations for gmo/hexadecane membranes the lifetime is more influenced by the ion occupancy of the channel than by variations in surface tension (Ring and Sandblom, 1988a, 1988b).

The occupancy at low voltages may be calculated using elementary equilibrium thermodynamics, whereas the occupancy dependence on the electric field is more

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complex. For K^+, theoretical modeling using empirical conductance data shows that the occupancy increases with the salt level and with the electric field. The lifetime also increases for the same experimental conditions, and these results are therefore consistent with the occupancy hypothesis. For H^+, the lifetime changes from ~1 s at 1 mM to ~50 s at 1 M, and decreases with the electric field. Both results are also consistent with occupancy changes obtained from modeling of permeability data.

For the same experimental conditions only minor changes in surface tension are observed. These results may be interpreted as support for the MSC models which predict little dependence on surface tension. The ENDH model, however, only makes predictions about lifetime when the membrane properties are changed. It does not exclude the possibility that the lifetime may also be perturbed through variation of occupancy.

In view of the difference between the prediction of the ENDH model, which may be more applicable to thick solvent containing membranes, and the MSC models, the earlier investigations of the dependence of lifetime on ion occupancy for the thin gmo/hexadecane membranes has now been extended to the gmo/decane system.

Gmo/decane membranes are relatively thick (4.8 nm) and decane occupies the interior of the membrane (White, 1978, 1980). This system is therefore at the other extreme as compared with the gmo/hexadecane membranes, which are thin and, where hexadecane is thought to be mainly excluded from the membrane interior.

The results for the dependence of the lifetime of gramicidin A channels in gmo/decane membranes on salt concentration, electric field strength, and ion-blockers are presented. It is also concluded that for this system ion occupancy is an important determinant of lifetime, and these findings confirm the earlier results for gmo/hexadecane membranes. It was also found, however, that the field dependence of the lifetime is consistent with an influence on membrane thickness.

MATERIALS AND METHODS

Bilayer formation

The method of measuring the average gramicidin lifetime (τ) in bilayer membranes has been described previously (Neher and Eibl, 1977; Ring and Sandblom, 1983). Briefly, the cylindrical chamber with a hole of ~0.2 mm diameter was mounted vertically in electrolyte contained in a glass chamber and the lipid was then applied to the hole using a micropipette. Bilayer formation was observed at ×80 magnification.

Chemicals

Salts were of analytical grade and the water was deionized in a Millipore system (Bedford, MA). Glycerol was a synthetic product for clinical applications (Apoteksbolaget, Sweden). The activities of the solutions with osmotic or blocker additives were measured using a potassium electrode (Radiometer Medical A/S, Copenhagen, Denmark) and a calomel reference electrode. The system was calibrated using KCl solutions.

Glycerylmonooleate (99%) was from Nu-Check-Prep (Elysian, MN), and was used in a concentration of 30 mg/ml *n*-decane (for gas chromatography; from Sigma Chemical Co., St. Louis, MO). The gramicidin A, purified using HPLC, was a kind gift from Dr. R. Koeppe (University of Arkansas). The experiments were performed at room temperature (21–24°C).

Instrumentation and signal processing

The cut-off frequency of the I-V converter (OPA-111; Burr-Brown Corp., Tucson, AZ) was ~150 Hz. The signal was conditioned using a 50-Hz notch-filter and a four-pole butterworth filter (cutoff at 10 Hz), recorded on paper and sampled at 50 Hz.

The data were processed off-line by (*a*) visual inspection and rejection of records with significant instabilities, (*b*) detrending 5-min portions, and calculating the average amplitude spectrum from overlapping 40-s segments, (*c*) averaging for two to three membranes and (*d*) correcting for the measured effects of the filters.

The method was evaluated using simulation runs of channel fluctuations. The effects of drifts, windowing functions, and weighting were assessed.

The system transfer function was obtained by sampling the step response. (It was also calculated theoretically and they compared well.) The effects of the filters have to be compensated for, because filtering even at ten times above or below the cutoff frequency can significantly influence the results.

A Lorentzian model (Zingesheim and Neher, 1974), $A/(1 + (\omega/\omega_0)^2)^{0.5}$, was used to fit the amplitude spectrum and τ was obtained from the corner frequency, $\tau = 2\pi/\omega_0$, after a two-step iteration where the upper cutoff frequency was set at ten times the value obtained at the first iteration.

The errors for the determination of τ (using three or more membranes) is of the order of ±10% with a slow convergence (inherent to the spectral methods) with the length of the recording period (Ring and Sandblom, 1983). The major error, however, is due to variabilities such as temperature and the large activation energy for the loss of conduction.

RESULTS

Fig. 1 *a* shows a typical record of channel fluctuations and Fig. 1 *b* shows the corrected amplitude spectrum of the record and the best fit Lorentzian. Also shown is the filter transfer function and the uncorrected spectrum of one quarter of the record.

The occupancy of the channel increases with concentration, and the dependence of τ on the salt level is therefore a general test for the occupancy hypothesis. Fig. 2 shows the dependence of τ and channel unit conductance (G) on the potassium activity of the solutions. The salt concentrations and the corresponding potassium activities for the mixed salts were measured

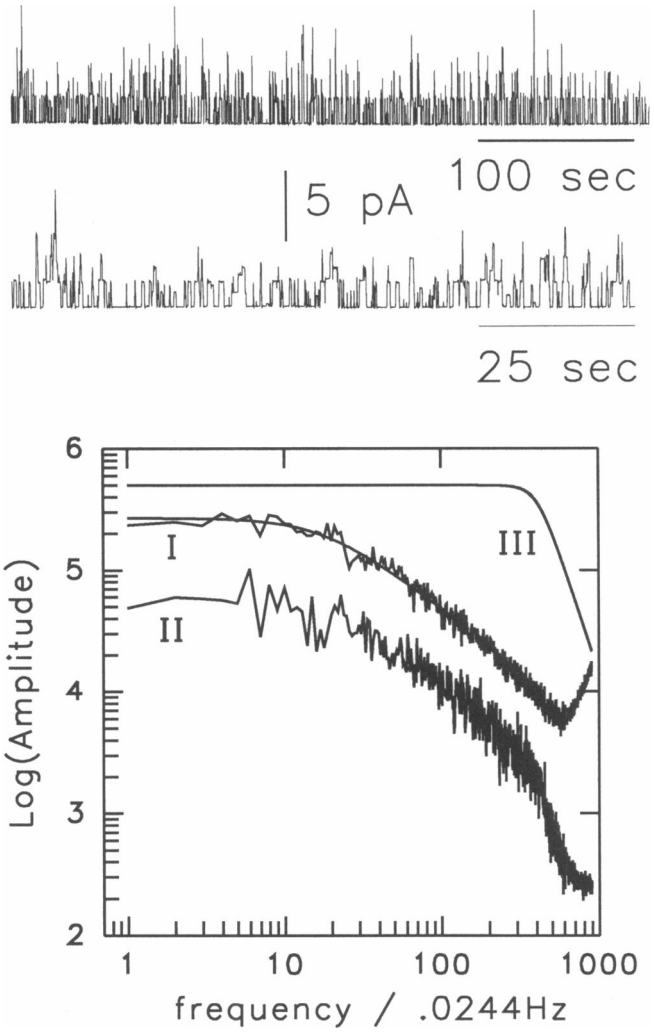

FIGURE 1 Amplitude channel noise spectrum for gramicidin A channel events. The upper part of Fig. *a* (*top*) shows the full recording (7 min) used to obtain spectrum I in Fig. *b* (*bottom*). The lower part of Fig. *a* is the first quarter (1.75 min) of the recording and the corresponding spectrum is denoted II. The transfer function of the low pass butterworth filter is also shown (III). The improvement obtained for the full recording may be noted. Spectrum I has been corrected for the effect of the filter, whereas II shows the uncorrected spectrum. The sharp drop of II at higher frequencies is due to the filter response. The rising tail of I is due to the "correction" of a digitization noise component which levels off as the Nyqvist frequency is approached. The sample time was 20 ms, 150 mM KCl, 125 mV, gmo/decane.

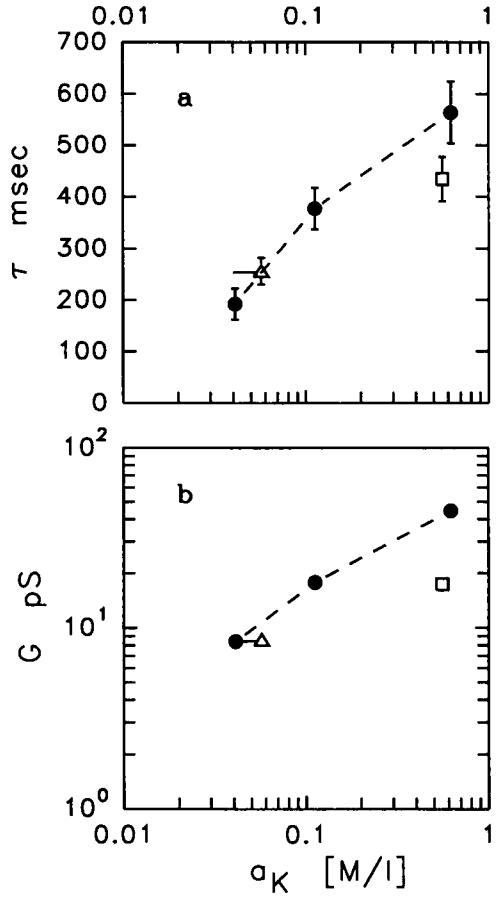

FIGURE 2 Average channel lifetime (●) (*a*) and unit channel conductance (●) (*b*) as a function of KCl salt activity, 50 mM, 150 mM, and 1 M. (□) Effect of a blocker (0.7 M KCl + 1 M $BaCl_2$), and (△) of salt-level stabilization (50 mM KCl + 2 M glycerol + 0.25 $MgCl_2$). The values for the salt levels are adjusted for the measured activities as discussed in the main text. The lifetime given is the average of measurements at 25 and 50 mV. The error bars are the standard deviations. The horizontal bars for the data of increased salt level of impermeants indicate the shift along the *x*-axes if those data were plotted as a function of concentration instead of activity (see text).

using a potassium-selective electrode and are given in Table 1.

τ is seen to increase with the salt level. Ba^{2+} decreases τ and G. Raising the salt level, keeping K^+ fixed, increases τ with no change in G.

Depending on the way data are plotted, erroneous conclusions may be drawn from the apparent shifts in τ and G. For example, if the data are plotted as a function of activities the lifetime at the lowest activity appears not to have been affected by addition of $MgCl_2$ whereas G is decreased. If instead the data are plotted as a function of concentration, the values will be shifted along the *x*-axis (the horizontal bars in Fig. 2 indicate the changes). Addition of $MgCl_2$ and glycerol then appears to give an increase in τ whereas G instead is unaffected.

Because of this uncertainty in the interpretation of the effects of $MgCl_2$ only the effect on the voltage dependence of τ is discussed, and no conclusions have been drawn from the change of τ on addition of $MgCl_2$. The shifts of the data along the concentration-axis on addition of $MgCl_2$ are small, however, and the arguments in the discussion section are not affected by this uncer-

TABLE 1 **Activities of salt mixtures**

Electrolyte	Activity	Equivalent	T°C
M/liter		*KCl (M/liter)*	
(1 M KCl)	(0.62)		
1 M KCl + 0.5 M $CaCl_2^{\ddagger}$	0.78 ± 0.04	1.28 ± 0.06	23.5
1 M KCl + 1.5 M glycerol*	0.75 ± 0.04	1.22 ± 0.06	22.5
0.7 M KCl + 1 M $BaCl_2$	0.56 ± 0.03	0.89 ± 0.05	22.5
0.05 mM KCl + 2 M glycerol + 0.25 mM $MgCl_2$	0.057 ± 0.006	0.072 ± 0.007	24

The activities were obtained (see Results) using a calibrated potassium selective electrode. The errors given are the maximum errors, and larger than the SEM, as calculated from the accuracy of the scale of the electrometer (1 mV). The value for pure 1 M KCl was obtained from the literature and is included for comparison. (This value was also one of the values used for the calibration). The temperature is the electrolyte temperature for lifetime determination. This would generally increase by up to 1°C during the time of an experiment). *Used on one side in the asymmetrical block experiments.

tainty. For the blocking experiments, performed at a high concentration of K^+, no significant shift will appear if the data are plotted as a function of concentration instead of the activity.

The permeabilities of gramicidin in various gmo/alkane membranes are almost equal (for a specified salt level and alkali cation). The behavior of occupancy should therefore also be similar for the different membranes, and the occupancy hypothesis then predicts that so should the behavior of τ. The voltage dependence of τ at various concentrations of KCl was therefore investigated. For 1 M KCl, τ is strongly dependent on the membrane voltage (Fig. 3 *a*) increasing by more than 50% when increasing the voltage from a low voltage to 200 mV. This result is qualitatively similar to that for gmo/hexadecane.

The voltage behavior of τ may be particular to permeants with a high probability of double occupancy and where the off-rate constant is rate limiting at higher electric fields, i.e., the voltage dependence of exiting is less than that of entry. It is also to be expected that the voltage dependence of τ will itself be affected by the salt concentration, because at lower salt concentration access diffusion limits the accessibility to ions (Andersen, 1983; Levitt and Decker, 1988). The voltage dependence of τ was therefore also investigated for lower concentrations of salt.

For 150 Mm KCl there was no change of τ with voltage (Fig. 3) a result consistent with that observed for 0.2 M CsCl in gmo/decane membranes (Ring, 1986). For 50 mM KCl a small increase of τ with voltage was found (Fig. 4). This finding was in contrast to the anticipated effects of access limitations and also to that observed with hexadecane membranes (Ring and Sandblom, 1988a).

The field dependence of τ here is likely to be due to membrane compression. The decrease in thickness will (both for the MSC and the ENDH models) increase τ. At low salt concentrations the gmo/decane membrane is thick (~ 4.8 nm at 0 mV) and the electric field decreases the thickness (to ~ 4.0 nm at 150 mV). Raising the total salt and osmotic level, using impermeants, also causes membrane thinning (Andrews et al., 1970). For gmo/hexadecane, the membrane thickness is not affected by the salt level and the electric field compression is also

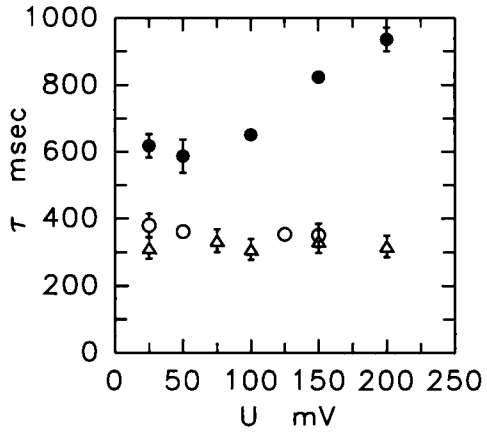

FIGURE 3 Voltage dependence of lifetime. (●) 1 M KCl, and (○, △) two experiments for 0.15 M KCl. (The temperature for the data corresponding to △ was probably larger than that of ○). At 1 M a marked increase (50%) is observed which is absent at 0.15 M KCl. Error bars are standard deviations when greater than the size of the symbols.

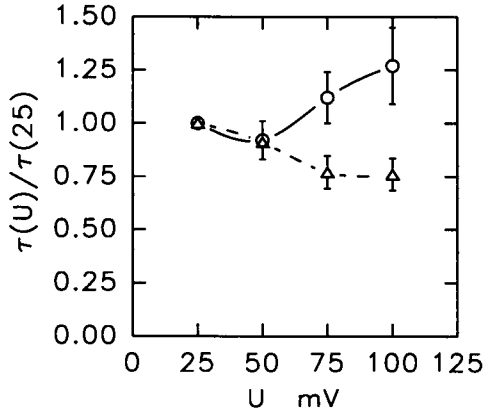

FIGURE 4 Voltage dependence of lifetime for 50 Mm KCl with (△) and without (○) salt level stabilization. (△) 50 mM KCl + 250 mM $MgCl_2$ + 2 M glycerol. The increase in lifetime for increasing field disappeared when the concentration of nonpermeants increased.

negligible, so membrane compression will not mask effects of field on occupancy.

To test the hypothesis that the increase of τ with voltage, for gmo/decane and at a low salt concentration, is due to membrane compression, the salt concentration of nonpermeations was increased using $MgCl_2$. Mg^{2+} was chosen because in contrast to Ba^{2+} and Ca^{2+} it has little blocking effect on the channel at the concentration used. (Stabilizations using nonpermeant ions such as TEA^+ have been used [Andersen, 1983] to investigate the effect of polarization on the channel conductance. Below 100 mV the effect of polarization is negligible even for a permeant salt level as low as 10 mM CsCl. $MgSO_4$ was used by Hainsworth and Hladky [1987].)

The salt level is moderate but, because the effects of increased salt concentration may also be partially an osmotic effect (Ring and Sandblom, 1988a), glycerol was used to further increase the osmotic pressure.

To facilitate comparison of the voltage dependence of τ with and without stabilization, τ in Fig. 4 is normalized by dividing with τ at 25 mV. τ is now seen to decrease with voltage (three experiments) (Fig. 4). The increase of τ with voltage at a low overall salt concentration indicates that membrane compression may be an important factor, but also, because the effect was reversed by stabilization with nonpermeants. Also, this field-compression effect is less important at a high concentration of permeant.

An important check of the consistency of the occupancy hypothesis is the influence of channel blockers, because the effects on the conductance and the field dependence of block have been well documented (Bamberg and Läuger, 1977).

The voltage dependence of τ in the presence of the divalent blocker Ba^{2+} is shown in Fig. 5. For 0.7 M KCl with 1 M $BaCl_2$, τ decreases with voltage in sharp contrast to the data of Fig. 3, where no blocker was present. This change in behavior on addition of a blocker may be attributed to the dependence of τ on ion occupancy (see Discussion).

If the change in behavior on addition of the blocker is indeed related to the ion occupancy and not to the membrane properties, then it should be possible to modulate τ for an experimental situation where the environmental conditions for the membrane are kept constant.

For example, with the blocker on only one side of the membrane the voltage dependence of the occupancy is anticipated to be asymmetric with respect to the direction of the field. The result of such an experiment is shown in Fig. 6. One side contains 1 M KCl + 0.5 M $CaCl_2$ and the other 1 M KCl + 1.5 M glycerol.

Here it is clear that τ decreases when applying fields in a direction corresponding to that of increasing the blocking of the channel (i.e., positive voltage on the side with Ca^{2+}). In contrast, when reversing the field, unblocking the channel, τ increases with the electric field.

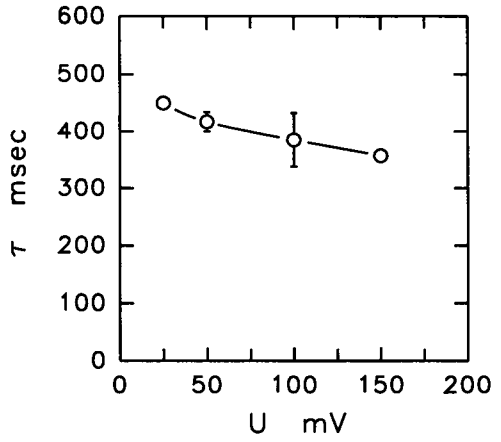

FIGURE 5 Voltage dependence of lifetime for 0.7 M KCl with 1 M $BaCl_2$. The lifetime decreases with voltage, in contrast to the case without a blocker (see Fig. 2). The activity of the electrolyte was about the same as that of 1 M KCl (Table 1). Error bars show standard deviations when greater than the symbol used.

DISCUSSION

Lifetime dependence on salt level

The average lifetime of gramicidin channels in gmo/*n*-decane membranes is seen to increase with the salt level. At 0.05 M KCl it is ~200 ms and for 1 M KCl ~600 ms.

The increase in τ is consistent with the occupancy hypothesis, i.e., the channel dissociation rate decreases with an ion in the channel. It should be noted, however, that the salt level also influences the membrane properties because there are strong effects on the critical micelle concentration of GMO in alkane solvents (White, 1973). Also, the membrane capacitance increases with salt concentration from ~0.32 $\mu F/cm^2$ at low concentration. Up to ~100 mM there is a tendency for stabilization at ~0.38 $\mu F/cm^2$, but at unimolar concentrations there is a further rise in the capacitance and this has been interpreted as being due to effects of the water activity on the state of GMO in the annulus (White, 1986).

This capacitance increase is probably related to membrane thinning (whatever the physical mechanism). It is therefore possible that the change in τ may be due to a thickness effect with changes in the destabilizing forces. As will be shown, however, the change in thickness is not sufficient to account for the changes in τ observed here.

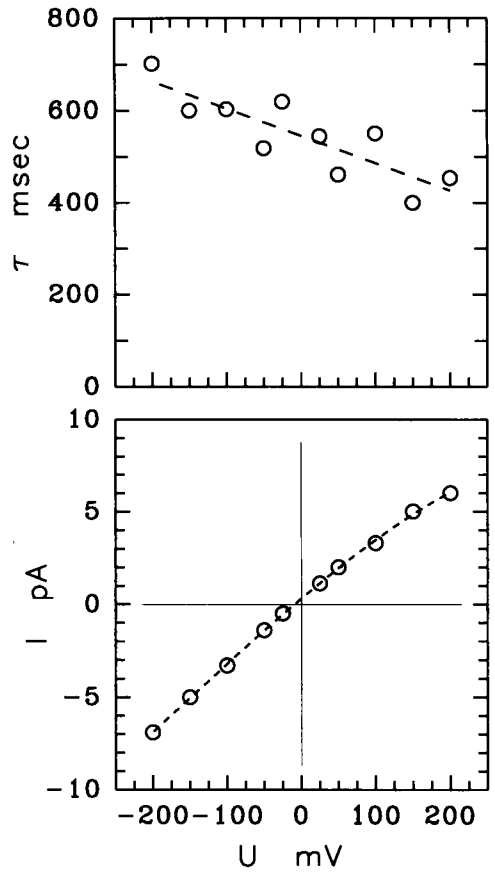

FIGURE 6 Voltage dependence of lifetime (*upper*) and single-channel current (*lower*) for asymmetric solutions. One side contains 1 M KCl + 0.5 M $CaCl_2$ and the other 1 M KCl + 1.5 M glycerol. The side with glycerol is here taken as the reference, i.e., positive voltages correspond to the blocker-containing side being positive.

The lifetime dependence on the electric field changes with salt concentration

The effects of electric field is interesting in that there is a difference in the field dependence of τ at high and low concentrations of permeant ion. For gmo/hexadecane membranes, which are thin ($\sim$3 nm), such a qualitative difference in the field dependence has been reported (Ring and Sandblom, 1988a). For gmo/hexadecane the solvent is largely excluded from the membrane and the electrical field cannot compress the membrane significantly. The relative membrane compression at 100 mV for a gmo/squalane membrane was calculated to be only $\sim$0.0003 (Hladky and Gruen, 1982, using the data from White, 1978). The influence of the electric field on surface tension is also small and may be calculated theoretically from the Lippman equation (Requena and Haydon, 1975).

In this case the field effects are more complicated. Since the decane may be "squeezed out" to the annulus (White, 1980) or into lenses (Requena et al., 1975b), it is reasonable that the electric field may influence τ due to effects on: (*a*) ion occupancy and (*b*) membrane thickness.

In addition, salt level variations complicate the voltage dependence of τ due to two separate factors:

(*a*) with a change in salt concentration the field dependence of ion occupancy also changes because the rate limiting barriers generally have different field dependencies at low and high salt concentration (e.g., due to access diffusion limitations); and

(*b*) the thickness of the membrane decreases with increasing salt concentration. This means that the field dependence of membrane thinning will be different at low and high salt concentrations.

One important difference between the salt concentration effects of cases *a* and *b* above is that the ion occupancy depends mainly on the salt concentration of only the permeant ion, whereas the membrane compression depends on the total salt level. Therefore, by varying the concentration of nonpermeants, it is possible to investigate the field effects of membrane thinning independently of those of ion occupancy.

The results of Fig. 3 can be understood as follows: at high salt concentrations increasing field increases ion occupancy. This assumption is not inconceivable because, for example, it may simply reflect that the ion entry rate has a greater field dependence than that of the exit rate (although the shape of the I-V curve must also be accounted for). At 0.15 M, however, no field dependence is observed; this can be due to a lower field dependence of entry rate at the lower salt concentration, with a retained field dependence of the rate of exiting.

For 50 mM KCl (Fig. 4), τ again increases with voltage although the field dependence is much smaller than at high salt concentrations. This increase in τ is in contrast to the result for gmo/hexadecane, where τ was found to decrease with voltage.

The change in salt concentration should have effects on the free energy barrier structure (osmotic-, screening-, or diffusion limitation-effects) (Andersen, 1983a, b, c; Finkelstein and Andersen, 1981). Nevertheless, assuming that the channel barriers and ion accessibility for gmo/hexadecane and gmo/decane membranes are similarly influenced by the salt level, it follows that the increase in τ with voltage for gmo/decane at a low salt level, different from that of gmo/hexadecane, is not explained by occupancy effects.

Modulation of lifetime by field compression of the membrane

In Fig. 4 it is clear that the field dependence on addition of $MgCl_2$ and glycerol is changed. τ now decreases with voltage, which is opposite to that expected for effects of membrane thinning but the same as the field effect obtained for the incompressible gmo/hexadecane membrane. The results are therefore consistent with the interpretation that for the nonstabilized gmo/decane membrane there were effects on τ due to membrane compression.

The influence of membrane compression is not predicted by the ENDH model (using the theory as an empirical model). From the correlation of τ with σ (Elliott et al., 1983) it follows that,

$$\ln(\tau(U)/\tau(0)) = -(z \cdot l \cdot \sigma/2 \cdot \cos\phi)/kT, \quad (1)$$

where $\cos\phi = (h - h0)^2 + 4R^2)^{0.5}$.

For saturated NaCl the calculated increase in τ is 4.25%. The values for R = 0.2 nm, $h0$ = 2.2 nm, z = 1.8 nm, l = 5 nm, $\sigma/2$ = 3.6 mN/m are from Elliott et al. (1983) and the voltage dependence of h was calculated from Fig. 2 of Andrews et al. (1970). The experimentally found increase of τ (see Fig. 3), however, is one order of magnitude larger. The "error" of the ENDH model is partially due to the small value of the deformation decay parameter R.

The voltage dependence at high salt levels is also not explained using the MSC models developed previously (Huang, 1986; Helfrich and Jakobsson, 1990). The deformation free energy is determined mainly by the incompressibility of the nearest neighbor lipid chains and should not be sensitive to the field compression which is a soft "egg-sandwich" (White, 1980) effect.

It may be noted that in these MSC models it was concluded that the surface tension is of little significance to the energy and shape of the deformation. Using new boundary conditions, however, the deformation energy was shown (Ring, A. Gramicidin channel-induced lipid membrane deformation energy. Influence of chain length and boundary conditions. Submitted for publication) to be dominated by surface tension for gmo/decane membranes. It was proposed that the lipids closest to the channel need not be fixed in a plane at the channel end. Due to the slope of the membrane at the channel rim, it is expected that the lipid molecules are displaced slightly upwards (i.e., in a direction along the axis of the channel). Boundary conditions, such that the initial slope of the membrane determines the displacement of the lipids, were used to numerically solve for the shape and energy of the deformation.

The calculated membrane shape predicts that for gmo/decane membranes, the deformation region extends for hundreds of Ångstroms and the deformation energies are smaller than those predicted using the ENDH model and are dominated by the surface tension, in part due to the high surface tension of gmo/decane membranes. From these calculations it appears that the membrane compression at low salt levels could have an effect on τ. The total deformation energy depends on both the surface tension and the difference in thickness of the membrane and the length of the channel.

At high salt levels the voltage dependence of τ is much larger. The surface tension is negligibly voltage dependent and it is not likely that the already compressed membrane should now be more voltage dependent. Thus, the MSC models do not provide an explanation for the voltage dependence of τ at high salt levels. The results are, however, consistent with the occupancy hypothesis, as discussed previously.

Blockers decrease the lifetime

Further evidence for the effects of ion occupancy comes from the result of the blocking experiment. Here (Fig. 5), at a high salt concentration (0.7 M KCl + 1 M $BaCl_2$), the dependence of τ on the electric field changes completely, now decreasing with voltage instead. This is easily understood in terms of a decreasing ion occupancy because the blocking action of Ba^{2+} may be expected to decrease the entry rate and increase the exit rate of ions; this conclusion should be of general validity, irrespective of the details of the barrier-site structure of the channel.

The saturating I-V curve in itself is an indication that the ion entry/exit rate decreases with voltage. This is consistent with the assumption of a decreasing ion occupancy.

The data do not exclude the possibility that Ba^{2+} has effects on the membrane or structural effects on the channel not related to occupancy. The channel may then be voltage dependent. Nevertheless, further support for the occupancy hypothesis is obtained from the results of the field dependence of τ in asymmetrical solutions (Fig. 6) with blocker on only one side of the membrane.

The dependence of τ on voltage for the asymmetric blocking case (Fig. 6) is markedly asymmetric, the shorter τ being obtained when the field is directed so as to promote blocking of the channel (cf. Fig. 5). For larger fields τ continues to decrease. In the reverse direction, however, the lifetime increases with voltage, consistent with the behavior associated with a high concentration of the permeant ion with no blocker present (cf. Fig. 3).

The magnitude of the electric field should be the same for positive and negative voltages because polarization effects are negligible for the voltage range used at high salt concentrations. At low concentrations of permeants,

addition of divalent cations increases the polarization at the membrane. The polarization reduces the extent of the space-charge region and therefore the access of ions to the channel (Andersen, 1983). Also, the viscosity at the channel entrance is increased. Both these effects, however, also reduce the channel entry rate and thus the occupancy, in line with the present assumption of the effects of the blockers on occupancy.

Physical mechanisms of occupancy stabilization

The physical mechanism of occupancy stabilization may be due to dielectric interaction between the ion in the channel and the gramicidin side-chains (Koeppe et al., 1990), or interaction with the carbonyl oxygens lining the channel, which may stabilize its structure or polarization of the dimerization region. These interactions give rise to changes of several kT in the permeation profile of the ion, and it seems plausible to suggest that the resulting stabilization of the channel would be of the same order of magnitude. The free energy lowering, due to interaction between the ions and the membrane, is also significant because this may be of the order of 10 kcal/mol (Åqvist and Warshel, 1989).

With the membrane compression water replaces lipid in the deformation region. The dielectric screening of ions in the channel therefore increases, which relieves the deformation energy. The decrease in deformation energy, for both the ENDH and the MSC models, results in a decrease in the "pull" on the dimer. The latter hypothesis of an interaction of the ion and the shape of the deformation region is of particular interest because it allows for a unification of the occupancy and MSC models, which are a priori independent.

It is interesting to note that the behavior of fluctuations and noise in the gramicidin channel has recently been explained (Heinemann and Sigworth, 1990) by using a model of the channel that fluctuates between two different states, where the high conductance state is stabilized by ion occupancy. This conclusion is supported by these findings and the occupancy hypothesis. The occupancy hypothesis also gains support in the finding (Roux and Karplus, 1988) that the conformation of the backbone of gramicidin is stabilized when the channel is occupied by a Na^+ ion.

There are candidates other than surface tension for the effects of membrane thinning on the channel. For example, the tryptophan on position 11 (Trp-11) is limited in its rotational degrees of freedom as the membrane is thinned (Takeuchi et al., 1990). This phenomenon results in a "pull" (owing to the hydrophobic interactions and the decrease in entropy) of each dimer half towards the middle of the membrane. As the membrane is thinned such an effect of the hydrophobic side chains might therefore give rise to a dimer stabilization. Similarly, it was recently found that the rate of gramicidin channel membrane incorporation is affected by tryptophan-lipid interactions (Easton et al., 1990). These other effects of membrane thinning on the gramicidin channel side-chains do not immediately explain, however, the correlations of τ to surface tension previously observed (Neher and Eibl, 1977; Elliott et al., 1983, 1985).

SUMMARY

It is concluded that the behavior of the lifetime of gramicidin A channels in gmo/decane membranes is consistent with an ion occupancy dependent lifetime, although the effects of the salt concentration and electric field are more complicated in this case than for the gmo/hexadecane system. At high salt concentrations the field dependence of lifetime is not explained by the ENDH or smectic crystal models, but measurements using blockers and asymmetric solutions favor the occupancy hypothesis.

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