MODULATION OF GRAMICIDIN A OPEN CHANNEL LIFETIME BY ION OCCUPANCY

AVI RING AND JOHN SANDBLOM
Department of Physiology and Medical Biophysics, Biomedical Centre, Uppsala University, S-751-23 Uppsala, Sweden

ABSTRACT The hypothesis that the gramicidin A channel stability depends on the level of ion occupancy of the channel was used to derive a mathematical model relating channel lifetime to channel occupancy. Eyring barrier permeation models were examined for their ability to fit the zero-voltage conductance, current-voltage, as well as lifetime data. The simplest permeation model required to explain the major features of the experimental data consists of three barriers and four sites (3B4S) with a maximum of two ions occupying the channel. The average lifetime of the channel was calculated from the barrier model by assuming the closing rate constant to be proportional to the probability of the internal channel sites being empty. The link between permeation and lifetime has as its single parameter the experimentally determined averaged lifetime of gramicidin A channels in the limit of infinitely dilute solutions and has therefore no adjustable parameters. This simple assumption that one or more ions inside the channel completely stabilize the dimer conformation is successful in explaining the experimental data considering the fact that this model for stabilization is independent of ion species and configurational occupancy. The model is used to examine, by comparison with experimental data, the asymmetrical voltage dependence of the lifetime in asymmetrical solutions, the effects of blockers, and the effects of elevated osmotic pressure.

INTRODUCTION

In earlier studies (1, 2) we concluded that the gramicidin A dimer stability, determined from measurements of the average lifetime of the channel, may be correlated with the ion occupancy[1] of the channel. The lifetime (τ) was found to increase with salt level with $\tau_{Cs} > \tau_K > \tau_{Li}$. To allow for a quantitative test of a possible model for the lifetime behavior, we then extended the investigations to include the effects of proton occupancy and to determine the effects of the electric field (3). Variation of pH (with no cations except protons present) results in a much greater range of conductivities and lifetime than for the alkali cations with clear and distinct features in the characteristics, particularly in the dependence of the conductance at zero-voltage and the current-voltage curves on proton concentration, and these features impose significant restrictions on the possible barrier structures of the permeation model. Protons are thought to pass through the channel by means of a Grotthus mechanism (4. 5). The interactions of the proton with the ligands of the channel, the binding sites, the binding and barrier energies, and the voltage dependence of the transition rates are therefore expected to be different from those of the alkali cations. The results for the proton are thus partially independent of the findings for the alkali cations and also serve as a check for a possible "coincidential" correlation of lifetime with ion occupancy.

Earlier reports (6, 7) have concluded that there is little or no voltage dependence of channel stability for the alkali cations. These studies, however, were primarily aimed at the characterization of channel permeability, and it has been shown (1) that the systematic study of lifetime inherently requires far greater sample registrations than investigations involving measurement of channel conductance. This difference arises because of the difference in the distribution of channel lifetimes (exponential distribution) and channel conductances (sharp, well defined distribution).

We have previously proposed that ion occupancy is a major determinant of gramicidin A channel stability (1–3). From the voltage dependence of the lifetime in asymmetrical solutions (different solutions on the two sides of the membrane), this occupancy hypothesis for the gramicidin channel was qualitatively established (3). It is therefore of interest to examine the quantitative predictions for the lifetime behavior from the degree of occupancy of the channel, and since the ion binding properties have been well characterized for the gramicidin channel, it is particularly suited for a quantitative analysis of the

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The present address of Dr. Sandblom is Department of Medical Physics, Gotenburg, Sweden.

[1] We use the term "ion occupancy" when referring to the number of ions in the channel. This occupancy may be greater than one. The occupancy of a state, however, refers in the conventional manner to the probability of finding the channel in that state. The context will define the usage and no ambiguity should arise.

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Volume 53 April 1988 549–559

lifetime properties. Such an analysis will be given in this paper based on experimental data for the voltage and concentration dependence of the lifetime for various ions.

In a Theoretical section, we describe the simplest Eyring permeation model that may explain the experimental findings—a four-barrier, three-site model with a maximum occupancy of two ions. We propose a simple mechanism for relating channel lifetime to ion occupancy and find that it can account for the experimental findings.

In the Discussion section, we show that simple assumptions may also account for the experimentally established asymmetry of the voltage dependence of the lifetime in asymmetrical solutions. Similarly, we examine the predictions of the model for conditions of blocking ions and elevated osmotic pressure.

There is no simple way of directly measuring the ion occupancy as a function of concentration or voltage. Nevertheless, for Eyring rate theory models with the channel visualized as a series of energy barriers (8, 9), once a best fit to the permeability properties has been obtained, the occupancy may be calculated from the model for any specific conditions of concentration and voltage. A problem with this procedure is that the estimated occupancies may be sensitive to small variations of the model used and also to small changes in the parameter values. In fact, it is common to view the models as lumped models for barrier permeation (i.e., not corresponding to the actual structure in a detailed manner) and even for simple multiple-barrier site-occupancy models the number of degrees of freedom for obtaining the best fit may grow large therefore enforcing the use of approximations and further simplifications. It is therefore not obvious that the calculated occupancies are reliable estimates of the actual occupancies.

Due to these limitations, it seems appropriate to attempt the correlation of occupancy and lifetime using lumped occupancies. A lumped occupancy is a sum of several state occupancies (for instance summing over those states where an ion is bound at a particular site). Similarly, we assume only two possible influences on open channel stabilization—either the open configuration is completely stabilized or the state is unaffected by ion occupancy.

It is noteworthy that two independent theoretical frameworks are required in the correlation of ion occupancy to channel lifetime. First, a barrier-site model estimating the occupancies and second, a model for the relationship between ion occupancy and lifetime.

Physically, the effect of ion occupancy on channel stability may have its origin in any of several mechanisms (see Discussion section). We have previously proposed that the dimer conformation may be stabilized with an ion in the channel (1–3). Such stabilizations are well known for proteins and enzymes in general. In particular, the approximation we use here of two "states" of the dimer, occupied and non-occupied, is analogous to the classical model for stabilization that was used to define the concept of allosteric interactions for a symmetrical dimeric enzyme (10).

THEORY

The state-occupancy of the ith state is the probability, p_i, that the system is in the ith state. The expectancy of the dissociation rate of the channel $\langle k_D \rangle$ is then given by

$$\langle k_D \rangle = \sum_i p_i k_i, \tag{1}$$

where k_i is the dissociation rate of the dimer in the ith state, corresponding to a certain configuration of ions in the channel. The average lifetime is then given by

$$\tau = 1/\langle k_D \rangle. \tag{2}$$

Eqs. 1 and 2 contain the parameters of the permeation model (p_i) and of the model for channel stability (k_i). We describe below the simplest models that are adequate in their fit to the data, where the model for the ion permeation relies on previously established properties of the gramicidin channel. The link between permeation and lifetime is obtained using only a single parameter, a normalization constant, common to all permeating ions and independent of activity and voltage.

The Permeation Model

The simplest channel permeation model that could explain the experimental data entailed three barriers with two internal and two external sites (see Fig. 1). The external sites are in equilibrium with the solutions. The model differs from that used previously (1, 11, 12) in two respects. It disallows the simultaneous occupation of neighboring sites, thus limiting the ion occupancy to a maximum total of two ($3B4S_2$). Also, the present model is less restricted with respect to the field dependence of transitions. The earlier model assumes that transitions between neighboring sites are not influenced by the number of ions in the channel.

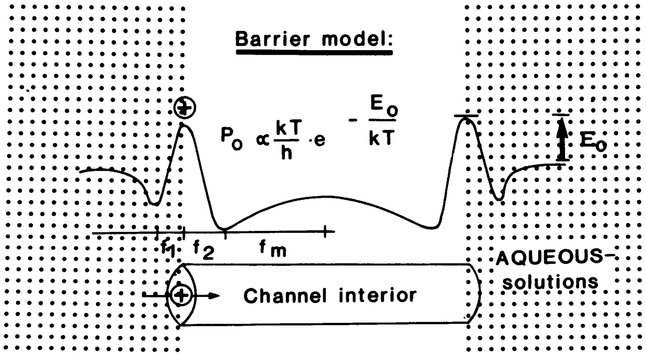

FIGURE 1. Eyring rate barrier model used to fit the data. The model shows two entry barriers and one central barrier, two internal and two external binding sites. The energy required to load an empty channel with an ion to the peak shown is here given by E_0, and the peak parameter P_0 is proportional to $\exp(-E_0/kT)$. The f parameters are the voltage dependences of the transitions. $f_m = -\frac{1}{2}(f_1 + f_2)$.

Lifetime Dependence on Ion Occupancy

Since ion occupancy appears in general to stabilize the channel, a simple postulate relating lifetime to ion occupancy is that an ion inside the channel completely stabilizes the channel (i.e., the channel can not dissociate). The same cannot apply to the situation when an outer site is occupied since then the lifetime would increase indefinitely as the concentration of permeant ion increases. This is clearly contradicted by experiment. It is also physically reasonable that a partially hydrated ion not occupying the peptide lumen is likely to have significantly less effect on the stabilization of the channel (see Discussion section). In this paper, as a first approximation, we therefore assume that an ion occupying an inner site will completely stabilize the channel, whereas an outer site has no effect on the lifetime.

In other words, the dissociation rate of a channel with an ion occupying an internal site is postulated to vanish (i.e., $k_i = 0$). The dissociation rates with ions situated in the outer sites (k_{xooo}, k_{ooox}, and k_{xoox}, indices corresponding to state configurations as shown in the Appendix), on the other hand, are equal to the dissociation rate of the empty channel (k_{oooo}). The average dissociation rate and the lifetime are then (in accord with Eq. 1) given by

$$\begin{aligned}\langle k_{\mathrm{D}}\rangle &= \sum_i p_i k_i\\ &= (p_{\mathrm{oooo}}k_{\mathrm{oo}} + p_{\mathrm{xooo}}k_{\mathrm{oo}} + p_{\mathrm{ooox}}k_{\mathrm{oo}} + p_{\mathrm{xoox}}k_{\mathrm{oo}})\\ &= p_{\mathrm{oo}}k_{\mathrm{oo}}\\ \tau &= 1/\langle k_{\mathrm{D}}\rangle = 1/p_{\mathrm{oo}}k_{\mathrm{oo}} = \tau_{\mathrm{o}}/p_{\mathrm{oo}},\end{aligned} \tag{3}$$

where p_{oo} is the probability that the channel is empty (i.e., the two internal sites not occupied), and k_{oo} is the dissociation rate in this case. It may be noted that the experimentally determined normalization constant $\tau_{\mathrm{o}} = 1/k_{\mathrm{oo}}$, which is the time constant in the limit of infinitely diluted solutions (i.e., the channel is always empty), is independent of voltage and the nature of the ion. Thus, given the occupancies of the channel, the model of lifetime contains no adjustable parameters (!).

Voltage Dependence of Channel Lifetime

For the $3\mathrm{B}4\mathrm{S}_2$ model, the relation between the τ-V curve and the I-V curve may be calculated explicitly from the basic postulate, Eq. 3 (see Appendix), and is given by

$$\frac{\tau(U)}{\tau(0)} = \frac{2\sinh(\psi/2)[1 + A(C)]\,kT}{[\cosh(\psi/2) + A(C)](I/G_{\mathrm{o}})e}. \tag{4}$$

Here $\tau(U)$ is the lifetime at a certain voltage, $\psi Ue/kT$, $e = 1.6 \cdot 10^{-19}$ Coulomb, k is the Boltzmann constant, U is the applied voltage, and I is the current in amperes through a single channel. $A(C)$ is an activity-dependent parameter given by

$$A(C) = \frac{P_{\mathrm{x}}C + P_{\mathrm{o}}}{P_{\mathrm{m}}}, \tag{5}$$

where P_{o}, P_{x}, and P_{m} are parameters that define peak energies similar to the way that the binding constants define site energies (11) (see Fig. 1 and Appendix). $A(C)$ may be interpreted in terms of relative transition rates in the channel. This is because the rate of exit from the channel is given by $p_{\mathrm{xo}}P_{\mathrm{o}}/K_{\mathrm{xo}} + p_{\mathrm{xo}}P_{\mathrm{x}}C/K_{\mathrm{xo}}$, the sum of the exit rates for the singly and doubly occupied channel. Here p_{xo} is the lumped occupancy of the channel with one inner site occupied, and K_{xo} is the equilibrium binding constant for this lumped state (see Appendix). The rate of crossing the channel is similarly given by $p_{\mathrm{xo}}P_{\mathrm{m}}/K_{\mathrm{xo}}$ and taking this into account, Eq. 5 may be recast as

$$\begin{aligned}A(C) &= \frac{P_{\mathrm{x}}C + P_{\mathrm{o}}}{P_{\mathrm{m}}}\\ &= \frac{(p_{\mathrm{xo}}P_{\mathrm{x}}C/K_{\mathrm{xo}} + p_{\mathrm{xo}}P_{\mathrm{o}}/K_{\mathrm{xo}})}{p_{\mathrm{xo}}P_{\mathrm{m}}/K_{\mathrm{xo}}} = \frac{\text{exit rate}}{\text{crossing rate}}.\end{aligned} \tag{6}$$

Eq. 4, which is valid for all models having three barriers (or less), predicts the voltage dependence of the channel lifetime from the current-voltage relationship (I-V) and from the ratio between exit and middle barrier crossing rates of the occupying ion. This provides a simple method for making a quantitative test of the occupancy hypothesis and will be used for this purpose.

The lifetime behavior for the case of a linear I-V curve predicted by Eq. 5 is depicted in Fig. 2 where the A parameter takes values from 0 to 10. As will be seen below for H^+, it turns out that the transition rate across the middle peak is very high as compared with channel entry/exit. Thus P_{m} is very large compared with the numerator

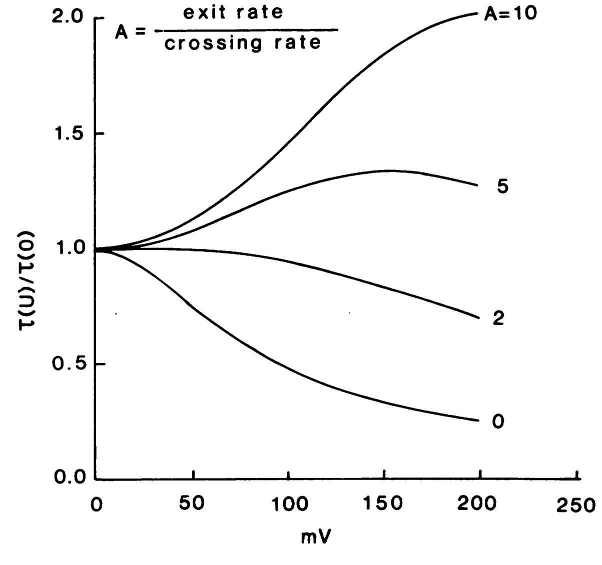

FIGURE 2. Theoretical voltage dependence of the time constant for the model of Fig. 1, for the case of an ohmic current-voltage relationship. Summing the rates from the states corresponding to the case of an ion in one of the inner sites (e.g., the left site), the voltage dependence is predicted from the ratio, A, of the total exit rate (here a jump to the left) to the crossing rate (a jump to the right). $A = 0$ and $A = 10$ correspond qualitatively to the respective cases of H^+ and K^+ occupancy.

and A is small, resulting in a lifetime strongly decreasing with voltage. The large value for the transition rate in the channel for the proton is in agreement with evaluations using other models (13).

For the opposite case, namely a slow rate of crossing the middle barrier (P_m small compared with the numerator), the lifetime increases with voltage and is a behavior seen in the presence of potassium (see below).

Since Eq. 3 provides the necessary connection between the lifetime and open channel properties, a quantitative test of the occupancy hypothesis will be carried out by fitting the $3B4S_2$ to conductance data as well as lifetime data.

RESULTS OF FITTING THE MODEL TO DATA

The best fit was obtained in three successive stages by fitting (*a*) the zero-voltage conductances as a function of concentration, (*b*) the zero-voltage lifetime as a function of concentration, and (*c*) the shape of the I-V curve as a function of concentration. The lifetime dependence on voltage is then predicted from Eq. 4, which permits a test between theory and experiment.

Fitting to G_o

The zero voltage conductance is given by (see Appendix)

$$G_o = \frac{\dfrac{e^2}{kT}\left(\dfrac{1}{P_m} + \dfrac{2}{P_x C + P_o}\right)^{-1} \cdot C}{[1 + 2(K_{xooo} + K_{oxoo}) \cdot C + (2K_{oxox} + K_{xoox})C^2]}, \qquad (7)$$

where K_{xooo} is the equilibrium constant for binding to the outer site of an empty channel and the other K parameters are similarly defined (11).

The fit was made using independent estimates of the value of P_o/P_m (15) obtained at low ionic strengths from "many channel" experiments, and four "lumped" parameters (P_o, P_x, and the coefficients of C and C^2 in the denominator) were determined using standard nonlinear regression techniques. It may be noted that different permeation models may give very similar functional dependences on activity, and thus the G_o data alone are not sufficient to distinguish between models. A further narrowing of the model is, however, accomplished using the lifetime data.

Fitting to Zero-Voltage Lifetime

The fit of the model to the dependence of G_o on salt level, as described above, gives only the sum of the inner and outer binding constants (e.g., $K_{xooo} + K_{oxoo}$, see Eq. 7). However, assuming the simple formulation of occupancy-dependent stabilization given above (Eq. 2) and using the lifetime vs. activity data, it is possible to determine the individual binding constants. This involves the regression of two additional parameters giving K_{oxoo}/K_{xooo} and K_{oxox}/K_{xoox}. The increase and behavior of the lifetime, as the salt level is increased, is determined by these ratios as can be seen by the following argument (for simplicity, we consider single ion occupancy only).

In the model for occupancy-dependent stabilization used here, it is assumed that binding to an outer site will not affect stability whereas binding to an inner site will completely stabilize the channel. With this assumption (approximation), the lifetime is determined by the magnitude of K_{oxoo}, the binding constant to an inner site. For example, $K_{oxoo}/K_{xooo} = 0$ implies that the lifetime will not change with concentration since no ion will bind internally. A large value for K_{oxoo}, on the other hand, means that a small increase in salt level results in a significant increase in inner ion occupancy and hence an increase in lifetime with concentration. Since $K_{oxoo} + K_{xooo}$ was previously determined, this means that the variation of τ with salt level determines K_{oxoo} and K_{xooo} uniquely. In other words, fitting to the lifetime determines the proportion of inner ($K_{oxoo}C$) and outer ($K_{xooo}C$) occupancy. In Fig. 3 these proportions, for the case of H^+, are expressed as percentages of the total single-ion binding constant.

For all the ions tested, it was apparent that the lifetime stabilizes and in a few cases even decreases at high salt levels (1, 3). This paradox, since occupancy is expected to increase with salt level, is resolved by considering the ratio of inner site to outer site binding constants at high salt levels. At very high salt levels, the outer sites, which are in equilibrium with the electrolyte, will almost always be occupied. The rate of entry of one ion when there is another

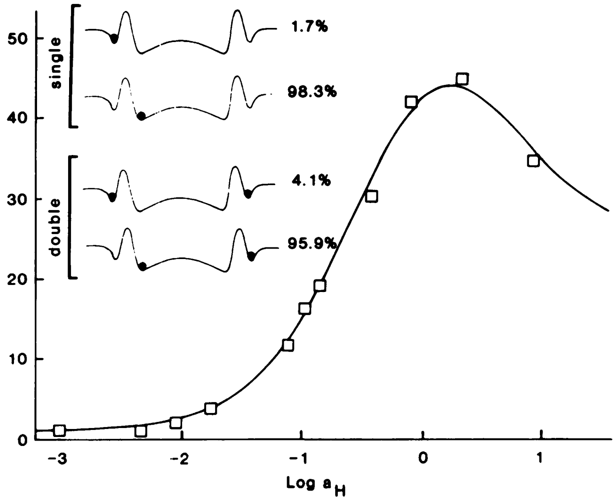

FIGURE 3. Fit of the model to the lifetime dependence on concentration. The data are for HCl (3). The curve is the best fit, given the single and double ion occupancies, when fitting the proportion of inner to outer site occupancies. The channel is assumed to break only when inner sites are empty and the lifetime is therefore dependent on the proportion of inner to outer site occupancy. The upper insert illustrates the two possible single-ion occupancies, i.e., outer and inner occupancy. The percentages are the best fit proportions of these at low applied fields (i.e., irrespective of salt level of the electrolyte, when the channel is singly occupied, a fixed proportion of the channels will be outer and inner occupancy). The lower inserts are the corresponding percentages for double-ion occupancy.

ion at the other end is not likely to be equal to the rate of entry when that site is empty. If K_{xoox}/K_{oxox} is greater than K_{xooo}/K_{oxoo}, then at high salt levels the equilibrium between inner site occupancy (proportional to $k_{oxoo}C + k_{oxox}C^2$) and outer site occupancy (proportional to $k_{xooo}C + k_{xoox}C^2$ is displaced towards the outer site. This will be observed as a decrease in lifetime due to the decrease in inner site occupancy. The physical mechanism of this shift from inner to outer occupancy may be due to the mutual repulsion of the ions and the interaction with the carbonyl oxygens in the channel. It should be noted that the total occupancy (the sum of inner and outer occupancy) increases monotonically with salt level.

Fitting to Voltage Dependence of Current

For K$^+$, because of the lack of distinguishing features in the G_0 and I-V curves, the τ-V curve is not predicted uniquely. Therefore, the distance parameters (f-parameters) were then obtained for K$^+$ from the simultaneous fit to the current-voltage and lifetime-voltage curves. The data for the shape of the I-V curves for K$^+$ were taken from reference 15. For H$^+$, on the other hand, the permeation data alone were sufficient to define completely the permeation model parameters. The voltage dependence of lifetime could therefore be predicted from the permeation data. The prediction (Fig. 4) is seen to be in excellent agreement with data.

Fig. 4 shows the fitted voltage dependence of channel lifetime for H$^+$, Cs$^+$, and K$^+$. It may be noted that for H$^+$ and Cs$^+$, τ decreases with increasing voltage, whereas that for K$^+$ increases. (These results contrast earlier reports that claim little or no dependence of gramicidin channel stability on voltage [6, 7, 16].) It is seen in Fig. 4 that the model is able to explain the qualitative differences in the voltage dependences of the lifetime.

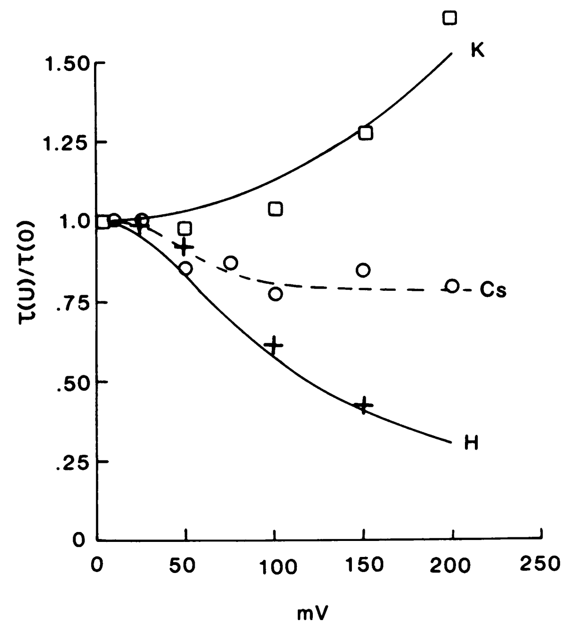

FIGURE 4. Voltage dependence of lifetime of gramicidin A for 0.5 M H$^+$, Cs$^+$, and K$^+$ (+, O and □). The lifetimes have been normalized by dividing with $\tau(0)$, the lifetime at low voltage. Continuous curves are theoretical predictions. The interrupted curve is drawn for visual aid only. Data from reference 3.

Also, it is clear from Eq. 4 that a hyperbolic I-V curve will be correlated to a lifetime increase with voltage (for a fixed value of the parameter A). For the concentration at which the data shown in Fig. 4 were measured, the corresponding I-V curve is indeed hyperbolic for Cs$^+$, where the lifetime decreases with voltage. The I-V curve is also hyperbolic for K$^+$ which, however, shows an increase in stability for increasing voltage. This unexpected behavior is useful in distinguishing between models that cannot be distinguished on the basis of permeability data alone, and is the reason why the comparison of K$^+$ and H$^+$ is particularly interesting as a test of our hypothesis.

The parameters obtained for K$^+$ and H$^+$ are given in Table 1.

DISCUSSION

The binding constants at the one ion occupancy level agree very well with the previously calculated values using the 3B4S″ model (14). This is interesting in view of the difference in the procedure used in obtaining the single-ion binding constants. In previous attempts (1) the binding constants were determined from the fit to I-V curves at various concentrations, whereas in this paper we have used the information contained in the lifetime behavior in order to extract the binding constants. The dependence of G_0 on concentration determined $K_{xooo} + K_{oxoo}$, and the lifetime dependence on concentration was then used to determine the proportion of inner to outer site occupancy.

A major obstacle in fitting even simpler models (such as the commonly used 3B2S model, which is satisfactory for many purposes) (15, 13) was the voltage dependence of lifetime and conductance of potassium. The basic requirement of the models is that of predicting a hyperbolic

TABLE I
PARAMETERS OBTAINED FROM BEST FIT OF THE 3B4S$_2$ MODEL

Parameter name	Unit	K$^+$	H$^+$
K_{xooo}	Molal^{-1}	2.25 (2.1)	1.6 (1.64)
K_{oxooo}	Molal^{-1}	7.97 (12.2)	92.5 (76.5)
K_{xoxo}	Molal^{-2}	2.96	9.01
K_{xoox}	Molal^{-2}	2.54	0.77
P_o	Molal^{-1}s^{-1}	$8.52 \cdot 10^7$ ($1.32 \cdot 10^8$)	$1.54 \cdot 10^9$ ($2.06 \cdot 10^9$)
P_m	Molal^{-1}s^{-1}	$4.37 \cdot 10^8$	$3.08 \cdot 10^{11}$
P_x	Molal^{-2}s^{-1}	$9.89 \cdot 10^8$	$6.04 \cdot 10^{10}$
f_1	%	8	9.7
f_2	%	12	19.5
f_3	%	2	8.35
f_4	%	0	2.35

Values in parentheses are from Eisenman and Sandblom (14).

(concave to the I-axis) I-V curve and, together with a simple occupancy-lifetime hypothesis, also predicting an ion occupancy increasing with the electric field.

As an example of model differences, Fig. 5 shows the predictions for channel occupancy of the 3B2S and $3B4S_2$ models for K^+ as a function of activity. The parameters for 3B2S were taken from reference 16. As can be seen the 3B2S model predicts an increase of channel occupancy at millimolar concentrations, inconsistent with the general behavior of the lifetime dependence on concentration. The predicted increase at millimolar salt levels is therefore difficult to fit with the hypothesis of occupancy related lifetime.

Figs. 6 and 7 show the dependence of G_0, the shape of the I-V curve, and the lifetime on the salt activity for HCl and KCl. No attempts to fit CsCl were made since, as explained above, it shows a behavior similar to that of H^+ (qualitatively), and since the permeation model for Cs^+ probably has to be extended to include multiple inner occupancies (12, 15, 17).

The change in voltage behavior with concentration, the contrasting behavior of K^+ and H^+, and the good agreement between theory and data are most clearly seen in the three-dimensional plots of lifetime as a function of both voltage and concentration (Figs. 8 and 9). The ordinate also represents the inverse of the probability of the channel being empty.

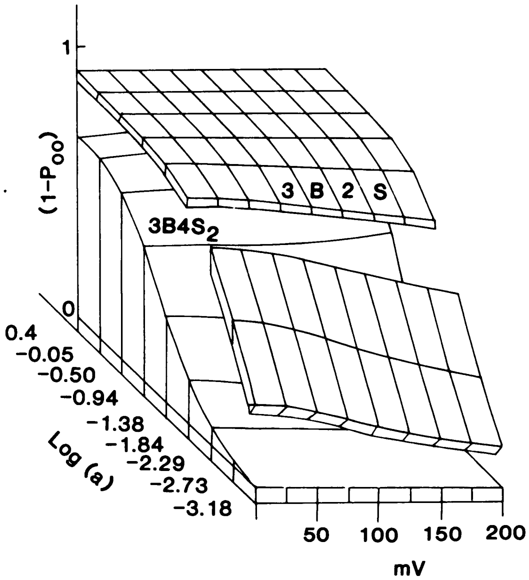

FIGURE 5. Theoretical predictions of occupancy dependence on electrolyte activity for the model used here (*lower*) and the 3B2S model (*upper*). The curves show the probability of the channel interior being at least singly occupied. The values for the 3B2S model were taken from reference 15. The occupancy rises steeply already at very low activities, which is incompatible with the postulate used here for predicting the dependence of lifetime on concentration. Also, the voltage dependence is qualitatively wrong for 3B2S, predicting a decrease of lifetime with voltage contrary to our findings.

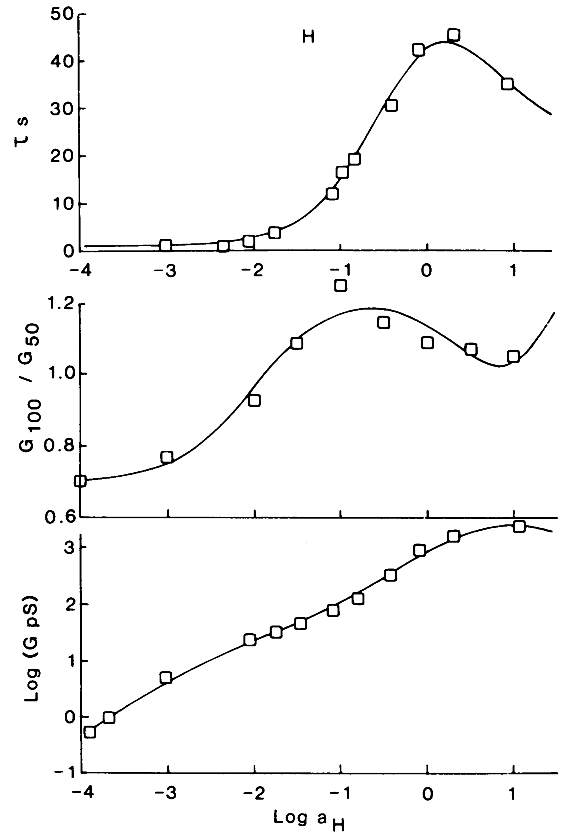

FIGURE 6 The dependence of lifetime, the shape of the I-V curve (plotted as the ratio of the conductance at 100 mV to the conductance at 50 mV), and the zero-voltage conductance for gramicidin A channels. The continuous curve is the best fit of the model to the data. HCl, 20°C gmo-hexadecane data from reference 3.

The measurement of lifetime for the case of asymmetrical solutions (3) was undertaken as a complementary test of the hypothesis of an occupancy-dependent stabilizing mechanism, and the $3B4S_2$ model was therefore used to fit the data. A good fit to the model was indeed obtained (see Fig. 10) although it is difficult to obtain a restriction of the eight "mixed" H^+/K^+ parameters from the experimental data presented here alone.

In obtaining the best fit of the model to the data in this case of mixed ion occupancy, the peak and well energies were restricted within bounds of the order of magnitude of the non-mixed binding energies. One bound is obtained on the assumption that the energy of loading the second ion is independent of the nature of the other ion present in the channel, e.g., the peak energy for loading a K^+ ion into a channel occupied with a proton is no different from the peak energy for loading the K^+ ion when a K^+ ion is in the channel. At the other extreme, it may be that an ion already in the channel determines completely the state of the channel and therefore also the energy barrier of loading a second ion, e.g., the peak-energy for loading a K^+ ion when a proton is in the channel (as previously), is equal to the peak energy of loading a proton when a proton is in the channel. The values of the parameters are likely to be of an order of magnitude lying within these bounds, which were

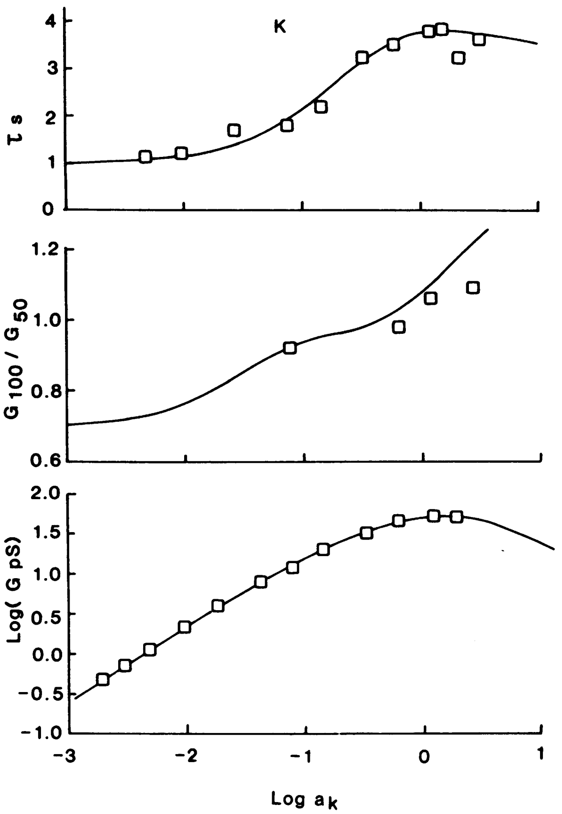

FIGURE 7 Same as Fig. 6, for KCl.

determined from the best fit in the non-mixed experiments.

Although the procedure above is not rigorous and, as noted above, the number of parameters is too large to allow a quantitative evaluation for the case of asymmetrical solutions, it is clear that the model is not contradicted by experiment and intuitively it is reasonable that if ion occupancy is the determinant of channel stability, then the lifetime should approach the lifetime of that for the "downhill" ion as the voltage is increased to extreme positive and negative values. This is certainly in complete agreement with the experimental results.

Similarly, blockers are expected to lower the ion occupancy (by decreasing the entry rate in the direction of the applied field without a corresponding decrease of exit rate on the "far field" side) and this also bears out qualitatively (3). Here also it is not possible to determine the model parameters from individual experiments, but in Fig. 11 a plot of the predicted lifetime and current versus voltage is made for the assumption that the multivalenced ionic blocker has a binding constant a factor of 20 higher than that of the proton, to the external site, all other parameters remaining unchanged. Remarkably this simple assumption, fitting only one parameter beyond those determined in earlier work, predicts both a rectification, an effect reported by Bamberg et al (18), and also a concomitant decrease of channel lifetime, as reported previously (3).

One may question whether a divalent ion, binding to an outer site of the channel, should not also have some stabilizing effect on the channel. Unfortunately there seems to be no way to settle this question until the precise molecular mechanism for the stabilization is clarified. If the mechanism is one of a simple steric hindrance, where the internal occupant ion restricts the rotational/vibrational modes of the channel, one would not expect an externally bound divalent to have any effect different from that of a monovalent. If, instead, the effect is due to electrostatic screening of the oppositely situated monomer, one might expect a stabilization by a divalent even if there is no stabilization from a monovalent. On the other hand,

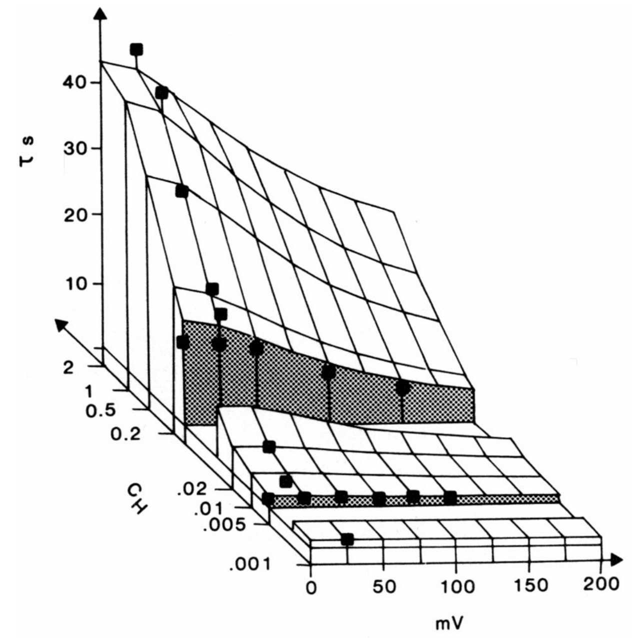

FIGURE 8. Plot of lifetime (τ_o/p_∞) as a function of voltage and concentration for HCl. τ_0 is the same for the plots of Figs. 8 and 9. Squares are experimental from reference 3.

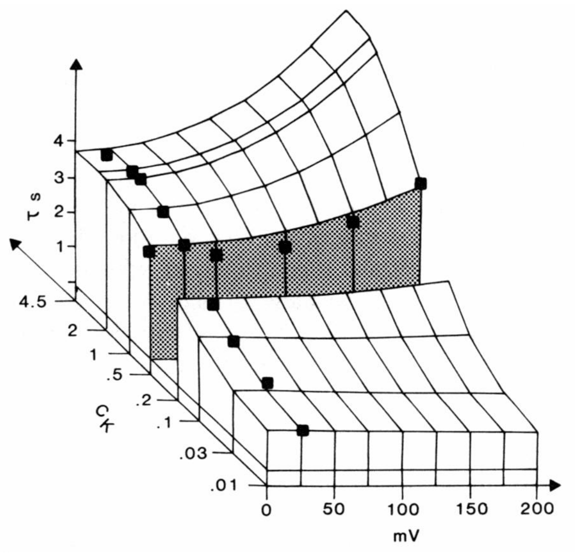

FIGURE 9 Plot of lifetime (τ_o/p_∞), for KCl.

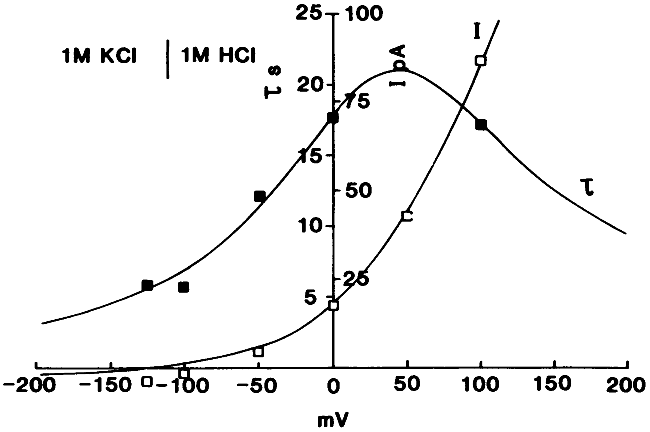

FIGURE 10. Best fit of the $3B4S_2$ model to the asymmetrical case of 1 M KCl on one side of the membrane and 1 M HCl on the other. The K^+ side is taken as reference (zero). 20°C, gmo-hexadecane. Data from reference 3. The parameters from the previous best fits (Figs. 8 and 9) were used for the single-ion occupancies. The theoretical curve shows a marked asymmetry at zero voltage.

even if there is such a stabilizing effect, it is then probably (as experimental data show) overridden by the lowering, due to the block, of the internal ion occupancy.

This suggests further lines of experimental investigation. One might see the stabilizing effects of the blockers at extremely low salt levels where the channel is anyway mostly empty, although effects of activity and anomalous influences of the blockers on the permeability at low salt levels have to be taken into account.

We have also reported (3) that the effect of the elevated osmotic pressure is to increase the lifetime and to decrease the conductivity. As we discussed there, although the mechanism of the effect is unclear, it is possible to model this behavior by postulating effects on the channel binding constants.

Fig. 12 shows an example of such calculations, for two different situations. Both curves are predicted using a single parameter change of the regular $3B4S_2$ model. The dotted curve is the effect of an increase in inner-site ion occupancy obtained by decreasing the outer-site affinity. This assumption results in an increase in lifetime and a decrease in conductance, as is seen experimentally for the osmotic effects. The dashed curve, on the other hand, is obtained using the assumption that if an ion blocker binds to an outer site (which is equivalent to reducing, competitively, the binding constant to the outer site for the permeant cation), it also interacts with ions inside the channel and thereby, in the extreme, excludes other cations from the channel.

The physical mechanism effect of ion occupancy on channel stabilization may be of a simple steric nature (19) or may involve interactions with water molecules and ligands (carbonyl oxygens) lining the channel. The stabilizing effect would then arise due to the restrictions of twisting movement or vibration of the monomer occupied with an ion. The stabilization may also come about due to one monomer screening the electrical field of the ion in the other (1, 16). Breaking the dimer would then increase the system energy more when an ion occupies the channel than if the channel is empty. Direct electrical effects of the ion in one monomer on the configurational stability of the other monomer can also not be excluded (13).

For the mathematical model of occupancy-related stabilization, several alternatives may be considered and at some level of theoretical complexity, and with a sufficiently large number of parameters one might obtain a fair fit to experimental data simply by allowing for (in the theory) multiple ion occupancy with a particular specific influence, on the lifetime, of each possible level of the occupancy (0, 1, 2 . . .). Or extending this, one may postulate distinct effects for each possible configuration of ions in the channel. The instantaneous dissociation rate of the dimer would then not be uniquely determined by the number of ions in the channel, but rather by the specification of the binding site for each of the ions in the channel.

Also, one may have the model take into account the nature of the ion. For example, due to the difference in size of Cs^+ and Li^+, they are expected to introduce different strains on the channel conformation (19). This would introduce additonal degrees of freedom in the theoretical model. It is clear that the number of "fitted" parameters may grow very large. We have described the simplest model adequate to explain the experimental data relying on a wealth of established properties of the gramicidin channel. The lifetime was then predicted from the calculated occupancies using only a single parameter.

The interactions of ion occupancy with physiological channels have not been systematically studied probably because the ion occupancy of a channel is not as easily

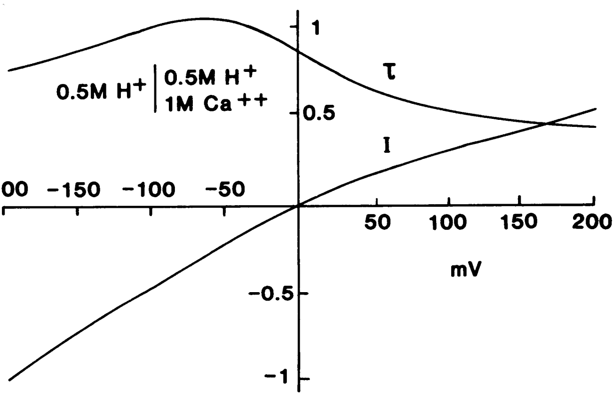

FIGURE 11. Theoretical prediction of the $3B4S_2$ model for the case of an asymmetric distribution of ionic blockers assumed to bind to external sites only (since the gramicidin channel is permeable only to single valued cations). Block-free side taken as reference (zero-voltage). The lifetime is asymmetric, decreasing for positive voltages, and the I-V curve shows a rectification.

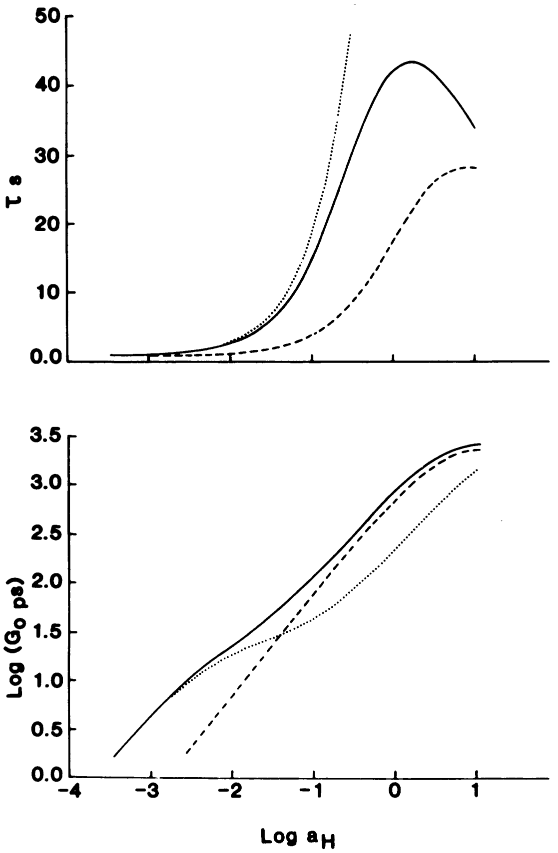

FIGURE 12. Effects of varying the affinities to the channel. Dotted curves (osmotic effects) result from the assumption that the outer binding energy is decreased (no other change!), and the dashed curves result from a similar decrease of the binding of a multivalenced ionic blocker, which then also excludes other (permeant) cations from the channel.

accessible as is the channel conductance and the membrane potential and generally requires additional information on the channel barrier-site structure. Nevertheless, the interaction of ion occupancy and channel gating has been independently implied by a number of authors for the acetylcholine receptor channel, the γ-aminobutonic acid-activated channel and for K channels (20–25). For example, Stühmer (24) found that increasing the extracellular K^+ concentration "slows down the decrease in K channel conductivity on repolarization" and suggests that the open-close transition may be blocked in K channels occupied by a K^+ ion. This seems to be in complete analogy to the conclusions of the present investigation.

In conclusion, specific models of ion permeation may be related to ion occupancy, and the relation between the lifetime and permeability selectivity may be quantified and tested for its predictions for ion channels in general. For this channel the fit of theory to data is remarkable considering that, given the ion occupancy of the channel, the lifetime is predicted using only an experimentally determined normalization constant independent of voltage and common to all ions.

APPENDIX

Derivation of State Occupancies and Channel Conductance

The state diagram for the modified 3B4S model, not allowing neighboring sites to be occupied, is shown in Fig. A1. The Eyring rate equations are easily derived using a method (13) where the rate constants are obtained as ratios of peak (P) to well (K) parameters. This method is particularly advantageous for models with sites in equilibrium with the solutions, allowing a reduced state diagram description (Fig. A1). Also, this method incorporates, in a self-evident manner, the constraints of microscopic reversibility and the field dependences for the various transitions.

The rate constants are obtained from the peak and well energies as follows (13). Each P_i and K_i is essentially the exponential of the energy required to load an ion to a peak (P) or well (K). The notation uses indices corresponding to the relevant configuration of ions in the channel, e.g., K_{oxox} is the binding constant for the state with the left inner and right outer sites occupied. In the P parameters, however, are included the universal frequency factor kT/h, as obtained from Eyring rate theory, so the dimensions of P_o and P_m are conc^{-1}s^{-1} and of P_x conc^{-2}s^{-1}. P_o/K_{xooo} is the rate constant for ion entry into an empty channel from the outer site, P_o/K_{oxoo} for a jump in the opposite direction, P_m/K_{oxoo} for a jump over the middle barrier, P_x/K_{xoox} for a jump from an outer site to an inner site when both outer sites are occupied, and P_x/K_{oxox} for a jump out of an inner site when the opposite outer site is occupied. The rate constants are then modified by voltage by multiplying with factors of exp (f_iUe/kT), where the f_i are expressed as fractions of the applied voltage U. $e = 1.6 \cdot 10^{-19}$ Coulomb, k is the Boltzmann constant, and T is the temperature in Kelvin.

The model used here (see Fig. 1) differs from the earlier 3B4S models in that f_3 and f_4 (not shown, representing a transition over the left barrier with an ion situated in the outermost site, on the right) are not set equal to f_1 and f_2 (same transition but with outermost site empty, see Fig. 1). Also, neighboring sites may not be simultaneously occupied in this model.

In general, if the rate constant of going from one state j to another state i is denoted v_{ij} (s^{-1}), then the peak parameter from the transition is defined by $v_{ij} = P_{ij}/K_j$. The rate constants are defined so that, in a

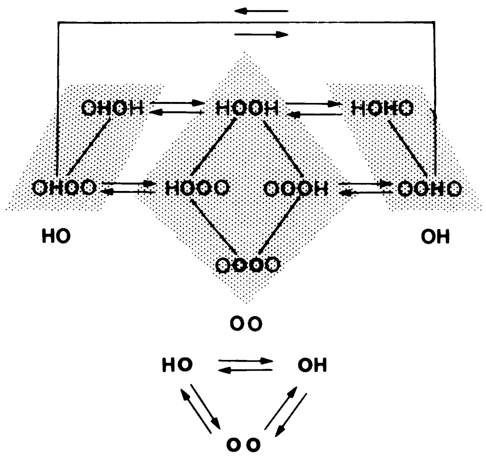

FIGURE A1 State diagram (*upper*) for gramicidin channel in symmetrical solutions. See also barrier model (Fig. 1) in main article. OOOO represents the empty channel, and this state is in equilibrium (also with a voltage applied) with all states with inner sites empty. The equilibrium states may be lumped, and this simplifies the state diagram (*lower*) and the mathematical analysis. In the reduced state diagram, HO represents the states with the inner left-site occupied. OO and OH are similarly defined.

conventional manner, the rate of ion jumps into the channel is given by the product of the electrolyte concentration (activity) and the rate constant. Given a set of P, K, and f parameters the lumped rate parameters, v_j^i (a transition from lumped state j to lumped state i), are given by

$$\begin{aligned}
v_{\mathrm{xo}}^{\mathrm{oo}} &= [P_{\mathrm{o}} \exp(f_1\psi) + P_{\mathrm{x}}C \exp(f_3\psi)]/K_{\mathrm{xo}} \\
v_{\mathrm{oo}}^{\mathrm{xo}} &= [P_{\mathrm{o}} \exp(f_1\psi) + P_{\mathrm{x}}C \exp(f_3\psi)]C/K_{\mathrm{oo}} \\
v_{\mathrm{ox}}^{\mathrm{oo}} &= [P_{\mathrm{o}} \exp(-f_1\psi) + P_{\mathrm{x}}C \exp(-f_3\psi)]/K_{\mathrm{ox}} \\
v_{\mathrm{oo}}^{\mathrm{ox}} &= [P_{\mathrm{o}} \exp(-f_1\psi) + P_{\mathrm{x}}C \exp(-f_3\psi)]C/K_{\mathrm{oo}} \\
v_{\mathrm{xo}}^{\mathrm{ox}} &= P_{\mathrm{m}} \exp(\tfrac{1}{2}\psi)/K_{\mathrm{xo}} \\
v_{\mathrm{ox}}^{\mathrm{xo}} &= P_{\mathrm{m}} \exp(-\tfrac{1}{2}\psi)/K_{\mathrm{ox}} \qquad \text{(A1)}
\end{aligned}$$

where $\psi = Ue/kT$ is the reduced membrane potential. Here the right-hand side of the channel is taken as the potential reference (i.e., a transition from xooo to oxoo is in the direction of the electrical field). The K parameters are given by

$$\begin{aligned}
K_{\mathrm{oo}} &= 1 + 2K_{\mathrm{xooo}}C + K_{\mathrm{xoox}}C^2 \\
K_{\mathrm{xo}} &= K_{\mathrm{oxoo}} \exp[(f_1 + f_2)\psi] + K_{\mathrm{oxox}}C \exp[(f_3 + f_4)\psi] \\
K_{\mathrm{ox}} &= K_{\mathrm{ooxo}} \exp[-(f_1 + f_2)\psi] \\
&\quad + K_{\mathrm{xoxo}}C \exp[-(f_3 + f_4)\psi]. \qquad \text{(A2)}
\end{aligned}$$

The occupancies p_{ss} are then calculated for the steady state using standard methods and the current I from the net transition rate over any one of the three barriers (Fig. 1). The average lifetime is similarly calculated, using the occupancies from the lifetime-occupancy correlation model, $\tau = \tau_{\mathrm{o}}/p_{\mathrm{oo}}$.

Zero-Voltage Conductance

The current may be calculated from the net transport rate over any one of the three barriers. From the three equations, p_{ox} and p_{xo} may be eliminated giving $I(\psi)$ and terms of $p(\psi)$,

$$I = \frac{v_{\mathrm{xo}}^{\mathrm{ox}} v_{\mathrm{oo}}^{\mathrm{xo}} v_{\mathrm{ox}}^{\mathrm{oo}} - v_{\mathrm{ox}}^{\mathrm{xo}} v_{\mathrm{oo}}^{\mathrm{ox}} v_{\mathrm{xo}}^{\mathrm{oo}}}{v_{\mathrm{xo}}^{\mathrm{oo}} v_{\mathrm{ox}}^{\mathrm{oo}} + v_{\mathrm{xo}}^{\mathrm{ox}} v_{\mathrm{ox}}^{\mathrm{oo}} + v_{\mathrm{xo}}^{\mathrm{oo}} v_{\mathrm{ox}}^{\mathrm{xo}}} \cdot p_{\mathrm{oo}}(\psi) \cdot e. \qquad \text{(A3)}$$

I is the current in ampere through a single channel, positive from left to right. Substituting for the v's (Eq. A1) and simplifying then gives

$$I = \frac{2 \sinh\left(\dfrac{\psi}{2}\right)(P_{\mathrm{x}}C + P_{\mathrm{o}})p_{\mathrm{oo}}(\psi) \cdot C \cdot e}{\left(2 \cosh\left(\dfrac{\psi}{2}\right) + \dfrac{P_{\mathrm{x}}C + P_{\mathrm{o}}}{P_{\mathrm{m}}}\right)K_{\mathrm{oo}}}. \qquad \text{(A4)}$$

G_{o} is obtained by dividing by $U = \psi\, kT/e$.

$$G_{\mathrm{o}} = \lim_{U \to 0} \frac{I}{U} = \frac{(P_{\mathrm{x}}C + P_{\mathrm{o}})p_{\mathrm{oo}}(0) \cdot Ce^2/kT}{\left(2 + \dfrac{P_{\mathrm{x}}C + P_{\mathrm{o}}}{P_{\mathrm{m}}}\right)K_{\mathrm{oo}}}. \qquad \text{(A5)}$$

$p_{\mathrm{oo}}(0)$ is obtained as follows. At zero voltage, from the definition of the binding constants, K_i,

$$\frac{p_{\mathrm{xooo}}}{p_{\mathrm{oooo}}} = K_{\mathrm{xooo}} \cdot C \qquad \frac{p_{\mathrm{oxoo}}}{p_{\mathrm{oooo}}} = K_{\mathrm{oxoo}} \cdot C$$

$$\frac{p_{\mathrm{oxox}}}{p_{\mathrm{oooo}}} = K_{\mathrm{oxox}} \cdot C^2 \qquad \frac{p_{\mathrm{xoox}}}{p_{\mathrm{oooo}}} = K_{\mathrm{xoox}} \cdot C^2 \qquad \text{(A6)}$$

and from the definition of the K_{ij} (Eq. A2),

$$\begin{aligned}
p_{\mathrm{oo}} &= p_{\mathrm{oooo}} + p_{\mathrm{xooo}} + p_{\mathrm{ooox}} + p_{\mathrm{xoox}} = K_{\mathrm{oo}}p_{\mathrm{oooo}} \\
p_{\mathrm{xo}} &= p_{\mathrm{oxoo}} + p_{\mathrm{oxox}} = K_{\mathrm{xo}} \cdot C \cdot p_{\mathrm{oooo}} \\
p_{\mathrm{ox}} &= p_{\mathrm{ooxo}} + p_{\mathrm{xoxo}} = K_{\mathrm{ox}} \cdot C \cdot p_{\mathrm{oooo}}. \qquad \text{(A7)}
\end{aligned}$$

Then using $p_{\mathrm{oo}} + p_{\mathrm{xo}} + p_{\mathrm{ox}} = 1$ gives p_{oooo} and then from the first of Eqs. A7,

$$p_{\mathrm{oo}} = \frac{K_{\mathrm{oo}}}{K_{\mathrm{oo}} + (K_{\mathrm{xo}} + K_{\mathrm{ox}}) \cdot C}. \qquad \text{(A8)}$$

Finally, inserting A8 into A5 one obtains Eq. 7.

Relation between the τ-V and I-V Curve

Dividing Eq. A4 by Eq. A5 gives

$$\frac{I(\psi)}{G_{\mathrm{o}}} = \frac{2 \sinh\left(\dfrac{\psi}{2}\right)\left(2 + \dfrac{P_{\mathrm{x}}C + P_{\mathrm{o}}}{P_{\mathrm{m}}}\right)\dfrac{p_{\mathrm{oo}}(\psi)}{p_{\mathrm{oo}}(0)}\, kT/e}{2 \cosh\left(\dfrac{\psi}{2}\right) + \dfrac{P_{\mathrm{x}}C + P_{\mathrm{o}}}{P_{\mathrm{m}}}}. \qquad \text{(A9)}$$

This shows that the shape of the I-V curve is related to the ion occupany of the channel in a simple manner. Also, in our model for occupancy-dependent stabilization of the dimer, we postulate that $\tau(\psi) = \tau_{\mathrm{o}}/p_{\mathrm{oo}}(\psi)$ (see main text and Eq. 3) and so substituting for $p_{\mathrm{oo}}(\psi)$

$$\frac{I(\psi)}{G_{\mathrm{o}}} = \frac{2 \sinh\left(\dfrac{\psi}{2}\right)[2 + A(C)]\tau(0)kT/e}{\left[2 \cosh\left(\dfrac{\psi}{2}\right) + A(C)\right]\tau(\psi)}, \qquad \text{(A10)}$$

where $A(C) = (P_{\mathrm{x}}C + P_{\mathrm{o}})/P_{\mathrm{m}}$ (defined by Eq. 5 in main text). On rearranging, one immediately obtains Eq. 4.

We thank Dr. D. Edmonds for valuable discussions and critical reading of the manuscript.

This work was supported by the Swedish Medical Research Council grants 7684 and 7900 (A. Ring) and 4138 (J. Sandblom).

Received for publication 28 July 1987 and in final form 13 November 1987.

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