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Channel Formation Kinetics of Gramicidin A in Lipid Bilayer Membranes

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Summary. Previous studies have given evidence that the active form of gramicidin A in lipid bilayer membranes is a dimer which acts as an ion channel; it has been further shown that the mean lifetime of the channel strongly depends on the membrane thickness. As the thickness slightly decreases when a voltage is applied to the membrane, the equilibrium between conducting dimers and nonconducting monomers may be displaced by a voltage jump. From the relaxation of the electrical current after the voltage jump, information about the kinetics of channel formation is obtained. For a dioleoyllecithin/*n*-decane membrane the rate constant of association is found to be 2×10^{14} cm^2 mole^{-1} sec^{-1}, which is by three orders of magnitude below the limiting value of a diffusion-controlled reaction in a two-dimensional system. The dissociation rate constant is equal to 2 sec^{-1}, a value which is consistent with the channel lifetime as obtained from electrical fluctuation measurements.

Valine-Gramicidin A is a linear pentadecapeptide with the structure HCO–L–Val–Gly–L–Ala–D–Leu–L–Ala–D–Val–L–Val–D–Val–L–Try–D–Leu–L–Try–D–Leu–L–Try–D–Leu–L–Try–$NHCH_2CH_2OH$ (Sarges & Witkop, 1965*a*). Characteristic features of this structure are the alternating sequence of D and L amino acids and the fact that all residues, with the exception of glycine in position 2, are hydrophobic. Gramicidin A has been shown to increase the cation permeability of biological membranes (Pressman, 1965; Henderson, McGivan & Chappel, 1969; Podleski & Changeux, 1969; Wenner & Hackney, 1969) as well as of artificial black lipid membranes (Mueller & Rudin, 1967; Liberman & Topaly, 1968; Tosteson, Andreoli, Tieffenberg & Cook, 1968; Goodall, 1970, 1971; Hladky & Haydon, 1970, 1971, 1972; Krasne, Eisenman & Szabo, 1971; Urry, Goodall, Glickson & Mayers, 1971; Myers & Haydon, 1972). In its ability to induce cation transport in bilayer membranes, gramicidin A is similar to the macrocyclic antibiotics valinomycin, enniatin B and the macrotetrolides. There is evidence, however, that the mechanism of action of gramicidin A is quite different from the

macrocyclic antibiotics which act as mobile ion carriers. The membrane conductance increases with the square of the gramicidin concentration in the aqueous phase (Tosteson *et al.*, 1968; Goodall, 1970; Hladky & Haydon, 1971), indicating that the conductance channel is a dimer, whereas in the case of valinomycin and the macrotetrolides the conducting unit is a single carrier molecule. Moreover, Hladky and Haydon (1970, 1972) were able to measure the conductance Λ of the single gramicidin A channel and obtained a value of $\Lambda = 4 \times 10^{-11}\ \Omega^{-1}$ (in 1 M potassium chloride), which corresponds, at a potential difference of, say, 100 mV, to a transport of $3 \times 10^7\ K^+$ ions per channel per second. This value is by three orders of magnitude higher than the turnover number of a single valinomycin molecule for K^+ (Stark, Ketterer, Benz & Läuger, 1971; Läuger, 1972). From the high value of Λ and from the observation that Λ is almost independent of the membrane thickness, Hladky and Haydon (1972) concluded that a carrier mechanism is very unlikely, but that gramicidin A probably forms a pore through the membrane. This conclusion is supported by the finding (Krasne *et al.*, 1971) that the high conductance of a gramicidin-doped bilayer membrane persists when the lipid film is solidified by lowering the temperature. Based on conformational energy studies, Urry (1971, 1972*a*, *b*; *see also* Urry *et al.*, 1971) proposed a detailed model of the gramicidin A channel. It consists of a dimer which is formed by head-to-head (formyl end to formyl end) association of two $\beta^6_{3,3}$-helices. The central hole along the helix axis has a diameter of about 4 Å and is lined by the peptide C–O moieties, whereas the hydrophobic residues lie on the exterior surface of the helix. The total length of the dimer is about 25 to 30 Å which is the lower limit of the hydrophobic thickness of a lipid bilayer membrane. This means that the bridging of the hydrocarbon core of the membrane by the gramicidin dimer must be associated with a local distortion of the bilayer structure. It is likely to assume that an equilibrium exists in the membrane between nonconducting monomers and conducting dimers. Hladky and Haydon (1970, 1972) have demonstrated that at very low gramicidin concentrations, discrete fluctuations of the membrane conductance occur, which presumably originate from the formation and the disappearance of single channels. Working with membranes of different composition, they found that the mean lifetime of a channel strongly increases with decreasing membrane thickness.

In the following, we report on an investigation of the kinetics of gramicidin channel formation by relaxation measurements. For the application of the relaxation technique it is necessary to create a sudden displacement of the equilibrium between monomers and dimers in the membrane. As an electric field exerts a compressive force on the membrane, the membrane

thickness slightly decreases when a voltage is applied between the external solutions (Babakov, Ermiskin & Liberman, 1966; Läuger, Lesslauer, Marti & Richter, 1967; Rosen & Sutton, 1968; Andrews, Manev & Haydon, 1970; White, 1970*a*, *b*). A change in the transmembrane voltage will therefore result in a shift of the monomer-dimer equilibrium. Although in most cases the thickness change is only a few per cent at a voltage of the order of 100 mV, the number of conducting channels may be increased in this way by a factor of two or more (Hladky & Haydon, 1972). As the current through the membrane is directly proportional to the number of conducting dimers, the rate constants for the formation and dissociation of the dimer may be obtained from the time course of the current after a sudden change of the voltage.

Theory

We assume that an equilibrium between monomers A and dimers A_2 exists in the membrane, which may be characterized by an association rate constant k_R and a dissociation rate constant k_D:

$$A+A \underset{k_D}{\overset{k_R}{\rightleftarrows}} A_2. \tag{1}$$

We further assume that the exchange of A and A_2 between membrane and aqueous phase is sufficiently slow, so that the total concentration N of gramicidin in the membrane remains constant during the relaxation experiment (*see* Appendix A). If N_1 and N_2 are the concentrations of monomers and dimers, respectively, in the film (expressed in moles/cm^2), then

$$N=N_1+2N_2. \tag{2}$$

At time $t<0$ the system is in an equilibrium state with concentrations N_1^0 and N_2^0:

$$\frac{N_2^0}{N_1^{0^2}}=\frac{k_R^0}{k_D^0}=K_0. \tag{3}$$

If at time $t=0$ a voltage is applied to the membrane, the rate constants are changed to new values k_R and k_D. After some time, the system reaches a new equilibrium with concentrations N_1^∞, N_2^∞:

$$\frac{N_2^\infty}{N_1^{\infty^2}}=\frac{k_R}{k_D}=K. \tag{4}$$

The rate of change of N_2 is given by

$$\frac{dN_2}{dt}=k_R N_1^2-k_D N_2. \tag{5}$$

Introducing the fraction y of dimers:

$$y \equiv \frac{N_2}{N} \tag{6}$$

and using the condition that $N = N_1 + 2N_2$ is constant, Eq. (5) may be written in the following form:

$$\frac{1}{k_D}\frac{dy}{dt} = 4NKy^2 - (1+4NK)y + NK. \tag{7}$$

The solution of this differential equation reads

$$y(t) = y_\infty - (y_\infty - y_0)\frac{q e^{-t/\tau}}{1+q-e^{-t/\tau}} \tag{8}$$

with

$$q = \frac{\sqrt{1+8NK}}{4NK(y_\infty - y_0)} \tag{9}$$

$$\tau = \frac{1}{k_D\sqrt{1+8NK}} = \frac{1}{k_D + 4k_R N_1^\infty}. \tag{10}$$

The second part of Eq. (10) is obtained from Eqs. (2) and (4). y_0 and y_∞ are the values of y at times $t=0$ and $t\to\infty$, respectively, and may be calculated from Eqs. (2)–(4):

$$y_0 = \frac{1}{8NK_0}(1+4NK_0 - \sqrt{1+8NK_0}) \tag{11}$$

$$y_\infty = \frac{1}{8NK}(1+4NK - \sqrt{1+8NK}). \tag{12}$$

At a given voltage, the current density $J(t)$ is proportional to the number N_2 of conducting channels, and therefore proportional to y. Using Eq. (8), the time course of the current may be expressed by

$$\frac{J-J_0}{J_\infty - J_0} = 1 - q\frac{e^{-t/\tau}}{1+q-e^{-t/\tau}} \tag{13}$$

where $J_0 \equiv J(0)$ and $J_\infty \equiv J(\infty)$. For large values of the parameter q, Eq. (13) reduces to

$$\frac{J-J_0}{J_\infty - J_0} \approx 1 - e^{-t/\tau} \quad (|q| \gg 1). \tag{14}$$

In this limit, the time course of J is purely exponential. As may be seen from Eq. (9), the condition $|q| \gg 1$ is fulfilled either for small displacements of the equilibrium, i.e., for $|y_\infty - y_0| \to 0$, or, at arbitrary displacements, if the gramicidin concentration is sufficiently low ($NK \ll 1$).

According to Eq. (10), the rate constants k_R and k_D may be calculated from the experimental values of the time constant τ, if τ is measured at different gramicidin concentrations. For this purpose, however, it would be necessary to know the monomer concentration N_1^∞ in the membrane, which is not directly measurable. This difficulty is circumvented in the following way. Using Eq. (4), N_1^∞ is expressed by the dimer concentration N_2^∞:

$$N_1^\infty = \sqrt{N_2^\infty / K}.$$

N_2^∞ is related to the stationary membrane conductance λ^∞ via the conductance Λ of the single channel (N^0 = Avogadro's number):

$$\lambda^\infty = N^0 N_2^\infty \Lambda. \tag{15}$$

Eq. (10) then assumes the form

$$\tau = \frac{1}{k_D + 4\sqrt{k_D k_R \lambda^\infty / N^0 \Lambda}}. \tag{16}$$

Thus, if $1/\tau$ is plotted as a function of $\sqrt{\lambda^\infty}$, a straight line should result. From the slope and the intercept with the $1/\tau$ axis the values of k_R and k_D may then be determined using the independently measured value of the single channel conductance Λ.

Finally, the relaxation amplitude α,

$$\alpha \equiv \frac{J_\infty - J_0}{J_0} = \frac{y_\infty - y_0}{y_0} \tag{17}$$

may be used to calculate the equilibrium constant K_0 at zero voltage. Using Eqs. (11) and (12), the following relation for the relaxation amplitude is obtained in the limit of small gramicidin concentrations ($NK \ll 1$, $NK_0 \ll 1$), i.e., in the limit $\lambda^\infty \approx 0$:

$$\alpha \approx \frac{K - K_0}{K_0} \quad (\lambda^\infty \to 0). \tag{18}$$

Materials and Methods

Optically black lipid membranes were formed from a solution of 1% (w/v) dioleoyllecithin in *n*-decane as described previously (Läuger *et al.*, 1967). The purity of the dioleoyllecithin (Supelco, Inc., Bellefonte, Pa.) was checked by thin-layer chromatography. Gramicidin was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio) and used without further purification. According to Glickson, Mayers, Settine and Urry (1972), the commercial product contains 72% gramicidin A, 9% gramicidin B, and 19% gramicidin C. Gramicidin B and C differ from A only in that L-tryptophan in position 11 is replaced by L-phenylalanine and L-tyrosine, respectively; in addition,

gramicidin A, B and C each contain 5 to 20% of an analogue in which L-isoleucine replaces the N-terminal valine (Sarges & Witkop, 1965*a–c*). Gramicidin was added from a methanolic stock-solution to the aqueous phase (1 M NaCl).

The aqueous solution contained always less than 1% methanol; it was checked that this amount of methanol has no influence on the electrical properties of the membrane. The temperature was 25 °C in all experiments.

The electrical relaxation measurements were carried out by applying a voltage of 100 to 200 mV to the membrane by means of an electronic switch (Ketterer, Neumcke & Läuger, 1971). The current was recorded with a Tetronix 549 storage oscilloscope as a voltage drop across an external resistor. The rise-time of the voltage was then given by the product of the membrane capacitance and the resistance of the external circuit and was in the range of 1 to 100 μsec. The experiments were performed with circular membranes of two different diameters, 1 mm and 3.2 mm. The relaxation time was found to be independent of the size of the membrane. It was important, however, to use only membranes with a small border torus (less than 10% of the total area). Otherwise, a slow increase in the area of the black film was observed after the voltage jump, which gave rise to an additional time-dependent current.

Silver-silver chloride electrodes, with an area of about 1 cm^2 each were used throughout. To check that polarization effects at the electrodes were negligible, control experiments were performed in which the membrane was replaced by its equivalent circuit (a parallel combination of a capacitor and a resistor of the appropriate magnitudes). In this case only the capacitive transient of the predicted time course was observed, which indicates that polarization effects may be neglected.

Results

An oscillogram of a relaxation experiment is shown in Fig. 1. After the decay of the capacitive transient which has a time constant of 2 μsec under the given experimental conditions and which is not resolved in the oscillogram, the current increases with time and finally reaches a stationary

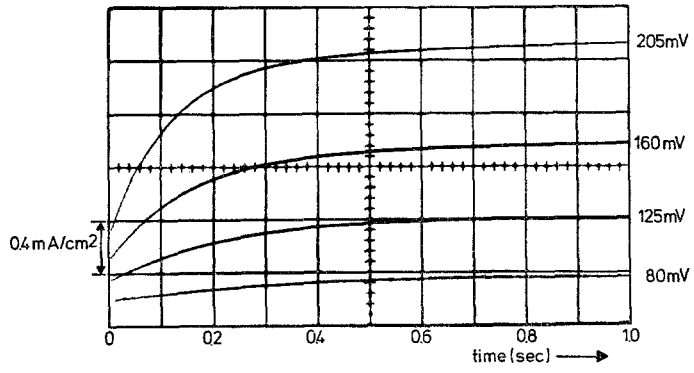


Fig. 1. Oscillogram of an electrical relaxation experiment with a dioleoyllecithin bilayer membrane in the presence of 1 M NaCl and 2×10^{-9} M gramicidin A (current referred to unit area of the membrane). A voltage of variable magnitude was applied at time $t=0$. The time constant for the charging of the membrane capacitance was about 2 μsec

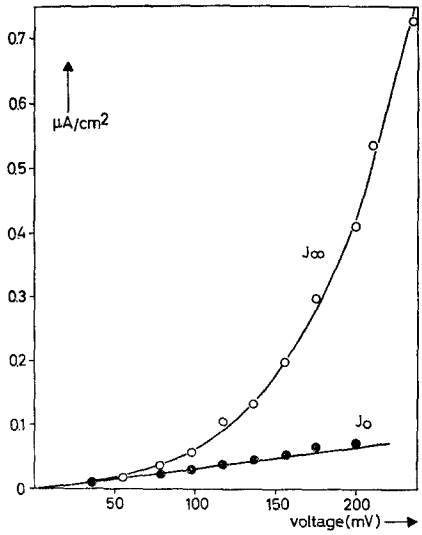


Fig. 2. Initial current J_0 and stationary current J_∞, referred to unit area of the membrane, as a function of the voltage. The aqueous solutions contained 1 M NaCl and 2×10^{-11} M gramicidin A

level J_∞. The initial current J_0 is obtained by extrapolation to time zero. As shown by Fig. 1, the relaxation amplitude strongly increases with the applied voltage V; for instance, at $V=205$ mV, the stationary current is 3.3 times larger than the initial current. From the finding of Hladky and Haydon (1971) that the current-voltage curve of the single gramicidin channel is almost linear, and in accordance with their interpretation, we may conclude that the ratio J_∞/J_0 directly reflects the increase in the number of conducting channels after the voltage jump.

This conclusion is further strengthened by Fig. 2 in which the current-voltage curves of the membrane in the initial and in the stationary state are compared. It is seen that J_0 is a nearly linear function of the voltage as expected from the linear $J-V$ characteristic of the single channel.

For an unambiguous interpretation of the results, it must be guaranteed that the rate by which the membrane is compressed under the influence of the electric field is fast compared with the relaxation process due to the dimerization reaction. The rate of compression of a bilayer membrane may be studied by measuring, at low levels of conductance, the additional capacitive current which flows during the thinning of the membrane. Thus, if a voltage jump is applied to a membrane without gramicidin, a "tail current" should be observed after the decay of the normal capacitive transient. (At higher levels of conductance the tail current is masked by the conductive current.) When such an experiment was performed with a pure

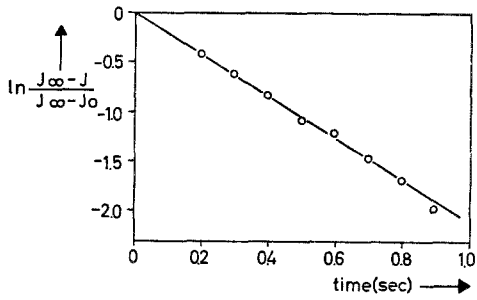


Fig. 3. Time course of the current J after a voltage jump of 205 mV. J_0 is the initial current and J_∞ the stationary current which is reached in the limit $t \to \infty$. The aqueous phase contained 1 M NaCl and 5×10^{-11} M gramicidin A. $\lambda^\infty = 1.5 \times 10^{-5}\,\Omega^{-1}\,\mathrm{cm}^{-2}$

dioleoyllecithin membrane and a voltage jump of $V = 185$ mV, a tail current was observed which decreased from an (extrapolated) initial value of 1.3 μamps/cm^2 to nearly zero with a time constant of about 3 msec. The total amount of transported charge, as obtained from the time integral of the tail current, was about 3.5×10^{-9} coulombs/cm^2, corresponding (for $V = 185$ mV) to a capacitance change $\Delta C_m = 19$ nF/cm^2, or 5% of the total membrane capacitance (360 nF/cm^2). This value of ΔC_m is close to the capacitance change which is found by independent bridge measurements (Läuger *et al.*, 1967) at comparable voltages. This gives strong evidence that the tail current indeed originates from the thinning of the membrane. In this way it is established that the thinning is much faster than the relaxation of the dimerization equilibrium which has a time constant of the order of 0.1 to 0.5 sec.

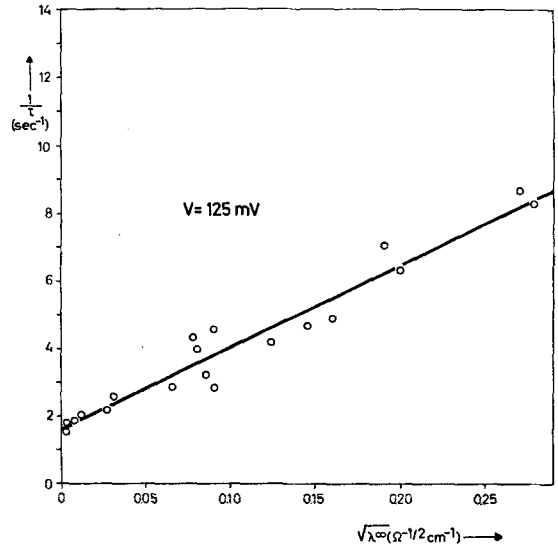
For the determination of the relaxation time τ, the quantity

$$\ln\left(1 - \frac{J - J_0}{J_\infty - J_0}\right) = \ln \frac{J_\infty - J}{J_\infty - J_0}$$

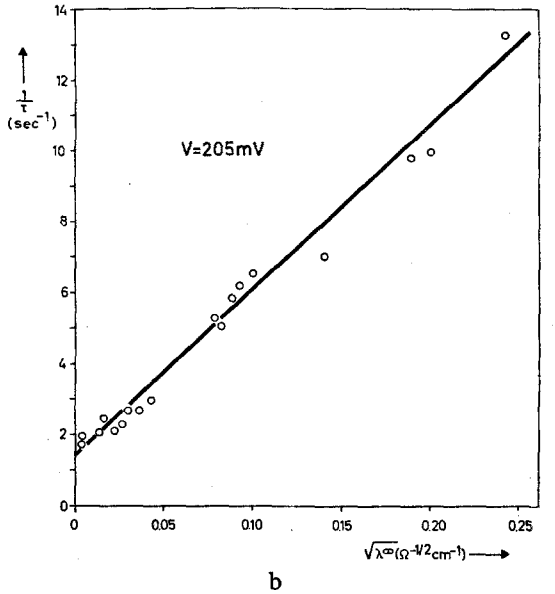
is plotted as a function of time t [*compare* Eq. (13)]. Under all experimental conditions a straight line is obtained within the limits of error (Fig. 3). This means that the parameter q is always large compared with unity, so that the approximative relation (14) is valid throughout. (For a check of the consistency of the results, q may be calculated using the relation

$$q = \frac{N^0 \Lambda}{4 \tau k_R (\lambda^\infty - \lambda^0)} \tag{19}$$

which is obtained from Eqs. (4), (6), (9), (10), (15) and $\lambda^0 = N^0 N_2^0 \Lambda$. If the numerical values of q are inserted into Eq. (13), it is found that the devia-



a



b

Fig. 4. Reciprocal relaxation time plotted as a function of the square root of the stationary conductance. (*a*) at a voltage of $V=125$ mV, (*b*) at $V=205$ mV. The experiments were performed in the presence of 1 M NaCl and different amounts of gramicidin A

tion from the approximative relation (14) is always within the limits of experimental error.)

The relaxation time τ has been measured over a wide range of gramicidin concentrations in the aqueous phase. The result is shown in Fig. 4 in which

Table 1. Rate constants k_R and k_D and equilibrium constant K of the dimerization reaction of gramicidin A

Voltage (mV)	k_D (sec^{-1})	k_R ($cm^2\ mole^{-1}\ sec^{-1}$)	$K=k_R/k_D$ ($cm^2\ mole^{-1}$)
205	1.4	1.4×10^{15}	1.0×10^{15}
125	1.6	3.4×10^{14}	2.1×10^{14}
0	1.6	2.4×10^{14}	$1.5\times10^{14}=K_0$

$1/\tau$ is plotted as a function of the square root of the stationary membrane conductance λ^∞. In accordance with Eq. (16), $1/\tau$ is found to vary linearly with $\sqrt{\lambda^\infty}$. The intercept with the $1/\tau$ axis gives directly the dissociation rate constant k_D. With this value of k_D, together with the experimental value of the single channel conductance Λ, the association rate constant k_R may be calculated from the slope of the straight line. From measurements with a variety of different membranes, Hladky and Haydon (1972) have found that the single channel conductance of gramicidin A is almost independent of the membrane composition and thickness. Although their measurements do not include dioleoyllecithin membranes, we may therefore nevertheless use their value of Λ in 1 M NaCl ($\Lambda\simeq2.4\times10^{-11}\ \Omega^{-1}$) for the calculation of k_R. In this way the following results are obtained which are summarized in Table 1. It is seen that only k_R varies appreciably between $V=125$ mV and $V=205$ mV, whereas k_D is much less sensitive to a change in the applied voltage. Since a direct measurement of the relaxation time at $V<100$ mV is difficult because of the small amplitude, the value of k_R at zero voltage has been calculated from the experimental value of K_0 (*see below*) under the assumption that k_D does not appreciably change between 0 and 125 mV.

For the determination of the equilibrium constant at zero voltage, K_0, the relaxation amplitude α has been plotted as a function of the membrane conductance for $V=205$ mV (Fig. 5). K_B may then be calculated according to Eq. (18) from the limiting value of α at low conductances using $K=1.0\times10^{15}\ cm^2\ mole^{-1}$ (*see* Table 1). Also plotted in Fig. 5 is the theoretical curve which has been calculated from Eqs. (11), (12) and (17) with this value of K_0. The total gramicidin concentration N which enters into Eqs. (11) and (12) has been determined from the relation

$$N=\frac{2\lambda^\infty}{N^0\Lambda}\left(1+\frac{1}{2}\sqrt{\frac{N^0\Lambda}{\lambda^\infty K}}\right) \tag{20}$$

[*compare* Eqs. (2), (4) and (15)]. As shown by Fig. 5, the relaxation amplitudes which are obtained from different membranes scatter over a relatively

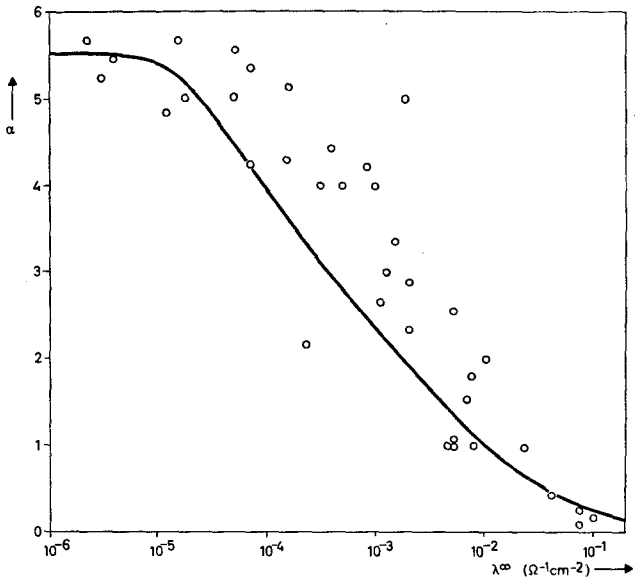


Fig. 5. Relaxation amplitude $\alpha=(J_\infty-J_0)/J_0$ as a function of the stationary membrane conductance λ^∞ (1 M NaCl, $V=205$ mV). Each point corresponds to a different membrane. The curve has been calculated from Eqs. (11), (12) and (17) using the K_0 value which gave the best fit in the limit $\lambda^\infty \to 0$ ($K_0=1.5\times 10^{14}$ cm^2 mole^{-1})

wide range. However, the qualitative predictions of the theory, namely the occurrence of a limiting value of α at low conductances and a decrease of α toward high conductances, are confirmed by the experimental results.

Discussion

The preceeding results demonstrate that electrical relaxation experiments are suitable to obtain information on the kinetics of ion channel formation in lipid bilayer membranes. For the interpretation of the experimental results with gramicidin A, we have assumed that an equilibrium between nonconducting monomers and conducting dimers exists in the membrane. This model has been previously proposed on the basis of the observation that the membrane conductance is roughly proportional to the square of the aqueous gramicidin concentration; however, as Hladky and Haydon (1971) have pointed out, the experimental support for the model is somewhat doubtful in view of the difficulty to establish a true equilibrium between membrane and aqueous phases. The electrical relaxation method is free from this difficulty, as the gramicidin concentration in the membrane

may be eliminated from the theoretical expression for the relaxation time by introducing the ratio of total conductance to single channel conductance [Eqs. (10) and (16)]. The consistency of our experimental results with the theoretical relations derived on the basis of the dimer model gives, therefore, additional evidence for this model. In particular, the fact that the relaxation time shows the characteristic dependence on membrane conductivity (Fig. 4), which has to be expected for a bimolecular reaction, strongly supports the view that the event which leads to the opening of a conducting channel is the association of two gramicidin monomers.

Alternative possibilities for the mechanism of the opening of a channel, for instance, a spatial rearrangement of a preformed dimer in the membrane, or a field-induced conformational change of the peptide, may be excluded from the linear dependence of $1/\tau$ on $\sqrt{\lambda^{\infty}}$.

The main result of this study is the evaluation of the rate constants for the formation and dissociation of the gramicidin A dimer. The dissociation rate constant is found to be $k_D = 1.6\ \text{sec}^{-1}$. This value has to be compared with the mean lifetime τ^* of a channel, as obtained from electrical fluctuation measurements. It is easily seen that the relation $k_D = 1/\tau^*$ holds. There are no published reports on fluctuation measurements with gramicidin-doped dioleoyllecithin membranes; however, we may compare our k_D value with the mean channel lifetime as measured by Hladky and Haydon (1972) with black glycerol monooleate films. For membranes formed from glycerol monooleate in *n*-decane, which have approximately the same thickness of the hydrocarbon core (48 Å) as dioleoyllecithin/*n*-decane membranes (Fettiplace, Andrews & Haydon, 1971), Hladky and Haydon obtained $\tau^* = 0.4$ sec, or $1/\tau^* = 2.5\ \text{sec}^{-1}$ which is roughly equal to our k_D value of $1.6\ \text{sec}^{-1}$. Within the limits of the assumption that k_D chiefly depends on membrane thickness and not very much on the other properties of the film, we may therefore state that the k_D value obtained from relaxation experiments is in agreement with the mean lifetime of the channel, as determined by electrical fluctuation measurements.

The association rate constant k_R is defined as the number of moles of dimers formed per cm^2 and second for unity interfacial concentration of the reactants [Eq. (5)]. It is therefore not possible to compare directly the numerical value of k_R with the more familiar association rate constant in a three-dimensional system. It is instructive, however, to compare k_R with the maximum value $k_R^{\max}$ of the association rate constant, which is reached in the limit of a diffusion-controlled reaction in a two-dimensional system. As shown in Appendix B, $k_R^{\max}$ may be calculated for a random-walk model

in a discrete lattice and is approximately given by the relation

$$k_R^{\max} = 16\sqrt{2}\, N^0 D \frac{r}{l} \tag{21}$$

where r is the radius of the monomer (which, for simplicity, is assumed to be of spherical shape), D the diffusion coefficient of the monomer in the film, and l the elementary jump-length in the lattice. A rough estimate of the diffusion coefficient D may be obtained from the microviscosity η in a lipid membrane, for which values in the range of 1 to 10 poise have been reported (Frye & Edidin, 1970; Cogan & Shinitzky, 1972; Cone, 1972). Using the Stokes-Einstein relation $D = kT/6\pi\eta r$, (k = Boltzmann constant, T = absolute temperature), $D = 9 \times 10^{-9}$ cm^2 sec^{-1} is calculated with $\eta = 3$ poise and $r = 8$ Å. A similar value of D has been obtained for the diffusion of spin-labeled androstane in synthetic lipid bilayers (Träuble & Sackmann, 1972). As an appropriate value for the jump length l, we choose the diameter of a hydrocarbon chain ($l \simeq 4$ Å). If D, l and r are inserted into Eq. (21), the maximal association rate constant becomes $k_R^{\max} = 2 \times 10^{17}$ cm^2 mole^{-1} sec^{-1}, a value which is by a factor of about 10^3 higher than the association rate constant of gramicidin in the lecithin membrane. The result that k_R is much smaller than $k_R^{\max}$ is not unreasonable, because, as mentioned above, the formation of a dimer presumably requires a local distortion of the bilayer structure; in addition, it has to be expected that a rather precise matching of the two monomers is needed for the formation of the hydrogen bonds involved in the stabilization of the dimer.

The variation of k_D and k_R with voltage results, at least in part, from the voltage-induced change of the membrane thickness. A direct influence of the electric field on k_D and k_R, however, cannot be excluded *a priori*. Upon application of a voltage of $V = 200$ mV, the membrane is compressed by about 5%, which corresponds, with an initial thickness of the hydrocarbon core of 50 Å, to a thickness change of 2.5 Å. The measurement of the channel lifetime as a function of membrane thickness (Hladky & Haydon, 1972) indicates a variation of k_D of the order of 0.13 sec^{-1} per Å. This would mean that the k_D value should be smaller by 0.3 sec^{-1} at $V = 200$ as compared with k_D ($V = 0$). The close agreement with the observed value ($\Delta k_D = 0.2$ sec^{-1}; *see* Table 1) seems to indicate that a direct influence of the electric field on k_D is small. The experimental accuracy, however, is not sufficient for a final answer to this question.

The equilibrium constant $K = k_R/k_D$ for the association of gramicidin in the membrane is found to be $K = 1.5 \times 10^{14}$ cm^2/mole (in the limit of zero voltage). The reciprocal of this value, $1/K = 7 \times 10^{-15}$ moles/cm^2 =

4×10^9 molecules/cm^2, gives the interfacial concentration N at which just half of the gramicidin in the membrane is present in the form of dimers, as may be seen from Eq. (12). According to Eqs. (12) and (15), this concentration corresponds to a membrane conductance of $\lambda^\infty = 2 \times 10^{-2}\ \Omega^{-1}\ \mathrm{cm}^{-2}$.

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Appendix

A. *Transport of Gramicidin from the Solution into the Film*

For the interpretation of the experimental results we have assumed that the exchange of gramicidin across the interface is slow. To test the validity of this assumption, we demonstrate in the following that the observed relaxation of the current cannot arise from the transport of gramicidin from the aqueous phase into the film; i.e., we wish to exclude the alternative model that the monomer-dimer equilibrium is established very fast after the voltage jump, but that additional gramicidin is subsequently taken up by the film with a time-lag corresponding to the observed relaxation time. We do this by showing that the concentration dependence of the relaxation amplitude, as calculated on the basis of the alternative model, is at variance with the experimental findings.

According to Eqs. (6) and (17), the relaxation amplitude is given by

$$\alpha = \frac{N_2^\infty - N_2^0}{N_2^0} \tag{A.1}$$

where N_2^0 is the dimer concentration in the membrane immediately after the voltage jump ($t=0$) and N_2^∞ the dimer concentration for $t \to \infty$. The equilibrium concentrations $\bar{N}_1, \bar{N}_2$ of monomers and dimers before the voltage jump ($t<0$) are related by the equations

$$\frac{\bar{N}_2}{\bar{N}_1^2} = K_0 \tag{A.2}$$

$$\bar{N} = \bar{N}_1 + 2\bar{N}_2 = \bar{N}_1 + 2K_0 \bar{N}_1^2 \tag{A.3}$$

where $\bar{N}$ is the total concentration and K_0 the equilibrium constant before the voltage jump. If exchange of the monomer across the interface takes place during the relaxation experiment, it is plausible to assume that the stationary concentrations of the monomer before and after the voltage jump are the same; i.e., that the partition equilibrium of the monomer is

independent of voltage:

$$\bar{N}_1 = N_1^{\infty}. \tag{A.4}$$

With the equilibrium constant K after the voltage jump, N_2^{∞} is given by

$$N_2^{\infty} = K N_1^{\infty 2} = K \bar{N}_1^2. \tag{A.5}$$

As we have assumed that the equilibrium is established immediately after the voltage jump, N_2^0 may be obtained from

$$N_2^0 = K N_1^{0 2} = K(\bar{N} - 2N_2^0)^2 \tag{A.6}$$

which gives

$$N_2^0 = \frac{1}{8K}(1 + 4\bar{N}K - \sqrt{1 + 8\bar{N}K}). \tag{A.7}$$

Using Eqs. (A.3), (A.5) and (A.7), the relaxation amplitude may be expressed by

$$\alpha = \frac{8\bar{N}_1^2 K^2}{1 + 4\bar{N}_1 K + 8\bar{N}_1^2 K K_0 - \sqrt{1 + 8\bar{N}_1 K + 16\bar{N}_1^2 K K_0}} - 1. \tag{A.8}$$

In the limit of high gramicidin concentrations ($\bar{N}_1 K \gg 1$, $\bar{N}_1 K_0 \gg 1$), this equation reduces to

$$\alpha \approx \frac{K - K_0}{K_0} \equiv \alpha^*. \tag{A.9}$$

On the other hand, at low concentrations ($\bar{N}_1 K \ll 1$, $\bar{N}_1 K_0 \ll 1$), Eq. (A.8) gives

$$\alpha \approx 4\bar{N}_1 K_0 \frac{K - K_0}{K_0} \ll \alpha^*. \tag{A.10}$$

Thus, the above model predicts that the relaxation amplitude should decrease with decreasing gramicidin concentration, in contradiction to the experimental findings.

B. *Association Rate Constant for a Diffusion-Controlled Reaction in a Two-Dimensional System*

An upper limit of the association rate constant k_R of a reaction $A + B \rightarrow AB$ is given if the reaction is diffusion controlled; i.e., if every encounter between A and B is successful. For reactions in three-dimensional space the diffusion-limited value $k_R^{\max}$ is calculated by the well-known Smoluchovski method in which the reaction rate is set equal to the stationary diffusional flux of molecules B toward a spherical reaction center A (Benson, 1960). This method is no longer applicable for a reaction in a two-dimensional

system, because a stationary state with nonzero flux does not exist in this case (Adam & Delbrück, 1968).

An estimate for k_R^{max} may be obtained on the basis of a random-walk model in a discrete lattice. We consider an infinite two-dimensional lattice with a random distribution of fixed molecules of type B. A molecule of type A is allowed to diffuse in the lattice in such a way that the molecule makes ν_A jumps of length l_A per second in either of two mutually perpendicular directions. The total path length s per second is then given by

$$s=\sqrt{2}\, l_A \nu_A. \tag{B.1}$$

The number Z_B of encounters per second between A and molecules of type B is equal to

$$Z_B=2(r_A+r_B)\, s\, N_B N^0 \tag{B.2}$$

where r_A and r_B are the radii of A and B and N_B is the concentration of B (expressed in moles/cm^2). If N_A moles of A are present per cm^2, the total number Z_{AB} of encounters per cm^2 and second is

$$Z_{AB}=Z_B N_A N^0. \tag{B.3}$$

Comparison with the definition of k_R^{max}:

$$\frac{Z_{AB}}{N^0}=k_R^{max} N_A N_B \tag{B.4}$$

gives

$$k_R^{max}=2\sqrt{2}\, N^0 (r_A+r_B)\, \nu_A\, l_A. \tag{B.5}$$

To introduce the diffusion coefficient D_A of A in the lattice, we may use the rate-theory expression for D_A (Glasstone, Laidler & Eyring, 1941):

$$D_A=\tfrac{1}{2}\nu_A\, l_A^2 \tag{B.6}$$

so that

$$k_R^{max}=4\sqrt{2}\, N^0 (r_A+r_B)\frac{D_A}{l_A}. \tag{B.7}$$

Finally, to account for the diffusion of B in the lattice, we may replace D_A/l_A by $(D_A/l_A)+(D_B/l_B)$ and obtain

$$k_R^{max}=4\sqrt{2}\, N^0 (r_A+r_B)\left(\frac{D_A}{l_A}+\frac{D_B}{l_B}\right). \tag{B.8}$$

For the dimerization reaction, where $r_A=r_B=r$, $D_A=D_B=D$, $l_A=l_B=l$, Eq. (B.8) reduces to

$$k_R^{max}=16\sqrt{2}\, N^0 D\frac{r}{l}. \tag{B.9}$$

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