ALAMETHICIN-INDUCED CURRENT-VOLTAGE CURVE ASYMMETRY IN LIPID BILAYERS

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ABSTRACT We have examined the causes of the asymmetry of the current-voltage curve induced by addition of alamethicin to one side of a black lipid membrane. We find that the alamethicin-induced current-voltage (I-V) curve has an inherent asymmetry. If it were possible to confine all alamethicin molecules to one side of the membrane, the I-V curve would exhibit a positive branch (voltage being measured with respect to the side of the membrane trans to the alamethicin addition) of steeper logarithmic slope than the negative branch and at a lower absolute value of potential. This condition is not usually realized, however, because alamethicin can leak through the membrane, so that, except at very high alamethicin concentrations and in certain kinds of membranes, the positive branch of the current-voltage curve has the same logarithmic slope as the negative branch and appears to arise from alamethicin which diffuses from the *cis* to the *trans* side of the membrane. We develop simple quantitative models for these two cases.

INTRODUCTION

Alamethicin, a 20–amino acid peptide antibiotic, originally isolated from a fungus, *Trichoderma viride* (Reusser, 1967), induces a strongly voltage-dependent conductance in lipid bilayers (Mueller and Rudin, 1968) and in biological membranes (Cahalan and Hall, 1982; Sakmann and Boheim, 1979). Because it can be synthesized, highly purified (Balasubramanian et al., 1981), and modified, it provides a convenient model for lipid-protein interaction. Considerable effort has been expended toward understanding the nature of the alamethicin-induced conductance (for reviews, see Hall, 1978; Latorre and Alvarez, 1981). There are, however, still problems concerning its mechanism of action. One question of fundamental importance is the origin of the current-voltage curve asymmetry, which occurs when alamethicin is added to only one side of the membrane. The degree of asymmetry varies with lipid composition, being maximal in membranes of bacterial phosphatidyl ethanolamine and essentially undetectable in membranes of monopalmitolein. We believe that the asymmetry of the I-V curve is a consequence of the way alamethicin and lipids interact. Thus, its study provides a convenient testing ground for ideas of how lipids and proteins interact.

In this paper, we examine the asymmetry of the alamethicin current-voltage curve as a function primarily of lipid composition. We introduce two simple and quantitative hypotheses, which we test experimentally, and we show how these hypotheses can be extended to cover more general cases than those we consider here in detail.

MATERIALS AND METHODS

The major fraction of alamethicin (denoted Fraction 4 from the number of its chromatography peak) obtained after purification of the natural product (Upjohn Co., Kalamazoo, MI) by high-pressure liquid chromatography (Balasubramanian et al., 1981) was used in these studies. Indistinguishable results are obtained with a synthetic derivative believed identical in structure to Fraction 4 (Balasubranmanian et al., 1981).

Phosphatidylethanolamine (PE) from *Escherichia coli* and dibromostearicphosphatidylcholine (DBrPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Phosphatidylserine (PS) (bovine) and PE (bovine) were purchased from Supelco, Inc. (Bellfonte, PA). Decane, *n*-pentane (Mallinckrodt, Inc., St. Louis, MO), and squalene (Albany International, Chemicals Division, Albany, NY) were passed through an alumina column to remove surface active impurities.

Our membrane formation method was similar to that described by Montal and Mueller (1972). We used a chamber made after a design of Shindler and Feher (1976). A 2-cm square piece of Teflon 20 μm thick with ~0.3-mm diam hole punched by a hypodermic needle was mounted between two half-chambers machined from a Teflon block as mirror images of each other. These two halves were then forced into a tapered hole in an aluminum block to clamp the chamber together and to provide an isothermal enclosure. Temperature was controlled by a specially designed bridge circuit using Peltier thermoelectric elements (Cambion, Cambridge, MA). A lipid mixture (10 mg/ml in *n*-pentane) was spread on two water/salt solutions separated by a thin Teflon partition. Membranes were formed by raising the water levels over the hole. A small drop of squalene (1 μl) was placed in the hole in the partition before raising the water levels. Lipid solution (10 μl) was added on the water surface (which had an area of 1 cm^2 on each side) using a glass microliter pipet, again before raising the water levels. An alamethicin derivative was added to

one side of the chamber after membrane formation. This side will always be referred to as the *cis* side, and the opposite side will always be referred to as the *trans* side. Formation of the membrane was monitored using capacitance, as measured by the current response to a 10-mV amplitude triangular voltage. Membrane area was $\sim 7 \times 10^{-4}$ cm^2.

A four-electrode (chlorided silver wire) system was used for measuring current-voltage (I-V) and current-time curves. Two electrodes provide inputs to a high-impedance electrometer. A third electrode was connected to virtual ground (summing point) of an AD42K (Analog Devices, Inc. Norwood, MA) operational amplifier usually operated with a 100 MΩ feedback resistor. The fourth electrode was driven by an appropriate voltage source. Voltage was generated by a computer-controlled 12-bit DAC (AD578, Analog Devices, Inc. Norwood, MA) buffered by an AD521 operational amplifier, or by battery driven potentiometer. By convention, voltage is measured relative to the *trans* side of the membrane. We used an *X–Y* recorder (HP7037A, Hewlett-Packard, Palo Alto, CA) to record I-V curves and current-time curves.

THEORY

First, we treat the case where asymmetry in the I-V curve arises from an asymmetry in the concentration of alamethicin adsorbed to the membrane surface. The positive voltage branch of the I-V curve arises from alamethicin on the *cis* side of the membrane, and the negative branch is attributed to alamethicin that has diffused from the *cis* side to the *trans* side. The relative rates of adsorption, translocation, and desorption of alamethicin will determine the concentration profile. We call this treatment the "translocation hypothesis," for short.

Second, we treat the case where alamethicin molecules are confined to one side of the membrane. Both branches of the I-V curve arise from alamethicin molecules on the same side of the membrane. If the I-V curve is asymmetric under this constraint, the negative-branch channels must be formed by a somewhat different process than the positive-branch channels, a possibility first suggested by Gordon and Haydon (1975). For simplicity, we attribute the difference between negative-branch channels and positive-branch channels to the orientation of the alamethicin molecules in the membrane. We call this treatment "inherent orientational asymmetry."

The translocation hypothesis and inherent orientational asymmetry are not mutually exclusive. In fact, if the I-V curve arising from alamethicin molecules confined to one side of the membrane is symmetric, it will never be possible to see an asymmetric I-V curve at all, regardless of the concentration difference of alamethicin across the membrane. Translocation of alamethicin across the membrane will thus tend to reduce the asymmetry of the I-V curve from a maximum asymmetry set by the inherent asymmetry for molecules confined to one side of the membrane.

For all experimental conditions reported in this paper, the positive branch of the I-V curve occurs at lower absolute values of voltage than the negative branch. Thus, positive voltage turns *cis* channels on more readily than negative voltage. We would like to know if the negative branch arises principally from *trans* alamethicin which has diffused across the membrane, or from *cis* alamethicin which is turned on by negative voltage. The way in which I-V curve asymmetry varies with aqueous concentration of alamethicin is predicted to be different for these two cases and will thus enable us to distinguish experimentally between them.

First we introduce the notion of "characteristic voltage," V_c. This is the voltage at which the membrane attains a given conductance, G_c, chosen for convenience by the experimenters and expressed in μS/cm^2. For example, V_{14} would be the voltage at which the membrane conductance attains a value of 14 μS/cm^2.

V_c is not strictly "characteristic," since its value depends on the choice of conductance used to determine it, but G_c, V_c, and V_e (see Eq. 1) completely determine the current-voltage curve under given conditions of alamethicin concentration, ionic strength, and membrane composition.[1] Shifts in V_c are the same regardless of the value of G_c chosen, provided only that the value of G_c chosen is the same for all experiments considered.

For conditions of fixed ionic strength, one can write the membrane conductance as

$$G = \Gamma_0 (C_{ala})^n \exp (V/V_e) \tag{1}$$

where Γ_o is a parameter depending on lipid composition and ionic strength, C_{ala} is the concentration of alamethicin in the aqueous phase, V is the applied voltage, and V_e the voltage that results in an e-fold increase in conductance. The alamethicin conductance obeys this empirical relationship under a large number of experimental conditions (Gordon and Haydon, 1975; Eisenberg et al., 1973; Roy, 1975; Vodyanoy et al., 1982).

Thus, if G is equal to G_c,

$$V_c = V_e \ln G_c - V_e \ln\Gamma_0 - n V_e \ln C_{ala}. \tag{2}$$

This can be written in the form

$$V_c = V_s - V_a \ln C_{ala} \tag{3}$$

where $V_s = V_e \ln G_c - V_e \ln \Gamma_o$, and $V_a = nV_e$.

The Translocation Hypothesis

The translocation of alamethicin across the membrane may be a very complicated process, much too complicated to model simply. Nonetheless, it is appropriate to exhaust the possibilities of a simple model before introducing unnecessary complications. For this reason, we consider a three-stage translocation process in which alamethicin monomers diffuse across the unstirred layer, undergo an adsorption/desorption step followed by a translocation and then a desorption/adsorption step and diffusion through the unstirred layer on the other side of the membrane. The reaction scheme is (see Fig. 1)

$$C'_a \rightleftharpoons C'_{us} \underset{K_D}{\overset{K_A}{\rightleftharpoons}} N'_m \underset{K_T}{\overset{K_T}{\rightleftharpoons}} N''_m \underset{K_A}{\overset{K_D}{\rightleftharpoons}} C''_{us} \rightleftharpoons 0 \tag{4}$$

[1] $G = G_c \exp [V - V_c)/V_e]$.

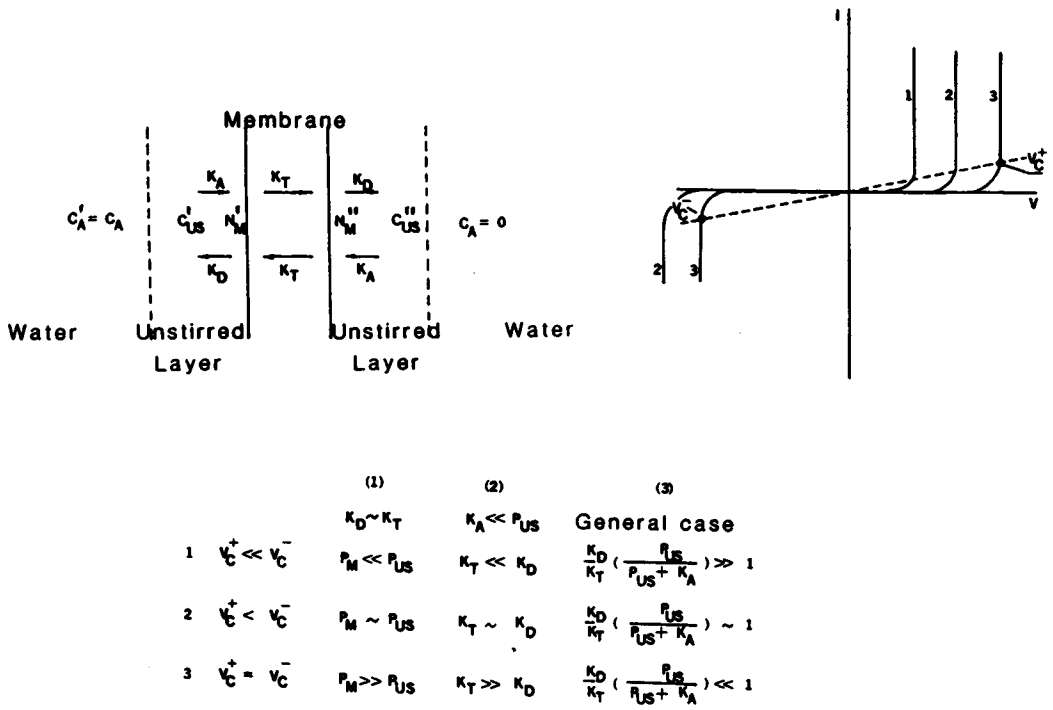

FIGURE 1 Illustration of the translocation hypothesis for I-V curve asymmetry. The left-hand side of the figure shows the general translocation model. C_A is the aqueous concentration of alamethicin monomer far from the membrane. C_{us} is the concentration of alamethicin monomer at the membrane side of the unstirred layer. N_M is the surface concentration of adsorbed alamethicin on one side of the membrane. Single primed quantities are on the *cis* side and double primed quantities are on the *trans* side of the membrane. The I-V curves on the right of the figure show how variations in the parameters of the model would be expected to change the asymmetry of the current-voltage curves. Three cases are shown: (*1*) extreme asymmetry ($V_c^+ << V_c^-$); (*2*) moderate asymmetry ($V_c^+ < V_c^-$); (*3*) no asymmetry ($V_c^+ = V_c^-$). Model parameters could be determined for each condition of asymmetry. Ranges of values leading to each kind of asymmetry are shown for two approximations: (*1*) unstirred layer permeability dominates, (*2*) translocation dominates, and (*3*) the general case.

where C'_a is the aqueous concentration of a alamethicin far from the membrane, C'_{us} the concentration in the aqueous phase close to the membrane, K_A the adsorption rate (units of cm/s), K_D the desorption rate (units of s^{-1}),[2] N'_m the surface concentration of alamethicin on the *cis* side of the membrane (to which alamethicin was added), N''_m the concentration on the *trans* side, K_T the translocation rate of alamethicin across the membrane, C''_{us} the aqueous concentration of alamethicin near the membrane on the *trans* side.

In the steady state,

$$K_D N''_m = (P_{us} + K_A) C''_{us}$$
$$K_T N'_m - N''_m (K_D + K_T) + C''_{us} K_A = 0 \quad (5)$$

where P_{us} is unstirred layer permeability so that

$$\frac{N'_m}{N''_m} = 1 + \frac{K_D}{K_T}\left(\frac{P_{us}}{P_{us} + K_A}\right). \quad (6)$$

This expression has two cases of interest. First, the unstirred layer permeability may be very large compared with the adsorption rate (which in our formulation implicitly contains the bulk/surface partition coefficient). In this case

$$\frac{N'_m}{N''_m} = 1 + \left(\frac{K_D}{K_T}\right). \quad (7)$$

Second, K_A may be very large compared with water permeability P_{us}. In this case

$$\frac{N'_m}{N''_m} = 1 + \left(\frac{P_{us}K_D}{K_T K_A}\right). \quad (8)$$

This expression is

$$\frac{N'_m}{N''_m} = 1 + \left(\frac{P_{us}}{P_m}\right)$$

and could be derived directly assuming no surface reactions, but only diffusion through the membrane and the unstirred layer. (P_m is the permeability of the membrane to alamethicin.

All of the above expressions, Eqs. 6–8, however, predict that ratio of N'_m to N''_m is independent of the bulk concentration of alamethicin.

If we assume that the relevant concentrations of alamethicin for calculating conductance are those at each surface of the membrane, then C_{ala} can be replaced by N'_m

[2] K_A and K_D are here assumed the same on both sides of the membrane. This need not be the case in membranes of asymmetric lipid composition.

or N''_m; and if both sides of the membrane are independent, Eq. 3 gives two characteristic voltages

$$V_c^+ = V_s - V_a \ln N'_m$$
$$V_c^- = V_s - V_a \ln N''_m \tag{9}$$

where the plus superscript denotes the characteristic voltage of the positive branch of the I-V curve and the minus superscript that of the negative branch. The characteristic voltages are the absolute values of the applied potentials at the characteristic conductance and decrease with increasing alamethicin concentration. Thus,

$$\Delta V_c = V_c^- - V_c^+ = V_a \ln \frac{N'_m}{N''_m} \tag{10}$$

Because N''_m/N''_m depends only on properties of the system and not on the aqueous alamethicin concentrations, Eq. 10 shows that all simple translocation models predict that the difference in characteristic voltages of the two branches of the I-V curve will depend only on the relative rates of unstirred layer permeability, adsorption, desorption, and translocation, and will be independent of alamethicin concentration.

There are three special cases of Eq. 10: rate limiting by unstirred layers, by desorption, or neither. In these cases, Eq. 10 becomes

$$\Delta V_c = V_a \ln \left(1 + \frac{P_{us}}{P_m}\right) \tag{11a}$$

$$\Delta V_c = V_a \ln \left(1 + \frac{K_D}{K_T}\right) \tag{11b}$$

$$\Delta V_c = V_a \ln \left(1 + \frac{K_D}{K_T} \frac{P_{us}}{P_{us} + K_A}\right). \tag{11c}$$

Because P_{us} is given by the diffusion constant of alamethicin in the aqueous phase divided by the unstirred layer thickness, stirring should be able to appreciably alter the asymmetry of the I-V curve if Eq. 11a is applicable. On the other hand, if adsorption is slow, compared with permeation through the unstirred layer, Eq. 11b should be applicable. If K_A and permeability are comparable, stirring should be able to alter the asymmetry of the I-V curve according to Eq. 11c.

Fig. 1 shows how the model parameters and I-V curve shape are related for these three limiting cases. The criteria for observing various degrees of asymmetry are shown for each case.

Inherent Orientational Asymmetry

We consider next a simple model of inherent asymmetry. It is consistent in some ways with models of alamethicin action previously proposed by others (Boheim, 1974; Eisenberg et al., 1973; Gordon and Haydon, 1975; Bauman and Mueller, 1974). We assume (see Fig. 2 *A*) that in the absence of an electric field most of the alamethicin molecules associated with the membrane have a conformation and orientation that has a net dipole moment of a particular magnitude and direction. We denote the dipole moment of a single monomer in this state $\vec{P}_{off}$. We further assume that those alamethicin molecules that contribute to the conductance (open pore) have a particular conformation and orientation, which has a net dipole moment in general different from that of the off state in both magnitude and direction. We denote the dipole moment of a monomer in this state $\vec{P}_{on}$. We further assume that only alamethicin molecules in this second state participate in the conducting structure and that n such molecules are required to create that structure. Then, if ΔE is the difference in energy between a molecule in the off state and one in the conducting unit, we can write the number of conducting units in the membrane as

$$N \sim [C^n_{ala} \exp n (\Delta G/kT)]$$
$$\Delta G = \Delta\mu - T\Delta S + (\vec{P}_{off} - \vec{P}_{on}) \cdot \vec{E} \tag{12}$$

where $\Delta\mu$ is the difference in chemical potential, $T\Delta S$ is entropic free energy difference, $\vec{P}_{off}$ is the dipole moment of monomers in the off, nonconducting state, and $\vec{P}_{on}$ the dipole moment of monomers in the on, conducting state, and E is the electric field across the membrane (see Fig. 2).

The present formalism has glossed over a number of important questions: We have assumed that alamethicin transfers as a monomer from water into membrane; we ignore those factors that determine the value of n; and, finally, we assume a simplified static model of the conducting entity, which may well be fluctuating in size about some average value. While these complicating factors are important in understanding other aspects of how alamethicin works, this simplified treatment will enable us to distinguish between the two classes of model for I-V curve asymmetry in a relatively simple way.

The germane feature of this treatment is that it provides a simple quantitative method for distinguishing between the effects of positive and negative applied voltages on alamethicin molecules on one side of the membrane. There are a number of physical models that would lead to formalisms identical to this one, and it is not the purpose of this paper to distinguish between these numerous possibilities.

Finally, we will not treat the kinetics of opening or closing of channels explicitly in this paper. It is clear from observation of single channels that a large activation energy separates the open-channel state from the closed-channel state. A physical picture satisfying this requirement is that the gating charge (in our simplified treatment, the positive end of the dipole) has its lowest energies at the extreme ranges of its travel. We might thus expect the kinetics of alamethicin to be faster in high than low dielectric constant membranes and that alamethicin might cross high dielectric constant membranes more rapidly

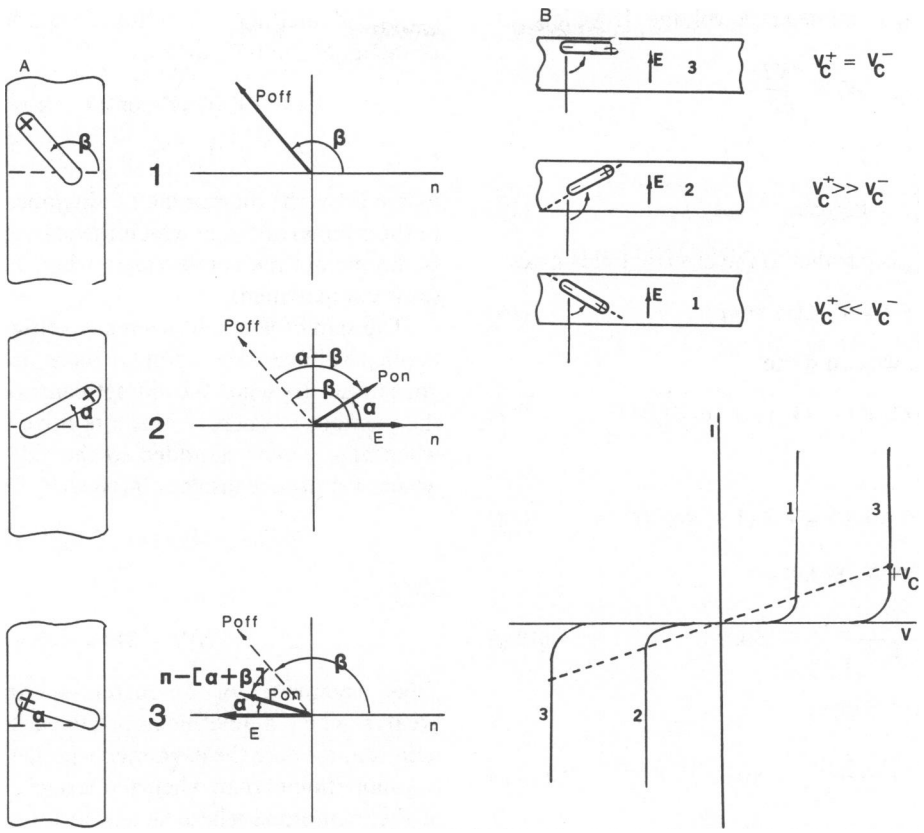

FIGURE 2 Inherent asymmetry of alamethicin hypothesis. *A*, The formal model. Alamethicin added to the left side of a membrane. *n* is a unit vector normal to the membrane pointing away from the side to which alamethicin was added. $\vec{P}_{off}$ and $\vec{P}_{on}$ are the dipole moment vectors for the off and on state, correspondingly. *E* is the electric field vector. β is the angle between the alamethicin molecule off dipole moment and the normal to the membrane, *n*. α is the angle between the on alamethicin dipole moment and the electric field vector. Case *1* represents the off state. Case *2* shows the on state with the electric field vector corresponding to a positive voltage by the usual sign convention (side opposite to alamethicin addition is ground). α–β is the angle between $\vec{P}_{off}$ and $\vec{P}_{on}$ vectors. Case *3* represents the on state with electric field vector corresponding to a negative voltage and an angle between $\vec{P}_{off}$ and $\vec{P}_{on}$ equal to $-(\alpha + \beta)$. *B*, Inherent asymmetry of alamethicin (simplified case). The net alamethicin dipole is assumed to make an angle β with the normal of the membrane in the off state and to be perpendicular to the membrane in the on state. If $\beta = 90°$, the field-induced reduction in energy is equal for both positive and negative direction of the electric field. The initial angle will affect the current voltage curve for three shown cases (three different angles). Alamethicin is added to only the top side of the membrane.

than low. We will later present data supporting the view that the alamethicin gating charge moves only part way across the membrane, but is affected by much the same forces that influence the carrier nonactin when crossing the membrane, electrostatic barrier and all. For the present, we consider only the energy differences between the on and off states.

For a positive applied voltage, we can write

$$(\vec{P}_{off} - \vec{P}_{on}) \cdot \vec{E} = -(P_{off} \cos\beta + P_{on} \cos\alpha) \cdot V/d \quad (13)$$

where β is the angle that the off-state dipole moment makes with the membrane normal directed away from the *cis* side (see Fig. 2 *A*), α is the angle of the on-state dipole moment with electric field vector, V is applied voltage, and d is the membrane thickness ($\vec{E}$ is assumed to be always normal to the membrane surface).

For ease of calculation (see Fig. 2 *B*), we will assume that the magnitudes of $\vec{P}_{on}$ and $\vec{P}_{off}$ are the same. This is very likely not the case, especially if a conformational change occurs in going from the off to the on state. Because it is the scalar product of $\vec{P}$ and $\vec{E}$, which appears in the energy change, this simplification only means that α and β cannot be rigidly interpreted as geometrically meaningful angles. Eq. 13 thus becomes

$$(\vec{P}_{on} - \vec{P}_{off}) \cdot \vec{E} = -P(\cos\beta + \cos\alpha) \cdot V/d. \quad (13\,a)$$

For a negative voltage

$$(\vec{P}_{off} - \vec{P}_{on}) \cdot \vec{E} = P(\cos\beta + \cos\alpha) \cdot V/d. \quad (14)$$

Thus, using Eq. 1, assuming that membrane conductance is proportional to the number of conducting structures, N, ignoring nonelectrical terms in ΔG, we can identify V_c with different terms for a positive and negative applied voltage. We define

$$V_c^{\pm} = \frac{dkT}{nP(\cos\beta \pm \cos\alpha)}$$

for the positive voltage and negative voltage. If we let

$$V_p = \frac{dKT}{nP},$$

then

$$V_c^{\pm} = V_p/(\cos\beta \pm \cos\alpha), \tag{15}$$

assuming $\alpha = 0$ (P_{on} is parallel to the electric field) gives

$$V_c^{+} = V_p/(1 \pm \cos\beta). \tag{16}$$

Using Eq. 1 and 16, we can write

$$G_c = \Gamma_0(C_{ala})^n \exp(V_c(1 \pm \cos\beta)/V_p) \tag{17}$$

and

$$V_c^{+}(1 + \cos\beta) = V_c^{-}(1 - \cos\beta). \tag{18}$$

Solving Eq. 18 for $\cos\beta$, we get

$$\frac{V_c^{-} - V_c^{+}}{V_c^{+} + V_c^{-}} = \cos\beta. \tag{19 a}$$

Solving Eq. 16 for $\cos\beta$ gives

$$\frac{V_c^{-} - V_c^{+}}{V_c^{+} + V_c^{-}} = \cos\beta. \tag{19 b}$$

Both of these relationships can be conveniently tested experimentally. Note in particular that inherent orientational asymmetry predicts that the difference between V_c^{+} and V_c^{-} depends on their sum. Because both V_c^{+} and V_c^{-} decrease as alamethicin concentration increases, orientational asymmetry predicts that $V_c^{-} - V_c^{+}$ should decrease with increasing alamethicin concentration. If the negative branch of the I-V curve is, however, due to alamethicin molecules which have diffused to the *trans* side of the membrane, $V_c^{-} - V_c^{+}$ should be constant with increasing alamethicin concentration.[3]

Inherent Asymmetry of the Membrane

The membrane may also be inherently asymmetric. In such cases, the alamethicin sees a voltage drop across the membrane that is equal to the sum of the applied voltage, V, and the asymmetry potential, Ψ. Ψ will, in general, depend both on the asymmetry of the membrane and on the nature of the conductance mechanism under study. If the only source of asymmetry is membrane asymmetry, Ψ will be equal in magnitude and opposite in sign to that voltage about which the current-voltage curve is symmetrical.

For alamethicin we write the conductance of the two branches of the I-V curve as

$$G^{+} = \Gamma_0(C_{ala})^n \exp[(V \pm \Psi)/V_c^{+}]$$
$$G^{-} = \Gamma_0(C_{ala})^n \exp[-(V \pm \Psi)/V_c^{-}] \tag{20}$$

where G^{+} is the membrane conductance when V is positive in the compartment to which the alamethicin is added, G^{-} is the membrane conductance when V is negative in the same compartment.

The sign of Ψ can be such as to either aid or oppose the applied voltage. Accordingly, there are two cases analogous to Eq. 10: when the alamethicin is added to the side of the membrane where Ψ aids a positive voltage (ΔV_c^{+}) and when alamethicin is added to the side of the membrane where Ψ aids a negative voltage (ΔV_c^{-})

$$\Delta V_c = -V_a \ln(1 + K_D/K_T) \pm 2\Psi \tag{21}$$

and[4]

$$\Delta V_c - \Delta V_c = 4\Psi. \tag{22}$$

Thus, asymmetry of the current-voltage curve should be greater when alamethicin is added to the side of the membrane where the asymmetry potential tends to turn on the alamethicin, than when alamethicin is added to the side of the membrane where Ψ tends to turn off the alamethicin.

Combined Permeability, Alamethicin Asymmetry, and Inherent Asymmetry

Assuming that the asymmetry of the current-voltage curve is due to all of the above described mechanisms, we can write the conductivity in general form as

$$G^{+} = \Gamma_0(N_m')^n \exp[(V \pm \Psi)(1 + \cos\beta)/V_p] + (N_m'')^n \exp[(V \pm \Psi)(1 - \cos\beta)/V_p] \tag{23}$$

for the positive branch, and

$$G^{-} = \Gamma_0(N_m')^n \exp[-(V \pm \Psi)(1 + \cos\beta/V_p] + (N_m'')^n \exp[-(V \pm \Psi)(1 + \cos\beta)/V_p] \tag{24}$$

for the negative branch.

RESULTS

We have measured I-V curves for a number of different membrane compositions using sweep rates shown by control experiments to be sufficiently slow to allow construction of an accurate I-V curve. Because membranes doped with alamethicin have a higher probability of breaking at voltages where the alamethicin conductance is turned on than at zero voltage, it is desirable to obtain I-V curves as rapidly as possible. But because the alamethicin conduc-

[3]This would also be true for an asymmetry that took the form $G^{+} = \Gamma_o^{+}(C_{ala})^n \exp(V/V_e)$, $V > 0$, and $G^{-} = \Gamma_o^{-}(C_{ala})^n \exp(-V/V_e)$, $V < 0$ where n and V_e must be the same for both signs of voltage. In this case, $V_c^{-} - V_c^{+} \ln \Gamma_o^{+}/\Gamma_o^{-}$. We rule this model out by showing that the slope of the positive and negative branches of the I-V curve are different at high alamethicin concentrations.

[4]Strictly speaking, Ψ might depend on which side the alamethicin is added to. Eq. 22 would then become $\Delta V_c - \Delta V_c = 2(\Psi_1 + \Psi_2)$.

tance requires time to turn on and off, sweeping the voltage rapidly results in considerable hysteresis. It is thus necessary to arrive at some compromise sweep rate that allows one to take an accurate I-V curve, but does not expose the membrane to elevated voltages for too long a period. By doing pulsed-voltage I-V curves and by sweeping at fixed rates of from 0.5 to 20 mV/s, we have found that for most purposes, sweeping at 10 mV/s is slow enough to allow construction of an accurate steady-state I-V curve. In most cases, a sweep rate of 10 mV/s is adequate to give a value of V_c within <5 mV of that which would be obtained at 0.5 mV/s. Fig. 3 shows a comparison of the I-V curve taken by interpolating between the positive-going and negative-going sweeps at a sweep rate of 10 mV/s (dashed line) and a pulsed I-V curve (solid points). There is essentially no difference between the two. Thus, while the hysteresis between the positively swept branch of the I-V curve and the negatively swept branch can be appreciable, the interpolated I-V curve is nonetheless an excellent approximation to the steady-state I-V curve if the sweep rate is <10 mV/s.

We measured current-voltage curves in symmetric membranes made of bacterial PE, brain PE (Bovine), PS, and DBrPC with and without organic solvent (*n*-decane or 1-chlorodecane) and asymmetric membranes made of bacterial PE/brain PE and bacterial PE/DBrPC.

Bacterial PE squalene membranes and bacterial PE with *n*-decane as a solvent membranes have current-voltage curves with only one detected branch—that where the voltage is positive on the *cis* side (see Figs. 4 and 5).

We varied membrane dielectric constant by changing both solvent and lipid (Dilger et al., 1979; Roseman et al., 1978). Bacterial PE membranes with 1-chlorodecane solvent (dielectric constant 4.5 at 25°C against 2 at 20° for *n*-decane) have a current-voltage curve with a negative branch (third quadrant) (see Fig. 5). The difference between positive characteristic voltage and negative characteristic voltage (called ΔV_c in Eq. 10) in bacterial PE/chlorodecane has a value of ~50 mV regardless of alamethicin concentration.

Increasing dielectric constant using halogen groups (Br) fixed to the lipid chains (dibromostearicphosphatidylcholine, DBrPC) also decreases asymmetry (Fig. 6), but

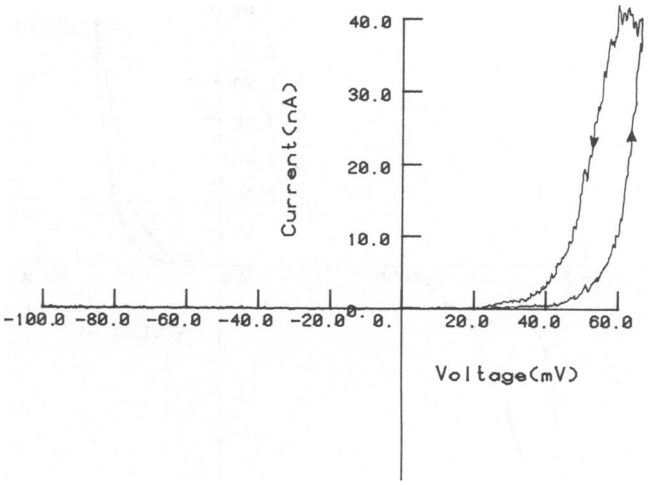

FIGURE 4 PE (bacterial) squalene membrane I-V curve in 1 M KCl unbuffered (pH 5.5). 6.5×10^{-7} g/ml of alamethicin was added to the *cis* side of the membrane (first quadrant). Voltage sweep rate 10 mV/s. Note that no increase in conductance is evident in quadrant three.

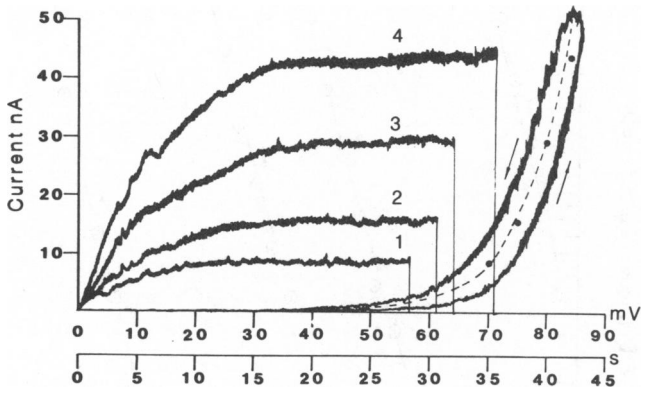

FIGURE 3 Diecosenoylphosphatidylcholine (squalene) membrane current-voltage curve and current responses to voltage steps. The membrane was formed in 1 M KCl unbuffered (pH 5.5) at 22°C and 1.4×10^{-7} g/ml alamethicin Fraction 4 was added to one (*cis*) side after membrane formation. The voltage sweep rate was 10 mV/s. Arrows show the record direction. The dashed line is an interpolation between the positive-sweeping curve and the negative sweeping curve. The time scale at the bottom is for the pulsed voltage (the same current scale) steps: 1–70, 2–75, 3–80, 4–84 mV. Solid points are the steady-state currents taken from current-time curves. Thus the swept current-voltage curve, even with considerable hysteresis, can be used to construct a curve equivalent to the steady-state current voltage curve.

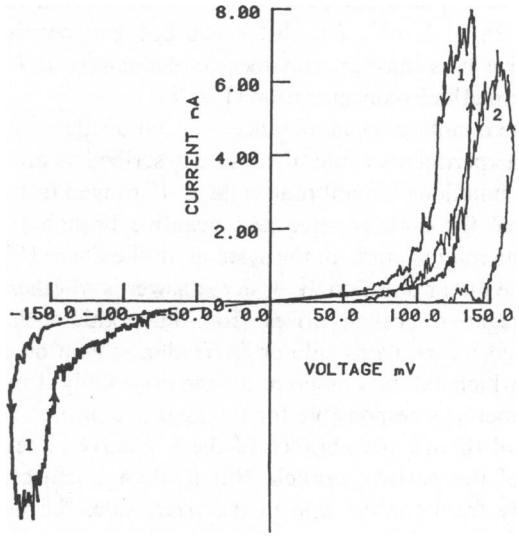

FIGURE 5 PE (bacterial) membrane I-V curves. Membrane was formed in 0.5 M KCl unbuffered (pH 5.5) with different solvents, and doped with alamethicin (2×10^{-7} g/ml). Antibiotic was added to the *cis* side of the membrane (first quadrant). Voltage sweep rate was 10 mV/s. (*1*) Membrane with 1-chlorodecane as solvent, and (*2*) membrane with decane as solvent. Note that the asymmetry in a PE-decane membrane is much more pronounced than in a PE-chlorodecane membrane.

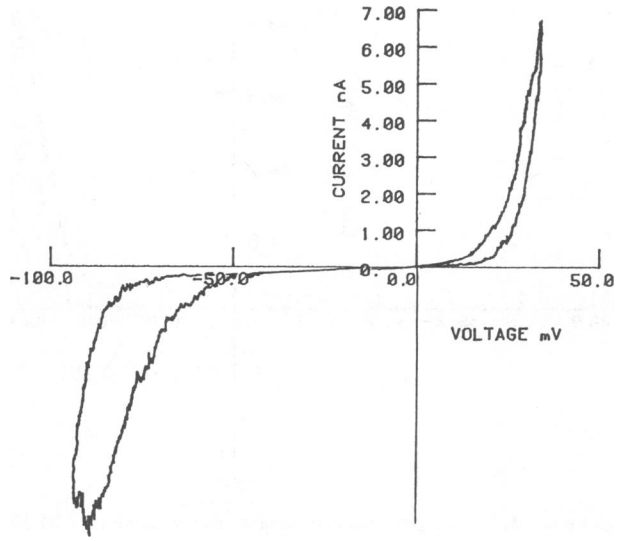

FIGURE 6 Dibromostearicphosphatidylcholine membrane (squalene) current-voltage curve in 1 M KCl unbuffered, (pH 5.5). 3×10^{-7} g/ml of alamethicin was added to the *cis* side of the membrane (first quadrant). Voltage sweep rate was 10 mV/s.

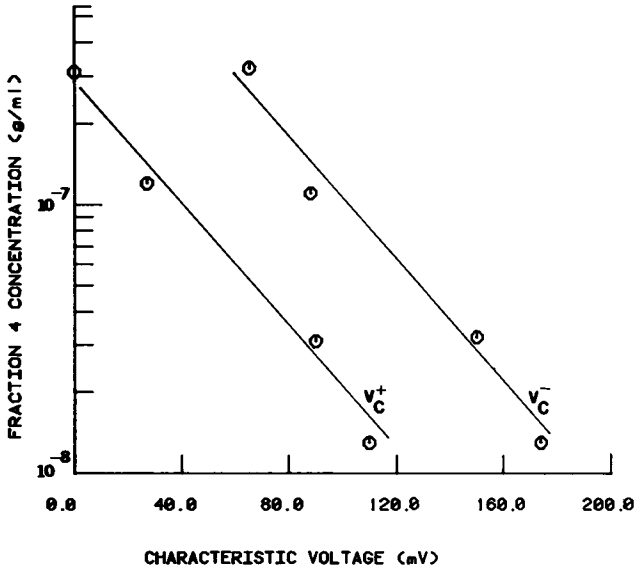

FIGURE 7 Characteristic voltage, V_{14}, for positive and negative branches of the dibromostearic phosphatidylcholine membrane (squalene) current-voltage curve as a function of concentration of alamethicin (Fraction 4). Membrane was formed in1 M KCl unbuffered (pH 5.5). Note that $\Delta V_c = V^+ - V^-$ is independent of alamethicin concentration.

the difference between positive and negative characteristic voltages (ΔV_c) is ~70 mV. This is consistent with the finding that DBrPC membranes have a lower dielectric constant than membranes made with 1-chlorodecane as a solvent (Dilger et al., 1979).

Characteristic voltages (V_{14}) for both positive and negative branches decrease ~38 mV per *e*-fold change of alamethicin concentration, but the negative branch of the curve is shifted. All systems studied (PE) bacterial, PS, PE brain, and asymmetric membranes) show the same dependence of V_{14} on alamethicin concentration, i.e., a value of V_a of 38 ± 5 mV. The difference between positive and negative V_c is constant and does not decrease with increasing alamethicin concentration (Fig. 7).

The membrane conductance induced by alamethicin in these experiments could always be described as an exponential function of membrane voltage. V_e ranged from 4.5 to 6.5 mV for both positive and negative branches of the current voltage curve in the systems studied here (Fig. 8).

One major question we wish to answer is whether or not the negative branch arises from alamethicin that has diffused to the *trans* side or from alamethicin on the *cis* side, which exhibits inherent asymmetry. Only if inherent asymmetry is responsible for the negative branch can the slope of the negative branch of the I-V curve be less than that of the positive branch. But if alamethicin can leak rapidly from the *cis* side to the *trans* side, the negative branch due to inherent asymmetry will be masked by that due to *trans* alamethicin. Thus only if the ΔV_c due to inherent asymmetry is less than that due to translocation will the asymmetry due to inherent orientation be observable.

Because ΔV_c due to translocation is constant and does not decrease with alamethicin concentration (Eqs. 10 and 11), although ΔV_c due to inherent asymmetry does decrease with increasing alamethicin concentration, the negative branch of the I-V curve is most likely to be due to inherent asymmetry at high alamethicin concentrations.

Accordingly, we added enough alamethicin to one side of a PE-PS (mixture 1:1 by weight) membrane to obtain

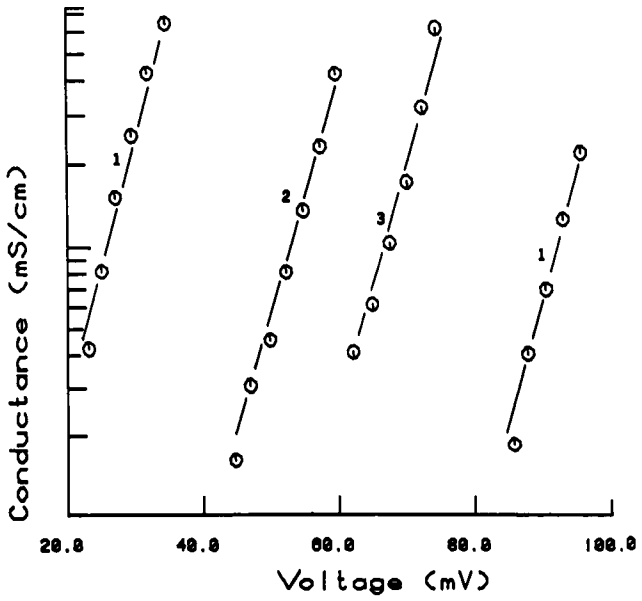

FIGURE 8 Membrane conductance vs. membrane voltage:(*1*) Dibromostearicphosphatidylcholine membrane (squalene) doped with 3×10^{-7} g/ml alamethicin. Real negative voltage taken as absolute value (1 M KCl); (*2*) PE bacterial (decane as a solvent) membrane doped with 1.2×10^{-7} g/ml alamethicin (0.5 M KCl); and (*3*) PE bacterial (squalene) membrane doped with 2.2×10^{-7} g/ml alamethicin (0.5 M KCl).

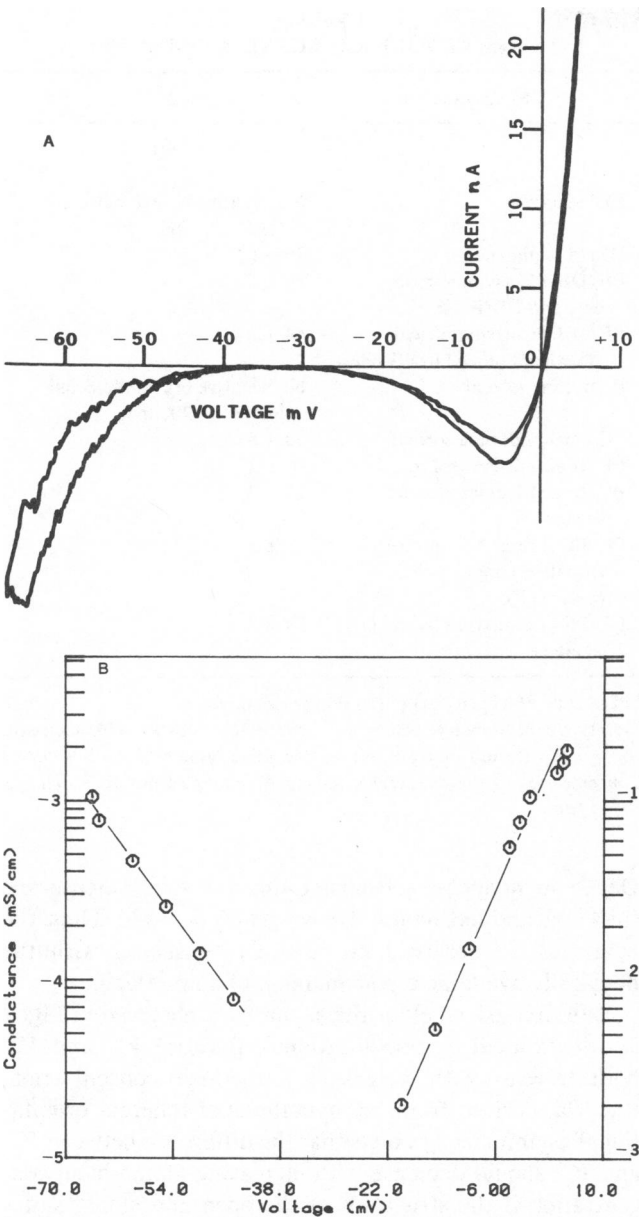

FIGURE 9 PE (bacterial)/PS (mixture 1:1 by weight) membrane (squalene) doped with 4×10^{-6} g/ml alamethicin, 0.1 M KCl (unbuffered, pH 5.5). *A*, I-V curve at voltage sweep rate of 1 mV/s. *B*, Membrane conductance vs. membrane voltage. Left curve and left scale are for the negative current-voltage curve branch. Right curve and right scale for the positive branch.

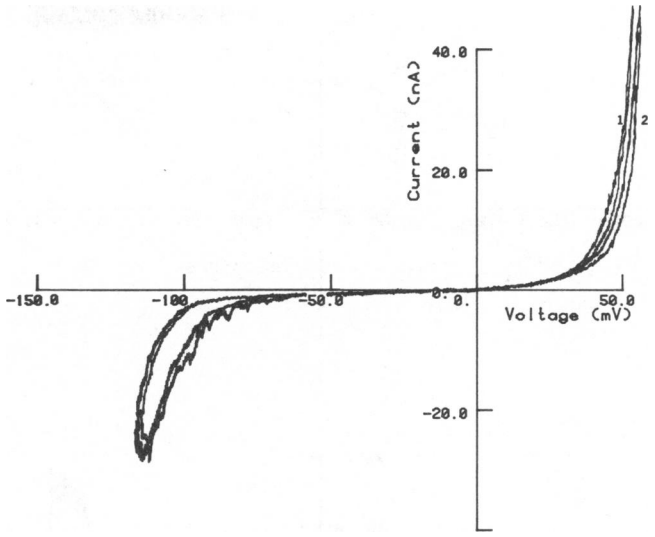

FIGURE 10 DBrPC membrane (squalene) current-voltage curve. Membrane was formed in 1 M KCl unbuffered (pH 5.5). 2×10^{-7} g/ml alamethicin. Curve *1*, current-voltage curve with vigorous stirring in both compartments. Curve *2*, no stirring in either compartment.

the I-V curve shown in Fig. 9. In the particular case of a PE-PS mixture and large alamethicin concentration, V_e for the positive branch of the current-voltage curve is 4.7 mV, and V_e for the negative branch is 6.4 mV. (Regression analysis gives $R^2 = 0.987$ for the positive branch and $R^2 = 0.997$ for the negative.) Possible reasons for the differing slopes will be discussed later.

We also wished to find out which case of Eq. 11 is applicable at lower alamethicin concentrations. To test whether or not unstirred layer permeability is rate limiting, we observed the shift in current-voltage curves that occurred when either compartment or both was well-stirred. Fig. 10 shows two I-V curves recorded from a DBrPC membrane with (curve *1*) and without (curve *2*) vigorous stirring. There is a very small, but reproducible increase in ΔV_c without stirring. This result indicates unstirred layer permeability is not the major barrier to alamethicin translocation.

Finally, we wish to consider the effects of membrane asymmetry on the asymmetry of the I-V curve. The I-V curve of an asymmetric membrane with bacterial PE on one side and DBrPC on another side is shown in Fig. 11 *B*. Membranes were formed in 1 M KCl. Then nonactin was added to both sides of the membrane to determine the degree of asymmetry (Hall and Latorre, 1976). Next, alamethicin was added either to the PE side or to the DBrPC side. In case of PE/DBrPS membranes, the origin of the I-V curve asymmetry is probably the difference in dielectric constant of the two phospholipids. Fig. 11 shows the nonactin I-V curve in this system is asymmetric with an asymmetry potential of ~50 mV (Hall et al., 1973; Latorre and Hall, 1976). Asymmetric membranes made of bacterial PE and brain PE are asymmetric due to both surface charge and the presence of plasmalogens (acyl chains ether—rather than ester—linked in the brain PE). Table I shows the ΔV_{14} for this membrane system and the systems previously described.

DISCUSSION

In this discussion, we wish to establish three central points. First, the asymmetry of the alamethicin I-V is regulated by the ratio of K_D to K_T (when K_T is large, asymmetry is small; when K_T is small, asymmetry is large). Second, for asymmetry to be manifest at all, there must be an inherent asymmetry of orientation. Finally, membrane asymmetry

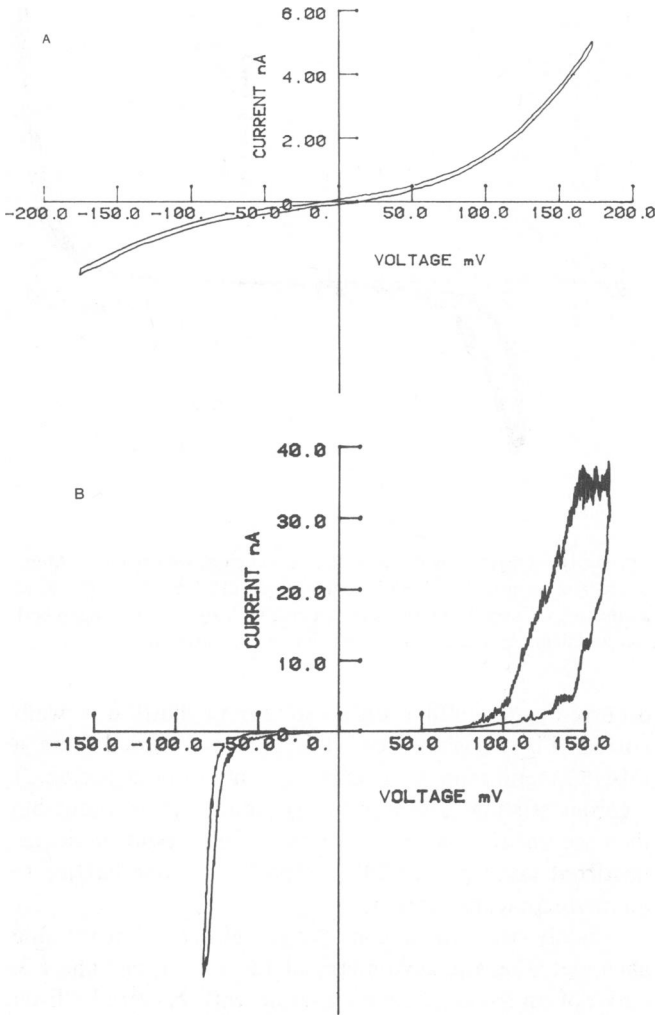

FIGURE 11 Current-voltage curves of asymmetric phosphatidylethanolamine on one side and dibromostearicphosphatidylcholine on the opposite side of the (squalene) membrane in 1 M KCl. *A*, 4×10^{-8} M nonactin-K was added to both sides of the membrane. *B*, 3×10^{-7} g/ml of alamethicin was added to the PE side of the same membrane. (This is the only case of alamethicin being added to the *trans* side of the membrane only.)

can itself alter the asymmetry of the alamethicin current-voltage curve.

The experimental results described above show that voltage-dependent conductance induced by alamethicin obeys the empirical equation (Eq. 1), where V_e is ~5 mV for all systems studied.

From our simple hypothesis, V_p can be expressed as

$$V_p = \frac{dKT}{nP}$$

where d is the membrane thickness, n is the number of monomers (dipoles), P is the dipole moment of the monomer [k and T have their usual meanings; remember that $V_e^{\pm} = V_p(1 \pm \cos\beta)$]. The alamethicin dipole moment has been measured by Yantorno (Yantorno et al., 1977; Yantorno et al., 1982; Schwartz and Savko, 1982) and is ~70

TABLE I
CURRENT-VOLTAGE CURVE ASYMMETRY

Membrane	ΔV_e
	mV
PE* squalene	No negative branch detected for V > −200 mV
DBrPC squalene	70 ± 6
PE/DBrPC asymmetrical, doped to DBrPC side	86 ± 3
PE/DBrPC asymmetrical (squalene), doped to PE side	44 ± 3
PE decane solvent	No negative branch detected for V > −200 mV
PE 1-chlorodecane solvent	50 ± 5
PE (brain) decane solvent	72 ± 4
PE (brain) 1-chlorodecane solvent	27 ± 3
PE/PE (brain) asymmetrical; (squalene) doped to PE (brain) side	48 ± 3
PE/PS (1:1 mixture by weight) squalene	78 ± 8

*PE means PE (bacterial) unless otherwise stated.
Membrane asymmetry taken as a difference between characteristic voltages V_{14} (in mV) for positive and negative branch of the I-V curve. Concentration of the alamethicin changed in range from 10^{-8} g/ml to 10^{-6} g/ml.

Debye in nonpolar solvents. Using 30 Å for membrane thickness and letting $n = 10$, we get $V_p = 5$ mV. Thus, the values of V_e observed are entirely consistent with the physically available dipole moment of alamethicin.

We first ask whether the asymmetry observed in Fig. 7 is orientational or translocational. Because V_c^+ and V_c^- both decrease with increasing alamethicin concentration, Eq. 19a, derived from the hypothesis of inherent orientational asymmetry, predicts that the difference between V_c^- and V_c^+ should decrease with increasing alamethicin concentration if the structures of the open and closed states remain unchanged. If orientation were the only cause of asymmetry, β would remain constant with increasing alamethicin concentration. But our results show that $V_c^- - V_c^+$ is independent of alamethicin concentration (Fig. 7). Because $V_c^+ + V_c^-$ decreases with increasing alamethicin concentration (see Fig. 7 and Table I), Eq. 19a is not adequate to describe I-V curve asymmetry as alamethicin concentration increases.

The permeability hypothesis, on the other hand, is consistent with the observed result: ΔV_c is constant with increasing alamethicin concentration (Eq. 10). We are thus left with deciding whether membrane permeability, unstirred layer permeability, or dissociation of alamethicin from the membrane is rate limiting. We first show that the permeability of the unstirred layer must be greater than the rate of dissociation from the membrane.

Consider the stirred and unstirred cases shown in Fig. 10. We know from previous work that in our chamber, we

can change the unstirred layer thickness by a factor of ~2 from something over 100 μm with no stirring to ~50 μm with vigorous stirring (Hall and Cahalan, 1982). Thus, we can estimate the relative value of P_{us} and K_A using Eq. 11c.

Because P_{us} can be calculated from D, the estimated diffusion coefficient of alamethicin in the water, and δ, the thickness of the unstirred layer, we can define α as

$$\alpha = \frac{K_A}{P_{us}}. \tag{26}$$

If the change in ΔV_c produced by stirring is small, α is small (equivalent to K_A, the adsorption rate being small compared with the permeability of the unstirred layer). Eq 11c then leads to the expression

$$\frac{\Delta(\Delta V_c)}{\Delta\alpha} = V_a$$

where $\Delta(\Delta V_c)$ is the change in asymmetry induced by a change in unstirred layer thickness. Because $\Delta(\Delta V_c)$ is less than ~5 mV, and because the change in unstirred thickness is effectively ~50 μm (or more) going from stirring to not stirring,

$$K_A \lesssim \frac{5\text{ mV}}{38\text{ mV}} \cdot \frac{D}{\Delta\delta} \approx 2 \times 10^{-5}\text{ cm/s}$$

where $\Delta\delta$ is the change in unstirred layer thickness. The fact that we observe very little shift in I-V curve with stirring (Fig. 11) thus argues that K_A is very small and that the appropriate description of translocation is Eq. 11b. The magnitude of the I-V curve asymmetry thus appears to be determined principally by the ratio of desorption rate, K_D to translocation rate, K_T.

Thus, I-V curve asymmetry is well described by Eq. 11b, because $P_{us}/(P_{us} + K_A) \approx 1$. The ratio K_D/K_T for DBrPC membrane evaluated from $V_c^- - V_c^+$ and Eq. 11b is ~5.3 and that for PE/chlorodecane membranes is ~2.8.

The constancy of $V_c^- - V_c^+$ suggests that the ratio of K_D to K_T for alamethicin is the principal determinant of the position of the negative branch of the I-V curve.

Under any given experimental conditions, the observed asymmetry will be due to the mechanism that results in the smallest ΔV_c. ΔV_c due to orientation asymmetry decreases with increasing alamethicin concentration. Eq. 19a shows this to be so. Because both V_c^+ and V_c^- decrease with increasing alamethicin concentration, the difference between V_c^- and V_c^+ must also decrease if cos β remains constant. Thus at very high alamethicin concentrations, the negative branch of the I-V curve may change from being due to translocated alamethicin to being due to inverse gating. Under this condition, the two branches of the current-voltage curve should have differing slopes and V_e^+ should be $< V_e^-$ according to Eq. 16.

While we were unable to find a high enough alamethicin concentration to see different slopes in the two branches of the I-V curve in DBrPC membranes, we did find different slopes at very high alamethicin concentrations in mixed PE/PS membranes.

For the I-V curve of Fig. 9, V_c^+ is 4.7 mV and V_c^- is 6.4 mV.

From Eq. 19b and these values, we calculate a value of 100° for β. Eq. 15 thus gives a value of 5.5 mV for V_p. If we multiply G, given by

$$G = \Gamma_0 (C_{ala})^n \exp [V(1 + \cos\beta)/V_p]$$

by V (potential), we get a calculated current-voltage curve

$$I = V\Gamma_0 (C_{ala})^n \exp [V(1 + \cos\beta)/V_p]$$

and then take the derivative of I with respect to V

$$\frac{dI}{dV} = \Gamma_0 (C_{ala})^n \exp [V(1 + \cos\beta)/V_p]$$

$$\cdot \{1 + [V(1 + \cos\beta)]/V_p\}.$$

The voltage V where the derivative is zero is

$$V^{\sim} = -\frac{V_p}{1 + \cos\beta} = V_e^+.$$

Such a calculation was first made by Muller and Finkelstein (1972). For the curve of Fig. 9, $V^{\sim}$ is 4.9 mV in good agreement with the value of V_e calculated from the slope of the G-V curve, 4.7 mV.

Thus, there is an inherent asymmetry apparent from the two slopes of the different branches of the I-V curve in PS/PE membranes. We can estimate the inherent asymmetry in DBrPC membranes by noting that it must be greater than or equal to that of translocation. At the highest alamethicin concentration shown in Fig. 7, $V_c^+ = 0$, $V_c^- = 70$ mV. Hence, cos $\beta = -1$ and $\beta = 180°$. This indicates that if alamethicin monomers form rigid rods that rotate under the influence of the electric field, the rods must be oriented so that their dipoles are perpendicular to the membrane in the off state. Alternatively, the magnitude of the dipole moment may increase under the influence of the electric field.

We do not believe that the rigid dipole picture used here for the sake of simplicity is correct, and we caution against using it as more than a convenient simplified picture capable of representing some of the effects of orientational asymmetry. We do believe our results show convincingly that some sort of orientational asymmetry exists, but what its detailed geometric interpretation is, we cannot yet say.

To further test the translocation hypothesis, we carried out an additional experiment. First, we measured the I-V curve (similar to that shown in Fig. 6) for DBrPC membrane with alamethicin added to only one side. Next, we added the same amount of alamethicin to the *trans* side.

If the alamethicin concentration on the membrane surface is additive (meaning that we can add alamethicin concentration profiles before and after doping the mem-

brane from the opposite side), then using the known ratio, K_D/K_T, the known value of V_a, and Eqs. 7, 9, and 10, we can predict from Fig. 7 the characteristic voltage shift. Thus, for 2.5×10^{-8} g/ml of Fraction 4 on only one side, V_c^+ is 100 ± 4 mV and V_c^- is 160 ± 6 mV. After adding the same amount of alamethicin to the opposite side, V_c^+ becomes 87 mV ± 4 mV and V_c^- is 105 ± 8 mV. Theoretical values calculated from assumed concentration profiles before and after addition are 88 mV for both V_c^+ and V_c^-. Thus, translocational asymmetry accounts for the most frequently observed asymmetry of the alamethicin I-V curves.

Finally, inherent membrane asymmetry alters the asymmetry of the I-V curve. For an asymmetric membrane made of bacterial PE on one side and DBrPC on the other, application of the translocation hypothesis alone cannot explain why the characteristic voltages obtained upon adding alamethicin to different sides of the membrane are different. But membrane asymmetry can account for the additional asymmetry observed in the I-V curve.

Experiment shows that $V_c^- - V_c^+ = (86 \pm 3)$ mV when alamethicin added to the DBrPC side and $V_c^- - V_c^+ = 44 \pm 3$ mV when alamethicin is added to PE side. Then from Eq. 20, we have an asymmetry potential seen by alamethicin equal to ~10 mV. Substituting known values of V_c, ΔV_c, and V_a in Eq. 19, and assuming that ratio K_D/K_T remained 5, we estimate there are ~7 monomers in the conducting unit. The asymmetry potential seen by nonactin-K in the same membrane (Hall and Latorre, 1976; Latorre and Hall, 1976) is ~50 mV. This fivefold difference can be accounted for if we assume that the movement of the effective alamethicin gating charge is confined to a limited region of the membrane and that the on state and off state are located asymmetrically in the membrane, a result already established from I-V curve asymmetry.

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