Voltage-dependent conductance for alamethicin in phospholipid vesicles

A test for the mechanism of gating

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ABSTRACT The ion currents induced by alamethicin were investigated in unilamellar vesicles using electron paramagnetic resonance probe techniques. The peptide induced currents were examined as a function of the membrane bound peptide concentration, and as a function of the transmembrane electrical potential. Because of the favorable partitioning of alamethicin to membranes and the large membrane area to aqueous volume in vesicle suspensions, these measurements could be carried out under conditions where all the alamethicin was membrane bound. Over the concentration range examined, the peptide induced conductances increased approximately with the fourth power of the membrane bound peptide concentration, indicating a channel molecularity of four. When the alamethicin induced currents were examined as a function of voltage, they exhibited a superlinear behavior similar to that seen in planar bilayers. Evidence for the voltage-dependent conduction of alamethicin was also observed in the time dependence of vesicle depolarization. These observations indicate that the voltage-dependent behavior of alamethicin can occur in the absence of a voltage-dependent phase partitioning. That is, a voltage-dependent conformational rearrangement for membrane bound alamethicin leads to a voltage-dependent activity.

INTRODUCTION

Membrane excitability is an essential feature of cells in muscle and the central nervous system. At a molecular level, this process is the result of electric field-dependent free energy differences between ion channel conformers. A determination of the structures of voltage-gated channels, such as the sodium and potassium channels of nerve, should lead to an understanding of the molecular basis for this process. Unfortunately, determining the structures of these large channels is a formidable task, and the mechanisms that lead to voltage-dependent gating remain largely uncharacterized. To advance our molecular understanding of membrane excitability, a number of small peptides are being studied that exhibit a voltage-dependent behavior in planar bilayer systems. While these are clearly simpler structures than the large ion channels of nerve and muscle, they nonetheless provide excellent models for protein-membrane electrostatic interactions and voltage-dependent conformational events.

Alamethicin, a 20 amino acid peptide obtained from the fungus *Trichoderma viride*, forms strongly voltage-dependent channels in planar lipid bilayers. Alamethicin is usually isolated as two components with the sequence Ac-Aib-Pro-Aib-Ala-Aib-Ala(Aib)-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol, where one component has Ala and the other Aib at position 6 (Pandey et al., 1977). In lipid bilayers, the peptide appears to be primarily α-helical (for example, see Vogel, 1987; Cascio and Wallace, 1988), and the conduction is thought to result from an aggregate of alamethicin monomers. Alamethicin is also observed to have a large molecular dipole that could interact with membrane electric fields (Schwarz and Savko, 1982; Yantorno et al., 1982). Several models have been proposed to account for the voltage-dependent activity of alamethicin. For example, in one model (Boheim et al., 1983), stable nonconductive aggregates of alamethicin are formed from α-helical monomers aligned in an antiparallel fashion. The application of a transmembrane potential, $\Delta\Psi$, produces a "flip-flop" in one of the monomers of the aggregate resulting in an unfavorable electrostatic repulsion between the helices. This repulsion then opens a channel in the center of the aggregate. In another model (Hall et al., 1984), alamethicin is bent in the closed state such that both the N and C terminal regions of the peptide lie on the same side of the bilayer. When aggregated, the monomers form a structure that resembles a β barrel. Application of a negative membrane potential forces the N terminal end of the peptide across the membrane and allows the β barrel to cross the bilayer forming an ion channel. In a third model (Schwarz et al., 1986; Rizzo et al., 1987), it is the partitioning of alamethicin to the bilayer that is voltage dependent. The

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0006-3495/91/08/380/09 $2.00

voltage-dependent conductance then arises simply from an accumulation and subsequent aggregation of alamethicin within the bilayer. In part, this third model is prompted by the high aqueous volume to membrane surface area normally present in a planar bilayer measurement. A characteristic feature of the first two models, not present in the third model, is that the voltage-dependent events leading to conduction result from structural changes that occur while the peptide is membrane bound.

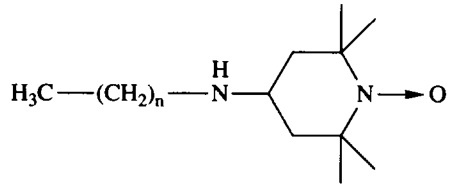
The work described here was carried out with the objective of delineating between the first two mechanisms and the third (voltage-dependent partitioning) mechanism described above. Because conditions for performing electrical measurements in vesicles can be arranged with virtually all of the alamethicin in a membrane-bound form, measurements of the current-voltage behavior in vesicles should permit an evaluation of the voltage-dependent partitioning mechanism. That is, the measurement of the activity of alamethicin in vesicles should allow a determination of the importance of the membrane-aqueous partitioning for the voltage-dependent gating activity of this peptide.

Another objective of the work described here was to obtain information on the conduction properties of alamethicin in vesicles. Although the ion channel activity of alamethicin has been well characterized in planar bilayer systems, the state of the peptide in these bilayers is virtually unknown. Structural studies on alamethicin are facilitated in vesicles; however, only a few measurements of activity have been performed in vesicles (Lau and Chan, 1976; Woolley and Deber, 1989) and these have not provided detailed information on the electrical activity of the peptide. A determination of the electrical properties of alamethicin in vesicle systems is necessary to facilitate structural studies that are directed at examining the mechanism of the peptide voltage dependence.

MATERIALS AND METHODS

Materials

Alamethicin was obtained from Sigma Chemical Co. (St. Louis, MO) and its chemical purity and sequence was characterized using HPLC and mass spectrometry. The peptide was used without further purification. Alamethicin from this source has Gln substituted for Glu at position 18 and also contains two main fractions with Ala or Aib at position 6 (see Archer et al., 1990). The ΔpH dependent, and the Δψ dependent spin probes (I and II, respectively), were synthesized after procedures similar to those described previously (Cafiso and Hubbell, 1978; Flewelling and Hubbell, 1986). Egg phosphatidylcholine (eggPC) was isolated from fresh hen eggs according to the procedure of Singleton et al. (1965), and was stored as a chloroform solution at −20°C under argon.



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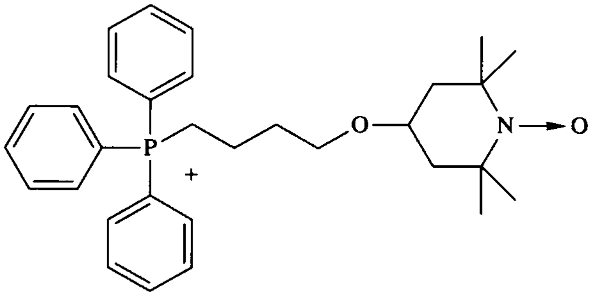

II

Preparation of vesicles and establishment of membrane potentials

Lipid suspensions for extrusion were prepared by measuring out the appropriate aliquot of the lipid/chloroform solution, removing the solvent under a stream of argon and drying the lipid overnight in a vacuum desiccator. The appropriate buffer solutions were added to the lipid, vortexed, and then freeze thawed five times in liquid nitrogen. This mixture was then extruded 10 times through 0.05 μM polycarbonate filters using a commercially available unit (Lipex Biomembranes, Inc., Vancouver, B.C.). Final lipid concentrations were determined using a modified Fiske-Subbarrow phosphate assay (Bartlett, 1959), and the trapped internal volume of the vesicle suspension was determined using a spin-label technique similar to one described previously (Todd et al., 1989). From measurements of the trapped volumes, the vesicles produced here had an effective diameter of 400 Å. Hence, this lipid preparation will be referred to as 400 Å extruded vesicles.[1]

Two procedures were used to establish transmembrane potentials across these lipid vesicles. In the first procedure, transmembrane pH gradients were used in the presence of the protonophores CCCP or S-13 to bring protons to rapid electrochemical equilibrium. In the second procedure, a transmembrane chemical potential difference of K^+ ions was brought to electrochemical equilibrium using valinomycin. To establish pH gradients, vesicles were formed in a buffered salt

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[1] Negative stain electron microscopy on these structures yielded larger structures with average diameters of 620 ± 150 Å. However, because the response of the spin probe depends solely on the effective trapped volume (See Cafiso and Hubbell, 1981), the effective diameter of 400 Å obtained from the entrapment studies is used here. The larger size obtained from electron microscopy is probably a result of some flattening of the vesicles on the electron microscope grid (note that half the circumference around a 400-Å vesicle is ~630 Å).

solution at low pH (125 mM Na_2SO_4, 25 mM MES, pH = 6.2) and then mixed with an isoosmotic solution of buffer at a higher pH (either MOPS, TES, or TAPS were used in the external solution depending upon the pH of the external solution). To form transmembrane K^+ gradients, vesicles were formed in a solution of 125 mM K_2SO_4, 10 mM MOPS, pH = 7, and passed down a gel filtration column (Bio-gel A0.5 M Bio-Rad Labs., Richmond, CA) that was equilibrated with a solution containing from 5 to 62.5 mM K^+. The external solution was osmotically balanced with the internal K^+ solution by the addition of Na_2SO_4.

EPR spectroscopy

EPR spectroscopy was carried out using either a Varian (Sunnyvale, CA) E-Line Century Series or a modified V-4500 series X-band spectrometer. Unless otherwise specified, spectra were recorded in a 70-μl quartz flat cell with a 1 G P-P cavity modulation and a 10-mW incident microwave power. Time-dependent experiments were initiated by rapidly mixing the sample preparation into the quartz flat cell using a computer-controlled mixing ram (System 1000 Chemical/Freeze Quench Apparatus; Update Instruments, Inc., Madison, WI). Processing and manipulation of the EPR spectra were carried out on an IBM compatible personal computer using the EPR Data Acquisition Package, Version 2.2 (Phillip D. Morse, II, University of Illinois College of Medicine, Urbana, IL).

Determination of transmembrane potentials

Membrane potentials were determined using the spin-labeled secondary amine or hydrophobic ion probe (I and II) shown above. These probes can be used to estimate electrical or pH gradients across lipid vesicles as described previously (Cafiso and Hubbell, 1981; Cafiso, 1989). Briefly, from the EPR spectra the ratio of membrane associated to aqueous probe, λ, can be determined. This partitioning is dependent upon the magnitude of these gradients and can be used to estimate their size from Eq. 1 below:

$$\frac{N_b}{N_f} = \left(\frac{V_{mi}}{V_i}\right) \frac{[K_i + K_o(V_{mo}/V_{mi})e^{\phi}]}{[1 + (V_o/V_i)e^{\phi}]} . \tag{1}$$

Here K_i and K_o are the internal and external probe binding constants, respectively, V_o/V_i is the ratio of external to internal aqueous vesicle volumes, and V_{mo}/V_{mi} is the ratio of volumes occupied by the probe on the external and internal membrane surface (this is approximately the ratio of membrane surface areas). The probe-binding constants and volume ratios were determined as described previously (Cafiso, 1989). In the case of the voltage-sensitive probe, ϕ is the reduced potential $zF\Delta\psi/RT$, and in the case of the pH sensitive probe $\phi = 2.3\Delta pH$.

As indicated above, protons were used to establish transmembrane potentials by bringing them to an electrochemical equilibrium with S-13 or CCCP. When protons are in electrochemical equlibrium across the membrane (that is, when $\Delta\psi = [RT/F] \ln x$, where $x \equiv [H^+]_{in}/[H^+]_{out}$) the partitioning of I as well as II can be used to estimate the membrane potential. Under these conditions, the initial ratio of internal to external $[H^+]$, x, decays slightly due to the number of protons needed to charge the membrane capacitance and the finite buffering capacity of the internal vesicle solution. To take this proton movement into account, and the small correction due to the presence of probe, the equation $f(x) = 0$ was solved to determine the expected values of x, where:

$$f(x) = \left(\frac{RT}{F}\right) \ln (x) + \left(\frac{c}{e}\right) \left[\frac{V_i K_a C_B (x[H^+]^i_{out} + [H^+]^i_{in})N}{(K_a + [H^+]^i_{in})(K_a + x[H^+]^i_{out})} + \frac{N_T}{1 + (V_o/V_i)(e_o/e_i)(1/x)}\right] . \tag{2}$$

Here, e is the electronic charge, c is the capacitance per vesicle, N is Avogadro's number, C_B is the concentration of buffer, the *initial* proton concentrations on the inside and outside of the vesicle are indicated by a superscript i, and N_T is the total number of probe molecules I or II per vesicle. The value for the specific membrane capacitance is taken as 0.9 $\mu F/cm^2$ (Montal and Mueller, 1972). In Eq. 2, $\epsilon_i = 1 + KV_{mi}/V_i$ and $\epsilon_o = 1 + KV_{mo}/V_o$, where the binding constants for the probe on the two surfaces are assumed to be approximately equal to K.

Determination of alamethicin induced ion fluxes

The ion currents produced by alamethicin were estimated under conditions of varied membrane potential and membrane concentration of peptide. In these experiments, CCCP or S-13 were added so that protons were maintained in an electrochemical equilibrium across the vesicle membrane. In most of the experiments described below, the pH gradient also serves as the driving force for the establishment of $\Delta\psi$. The determination of the current due to alamethicin assumes that protons are maintained in an electrochemical equilibrium, a condition that was experimentally verified under the conditions used here (see Results). The ion conduction produced by alamethicin was then measured by estimating the initial rate of change in ΔpH (hence the rate of change in $\Delta\psi$) using probe I, a rapidly equilibrating ΔpH sensitive spin probe. The voltage-sensitive spin probe, II, was not used for direct time-dependent voltage measurements, because of its slower transmembrane equilibration time.

In most of the experiments described here, membrane vesicles have an inside negative transmembrane potential that is produced by an inside acidic pH gradient. The addition of alamethicin promotes an inward flux of cations, i_{ala}, that diminishes the magnitude of $\Delta\psi$. Assuming that the buffer ions and anions (SO_4=) are not permeable, the change in the membrane potential is related to the inward flow of cations (Na^+) and the outward flow of protons by:

$$c(\partial\Delta\psi/\partial t) = -i_{ala} + i_{H^+} \tag{3}$$

again, c is the membrane capacitance, and i_{H^+} is the outward proton current. In the vesicle systems used here the external volumes are much larger than the vesicle internal volumes, and a change in ΔpH effectively represents a change in the internal pH (that is, $\partial\Delta pH = \partial pH\textit{in}$). The proton current, i_{H^+}, changes the internal pH in a manner determined by the internal buffer capacity, and the following expression can be derived for the current i_{ala}.

$$i_{ala}(t = 0) = \left[\frac{V_i 2.3 C_B K_a [H^+]_{in}}{(K_a + [H^+]_{in})^2} + \frac{2.3cRT}{F}\right] \left(\frac{\partial pH_i}{\partial t}\right)_{t=0} . \tag{4}$$

Here, $(\partial pHi/\partial t)_{t=0}$ is the initial rate of change in the internal vesicle pH which is equivalent to the initial change in ΔpH, $(\partial\Delta pH/\partial t)_{t=0}$. The quantities $(\partial pHi/\partial t)$ and i_{ala} are expressed in terms of initial rates, which are the experimentally determined values. The first term in this expression includes the contribution to the current made by the buffer capacity, and the second term includes the contribution due to the membrane capacitance.

RESULTS

Vesicle depolarization by alamethicin can be monitored using probe I

In the presence of S-13 or CCCP, transmembrane potentials are established across lipid vesicles and rapidly come to equilibrium with the pH gradient. Shown in Fig. 1 are the pH gradients and potentials that are estimated from the partitioning of probes I or II after the establishment of pH gradients across the extruded vesicles. The solid line represents the predicted values of potential or ΔpH based on Eq. 2. The data clearly indicates that probe II is monitoring a transmembrane potential that is in equilibrium with the expected pH gradient. Under the equilibrium conditions used here this data also indicates that probe I can be used as an effective measure of the membrane electrical potential. As expected, at 25 mM buffer the initial value of ΔpH drops slightly due to the proton flow needed to charge the membrane vesicle capacitance.

When alamethicin is added to these suspensions, either before or after the establishment of the membrane potential, a time-dependent decrease in the value of ΔpH is observed. Fig. 2*a* shows a recording of the high field resonance ($m_I = -1$) for the ΔpH-sensitive spin probe I after the rapid establishment of a pH gradient in the presence of alamethicin and S-13. The amplitude increase of this high-field resonance occurs as a result of a time-dependent decay in the value of ΔpH, and it is used to estimate $\partial pH_{in}/\partial t$ according to Eq. 1. Fig. 2*b* shows the ΔpH calculated from data in Fig. 2*a* using Eq. 1. Because Na^+ is not at electrochemical equilibrium in the vesicle system, it is likely that the addition of alamethicin promotes an inward flux of this cation. The decay in ΔpH (and Δψ) occurs to maintain protons in an electrochemical equilibrium. From Eq. 4, above, this change in internal pH yields the initial inward cation current, i_{ala}, produced by alamethicin. For the data shown in Fig. 2, this current is 21.5 nA/cm^{-2}.

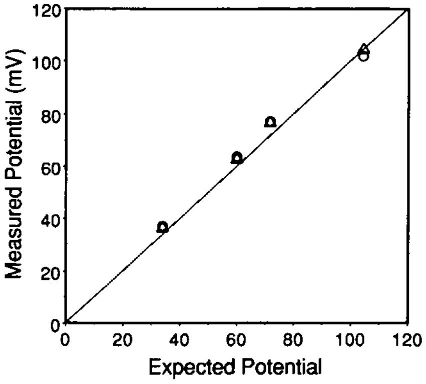

FIGURE 1 An electrochemical equilibrium of protons is demonstrated in extruded egg PC vesicles in the presence of S-13. Membrane potentials predicted using Eq. 2 are plotted versus the pH gradients and potentials calculated from the phase equilibria of the spin-labeled amine (Δ) and phosphonium probes (O), I and II, respectively. The solid line simply represents an agreement between the expected and experimental values. In these samples, eggPC was at a concentration of 15.7 mg/ml and S-13 was at a concentration of 5 μM. Probes I and II were both used at concentrations 20 μM.

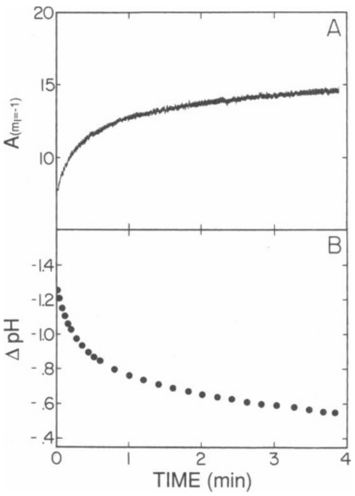

FIGURE 2 (*A*) A recording of the high-field resonance ($m_I = -1$) amplitude of probe I in the presence of eggPC vesicles (12.3 mg/ml eggPC) after the establishment of a pH gradient of 1.3. Alamethicin was added to the vesicles before the experiment at a concentration of 3 μM or 2.1 peptides/vesicles. (*B*) Values of the transmembrane pH gradient calculated from the data in part *A* using Eq. 1. The initial rate of the decay in the pH gradient can be used to calculate the ion flow through the alamethicin channel as described in the text. For the data shown here, the current can be calculated using Eq. 4 and is ~21.5 namp/cm^2 at a membrane voltage of −78.8 mV.

Protonophores CCCP or S-13 maintain protons in an electrochemical equilibrium

An important assumption in the analysis of this data is that the protonophores, S-13 or CCCP maintain protons in an electrochemical equilibrium. Thus, the proton current due to the protonophore must have the capacity to exceed that due to alamethicin. To test this assumption, the dependence of $\partial pH_{in}/\partial t$ upon the concentration of the protonophore was examined under the highest levels of alamethicin and most negative membrane potentials used. The data in Fig. 3 are plots of the initial rate of $\partial pH in/\partial t$ vs. S-13 concentration. At low levels of

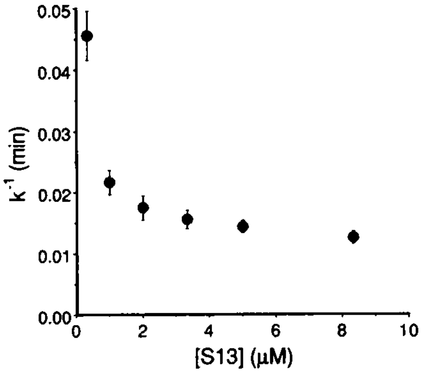

FIGURE 3 The rate of decay in ΔpH is examined as a function of the concentration of the protonophore S-13. The experiment is similar to the experiment described in Fig. 2 with alamethicin at a concentration of 6.7 μM (or 3.85 peptides/vesicle) and an initial transmembrane potential of -110 mV. Above 2 μM S-13, the rate of decay in ΔpH is relatively independent of S-13 concentration. This result indicates that under the most extreme conditions used, protons can be maintained in electrochemical equilibrium.

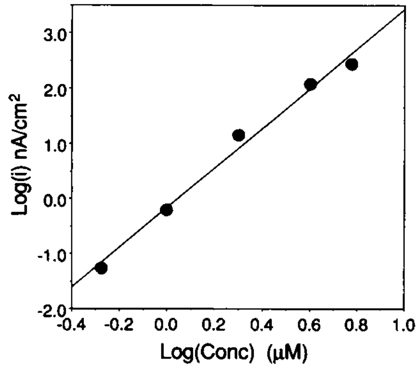

FIGURE 4 A log-log plot of the alamethicin-induced current vs. the concentration of alamethicin (●). The current is measured in 400 Å extruded vesicles at a lipid concentration of 12.5 mg/ml, and is calculated (see Methods) using Eq. 4. This current is expected to represent an inward flux of Na^+ ions through the alamethicin channel. The line represents a fit to the data points ($R = 0.988$) and has a slope of 3.6.

S-13, the rates are highly dependent upon the protonophore concentration, and protons are probably not at equilibrium. However, above 2 μM S-13 the rates are virtually independent of the protonophore concentration, indicating that proton movement is no longer rate limiting in these vesicle systems. The ability of CCCP to maintain a proton electrochemical equilibrium was also tested. Although it is less active than S-13, the levels of CCCP used here (4 μM) were sufficient to maintain the proton equilibrium. For most of the experiments described here, S-13 was used at a concentration of 5 μM. The rate of proton transport by the carriers was also examined by measuring the rate of change in the proton gradient, $\partial \Delta pH/\partial t$, after the establishment of the gradient in the absence of alamethicin. In this case a small number of protons flow, charging the vesicle capacitance and diminishing the initial value of ΔpH. At the concentrations of protonophore used in our experiments, these carriers bring protons to electrochemical equilibrium faster than could be detected with our current experimental arrangement (~ 50 ms).

Ion currents depend on the fourth power of the alamethicin concentration

The dependence of the inward cation current, i_{ala}, was examined as a function of the alamethicin concentration. Shown in Fig. 4 are the initial ion currents, calculated using Eq. 4, measured over a range of alamethicin concentrations. The data in Fig. 4 is plotted on a log–log plot and the solid line represents a linear least squares best fit of this data. The data is fit well with a line having a slope of ~ 4 and indicates that over the concentration range examined here the current increases approximately with the fourth power of the peptide concentration. This dependence changes with the membrane potential showing a slightly higher concentration dependence at lower inside negative membrane potentials. Plots, such as those in Fig. 4, were used previously to determine the molecularity of gramicidin and colicin (Bruggemann and Kayalar, 1986). The data shown here provides a strong indication that the molecularity of the alamethicin channel giving rise to the conductance in vesicles is 4.[2]

Current-voltage curve for alamethicin currents is highly nonlinear

When the voltage dependence of the alamethicin-induced current is examined as a function of the membrane voltage, a highly nonlinear current-voltage curve is found. Fig. 5 shows the initial alamethicin-induced currents that are obtained when vesicles are polarized to different values of $\Delta\Psi$ using a range of ΔpH values. The procedure for obtaining these currents is identical to the procedure used to obtain the data in Fig. 1. The lipid concentration used for the experiment shown in Fig. 5

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[2] If alamethicin is not monomeric in solution (for example, if it were dimerized in aqueous solution), the molecularity indicated in Fig. 4 would represent an underestimate of actual channel molecularity. Measurements using a spin-labeled derivative of alamethicin indicate that it is monomeric in aqueous solution under the conditions used here (Archer et al., 1990).

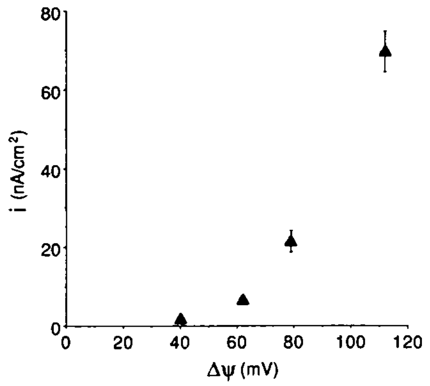

FIGURE 5 A current-voltage curve for alamethicin incorporated into 400 Å extruded vesicles (12.3 mg/ml eggPC). Under the conditions of this experiment, alamethicin is at a concentration of 1.2 peptides/vesicle (3 μM) and all the peptide is membrane bound. Transmembrane potentials and a proton electrochemical equilibrium were established and maintained using S-13. The currents were calculated using Eq. 4 and represent the inward Na^+ current after the establishment of Δψ.

was 12.3 mg/ml and direct binding measurements using optical techniques (Schwarz et al., 1986) or spin-labels (Archer et al., 1990) indicate that virtually all the alamethicin is membrane bound at lipid concentrations >1 mg/ml.

The current-voltage behavior was examined using a second procedure described above (see Methods) by polarizing membranes with a K^+ gradient and valinomycin. The initial net ion current induced by alamethicin is plotted as a function of ΔΨ and is shown in Fig. 6. The data is qualitatively similar to that shown in Fig. 5 and also indicates that alamethicin in vesicles has a highly nonlinear current-voltage behavior. Because of the different experimental conditions, the currents that are plotted in Fig. 6 represent a net ion current and are not quantitatively comparable to those in Fig. 5.

After the addition of alamethicin to polarized lipid vesicles, the vesicles depolarize and rapidly reach a steady-state potential that is maintained for many minutes. Shown in Fig. 7 are plots of the potential versus time after the addition of alamethicin as a function of the initial membrane potential. In spite of the different initial voltages, these samples approach steady-state potentials that are relatively similar. This behavior is expected if the conductance is strongly voltage dependent and provides additional evidence for a voltage-dependent gating of alamethicin in vesicles.

The current voltage behavior produced by alamethicin was examined at several membrane concentrations of the peptide. Over the limited range of concentrations examined (from 2 to 4 μM), the shapes of the current-voltage curves did not change. That is, curves obtained at different protein/lipid ratios could be superimposed by multiplying the data by a constant value.

Alamethicin activity rapidly exchanges between vesicles

To test for the exchange of alamethicin between vesicles, a population of vesicles that contained alamethicin (and had been allowed to depolarize) was injected into a second population of vesicles that did not contain alamethicin and was fully polarized. A subsequent decay

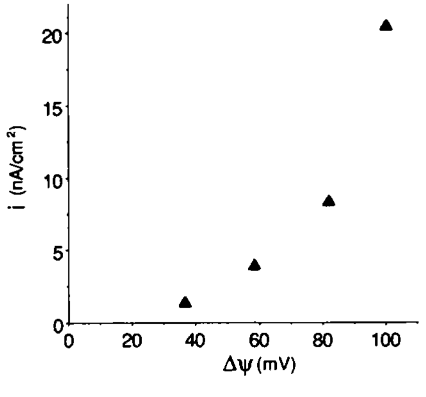

FIGURE 6 A current-voltage curve for alamethicin incorporated into 400 Å extruded vesicles (12.3 mg/ml). The membrane potential is established using K^+ valiomycin (see text), and the current is monitored using probe I in the presence of 10 μM CCCP. Alamethicin is at a concentration of 1.5 peptides/vesicles (4 μM) and all the peptide is membrane associated. The current represents the net current flow across the vesicle membrane.

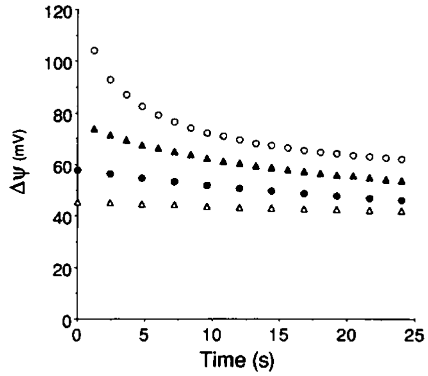

FIGURE 7 Plots of the potential vs. time after the establishment of a transmembrane potential across 400 Å extruded vesicles (12.3 mg/ml) that contain alamethicin at a concentration of 3 μM (1.2 peptides/vesicles): (○) −112 mV; (▲) −78 mv; (●) −58 mV; and (△) −46 mV. The potentials are obtained with probe I and Eq. 1, using S-13 to establish a proton electrochemical equilibrium.

in ΔpH (and ΔΨ) is observed, indicating that the population which did not previously contain alamethicin was depolarized by the peptide bound to the first population. When this data was analyzed assuming two populations of vesicles, the currents and potentials reached for the second vesicle population were identical to those shown in Figs. 2, 5, and 7. This observation indicates that alamethicin exchanges rapidly between vesicles on the time scale of the voltage and ionic changes measured here.

DISCUSSION

Transmembrane potentials were estimated in the vesicle systems described here by the use of a probe that is sensitive to the transmembrane pH gradient, ΔpH. Because this probe responds rapidly to changes in ΔpH and is not rate limited on the time scales studied here, changes in the pH gradient could be monitored within the time resolution of our instrumentation.[3] To estimate the transmembrane potential from ΔpH, protons must be in an electrochemical equilibrium during the experiment, a condition that is experimentally demonstrated by the data shown in Fig. 3. The current due to the carriers CCCP and S-13 is sufficient under the conditions used here to maintain protons in an electrochemical equilibrium.

In vesicles, alamethicin clearly exhibits a highly nonlinear current-voltage behavior, seen in Figs. 5, 6, and 7, that cannot be accounted for by diffusion across a simple barrier.[4] Although this voltage dependence is similar to that seen in planar bilayers, the voltage dependence is not as steep. At a fixed peptide concentration, V_e is the voltage required to produce a change in conductance of *e*-fold, and this value for alamethicin in planar bilayers is reported to be 4 mV (Hall et al., 1984). In the vesicle measurements described here, this value is closer to 20 mV. Several important differences between vesicle and planar bilayer measurements are discussed below that are possible sources for this difference.

The data in Figs. 5, 6, and 7 was taken under conditions where all the peptide was membrane bound; thus, the concentration of peptide in the membrane remained constant over the voltage range examined. A voltage-dependent binding of alamethicin was recently proposed to account for its voltage gating in planar bilayers (Schwarz et al., 1986; Rizzo et al., 1987). Clearly, the results presented here do not support this model and indicate that a voltage-dependent gating can occur in the absence of a voltage-dependent partitioning. That is, membrane bound alamethicin can undergo voltage-dependent structural rearrangements leading to gating of the alamethicin channel. The results presented here do not rule out the possibility of a voltage-dependent partitioning for this peptide; conceivably, there might be several processes leading to the overall voltage dependence seen in planar bilayers. However, measurements that were recently made with a spin-labeled derivative of alamethicin in isolated chloroplasts also show no evidence for a voltage-dependent partitioning (Wille et al., 1989). This result is consistent with the simplest interpretation of our data.

In the measurements described above, a large population of vesicles is being examined, typically on the order of 10^{15} vesicles/ml. In the interpretation of this data, it is assumed that the measured potentials approximate a mean potential for the vesicle population. However, because of the relatively low peptide concentrations being used (and the expectation that several alamethicin monomers must aggregate to form a channel) a slow exchange of the peptide between vesicles could result in two vesicle populations. For example, the steady-state potentials that are reached in Fig. 7 could be the average of a vesicle population that is completely depolarized and a population that retains the initial membrane potential. In addition, the kinetic data shown in Fig. 2 could be influenced by the rate of peptide exchange between vesicles. There are several observations which provide strong evidence that the vesicle measurements are not limited or influenced by a slow exchange of alamethicin between vesicles. First, when a spin-labeled alamethicin derivative is used to measure the rate of exchange of this peptide between vesicles, the process is rapid with an apparent rate of 2–4 s^{-1} (Archer, 1990). This is consistent with the previous findings obtained using a fluorescent derivative of alamethicin where the dissociation rate of alamethicin is $\sim 9\ s^{-1}$ (Schwarz et al., 1987). In addition, the shape of the current-voltage curve is independent of alamethicin concentration over the range examined here. If the kinetics of alamethicin exchange were rate limiting, the shape of the I-V curve would be strongly concentration dependent. Finally,

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[3] The ΔpH sensitive probe, I, equilibrates rapidly with transmembrane pH gradients when used near a neutral pH range. Under the conditions of our experiments, the approach of this probe to equilibrium cannot be measured and is faster than 10 ms (for a description and estimate of the rate constants, see Cafiso and Hubbell, 1978). Probe I was selected for these measurements, rather than a potential-sensitive hydrophobic ion probe, because of the need for a probe that could follow the rapid electrical changes produced by alamethicin in vesicles.

[4] A nonlinear current-voltage behavior is expected for the diffusion of any ion that passes over one or several barriers that are located within the membrane interior. However, the nonlinearity that is observed in the current-voltage behavior in Fig. 5 is much greater than can be accounted for by a simple diffusion process.

alamethicin can be delivered to a suspension of polarized vesicles in several ways: it can be added from solution during mixing in the EPR spectrometer, incubated with lipid vesicles before addition to polarized vesicles, or incorporated into vesicles before the establishment of a membrane potential. In each case, the rates of depolarization that are measured using probe I are identical, within experimental error.

In general, the behavior of alamethicin observed here compares with that seen in planar bilayers: the current has both a strong voltage and concentration dependence. However, there are some interesting differences that deserve comment. For the vesicle data obtained here, the ion currents increase with a constant fourth power concentration dependence. This dependence is less than that observed in planar bilayer systems, where the currents have been reported to depend on the sixth to the tenth power of the alamethicin concentration. These results imply a lower molecularity in vesicle systems. In addition, the concentration dependence remains constant in vesicles (at least over the limited concentration range examined). In planar bilayer systems this concentration dependence varies, and the single channel data indicate that the molecularity of the channel increases with concentration (Boheim and Kolb, 1978). There are several possible reasons for these differences. In the vesicle systems used here, all the alamethicin is membrane bound, and this is probably not the case in the planar bilayers, where both solution- and membrane-bound alamethicin are present. Because the binding of alamethicin is known to increase as the peptide concentration increases (Schwarz et al., 1986), the membrane bound concentration of alamethicin will not necessarily increase linearly with the total peptide concentration in the planar bilayer experiment. This could result in a higher estimate of the channel molecularity. Another important difference between the two systems is that planar bilayer experiments are usually carried out under steady-state conditions. That is, the peptide is allowed to come to equilibrium under constant voltage and ionic conditions. In the vesicle measurements this is not possible due to the relatively small vesicle size and hence the limited number of ions in the vesicle interior.

The currents produced by alamethicin in vesicles could be compared with the currents reported for planar bilayers. From the membrane area, aqueous volumes and binding constant of alamethicin in vesicles, the currents at similar membrane concentrations of peptide were compared. Most planar bilayer studies on alamethicin yielded currents that were about an order of magnitude higher than those obtained in vesicles (see for example, Eisenberg et al. 1973). However, the published data yielded a wide range of comparisons and some planar bilayer studies actually yielded currents smaller than those obtained here (see for example, R. J. Cherry et al., 1972). Given the differences between the two systems and the variability seen even among the planar bilayer experiments, the differences seen between planar bilayers and vesicles may not be significant.

In conclusion, the ion currents promoted by alamethicin in lipid vesicles have been examined as a function of voltage and concentration. Alamethicin produces a highly nonlinear current voltage curve similar to that seen in planar bilayer systems under conditions where the peptide remains entirely membrane associated. This suggests that a voltage-dependent partitioning of alamethicin does not account for its voltage-dependent gating in planar bilayers. The alamethicin-induced currents also vary approximately with the fourth power of the concentration. This suggests that four alamethicin monomers form the active ion-conductive channel in vesicle systems.

We would like to acknowledge Dr. Donald F. Hunt and members of his research group who carried out the mass spectrometry on alamethicin.

This work was supported by a grant from the National Institutes of Health (GM-35215) and a grant from the National Science Foundation (BNS 8908692).

Received for publication 19 September 1990 and in final form 19 April 1991.

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