Inactivation of the Alamethicin-Induced Conductance Caused by Quaternary Ammonium Ions and Local Anesthetics

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ABSTRACT Long alkyl chain quaternary ammonium ions (QA), the local anesthetics (LA) tetracaine and lidocaine, imipramine, and pancuronium cause inactivation of the alamethicin-induced conductance in lipid bilayer membranes. The alamethicin-induced conductance undergoes inactivation only when these amphipathic compounds are added to the side containing alamethicin. The concentration of QA required to cause a given amount of inactivation depends on the length of the hydrocarbon chain and follows the sequence C9 > C10 > C12 > C14. LA and imipramine, in contrast to QA or pancuronium, are able to promote appreciable inactivation only if the pH of the alamethicin-free side is equal to or lower than the pK of these compounds. The membrane permeability to QA, LA, or imipramine is directly proportional to the alamethicin-induced conductance and is larger than the one for potassium. The observed steady state and time-course of the inactivation are well described by a model similar to that proposed by Heyer et al. (1976, J. Gen. Physiol. 67:703-729) and extended for any value of the diffuse double layer potential and for LA and imipramine. In this model QA, LA, or imipramine are able to permeate through the membrane only when the alamethicin-induced conductance is turned on. The amphipathic compounds then bind to the other membrane surface, changing the transmembrane potential and turning the conductance off. For a given concentration of QA, LA, or imipramine the extent of inactivation depends on two factors: first, the binding characteristics of these compounds to the membrane surface and second, their ability to permeate through the membrane when the alamethicin-induced conductance is turned on. The several possible mechanisms of permeation of the amphipathic molecules tested are discussed.

INTRODUCTION

In excitable cells ionic permeability is regulated by the opening and closing of channels that are highly voltage sensitive. A property that is common to several voltage-dependent channels is inactivation. A prime example of inactivation is provided by the sodium channel in nerve (Hodgkin and Huxley, 1952; Bezanilla and Armstrong, 1977). In response to membrane depolarization the membrane sodium conductance reaches a peak and then decreases with a time constant of milliseconds. Although the potassium conductance in nerve does not inactivate in the same time span as the sodium system does, it presents a long term inactivation upon prolonged depolarization (Ehrenstein and Gilbert, 1966).
Moreover, Armstrong (1971, 1975) has found that when quaternary ammonium derivatives containing a long alkyl chain are present inside the axon, the time-course of the potassium current changes drastically: it rises to a peak and then decreases to a low value. Thus, in the presence of these compounds potassium channels “inactivate.”

In artificial lipid bilayer membranes several substances have been shown to induce voltage-dependent conductances; among these, excitability-inducing material (Lecar et al., 1975; Ehrenstein et al., 1978), suzukacillin (Boheim et al., 1976) and monazomycin (Heyer et al., 1976) also show inactivation under the appropriate conditions. In particular, Heyer et al. (1976 b) have shown that the voltage-dependent conductance induced by monazomycin “inactivates” when quaternary ammonium ions having long alkyl chains are introduced in the compartment containing monazomycin.

This paper concerns the interaction of quaternary ammonium ions, local anesthetics, and other tertiary amines with the alamethicin channel. Alamethicin, isolated from the fungus *Trichoderma viride*, is a peptide consisting of 19 amino acids, with a high content of hydrophobic residues. The structure of alamethicin, once thought to be cyclic (Payne et al., 1970) has recently been reported to be linear (Martin and Williams, 1976). When present in micromolar amounts on one side of a lipid bilayer membrane, alamethicin is able to induce a strong voltage-dependent conductance (Mueller and Rudin, 1968) that can mimic in many aspects those found in excitable cells. The membrane conductance is an exponential function of voltage, increasing an e-fold every 4-6 mV (Eisenberg et al., 1973; Gordon and Haydon, 1975; Gisin et al., 1977). Alamethicin-induced steady-state conductance ($G_s$) is also strongly dependent on both salt and polypeptide concentration and can be represented by (Eisenberg et al., 1973; Gordon and Haydon, 1975).

$$G_s \sim C_{ala} \cdot C_{salt} \cdot \exp(\gamma eV_m/kT),$$

where $C_{ala}$ and $C_{salt}$ are respectively the alamethicin and salt concentrations, $e$ is the electronic charge, $V_m$ is the transmembrane potential, and $k$ and $T$ have their usual meanings. The parameters $\alpha$, $\beta$, and $\gamma$ depend to a certain extent on membrane composition. For membranes made from phosphatidylethanolamine-decane (Eisenberg et al., 1973) or from glycerolmonoooleate-hexadecane (Gordon and Haydon, 1975), the data can be described with $\alpha \approx 9 \pm 1$, $\beta = 4 \pm 1$, and $\gamma = 6$. There is strong evidence that alamethicin-induced conductance arises as a consequence of ion channel formation through the membrane. The main evidence supporting this conclusion comes from records of conductance fluctuations taken at low alamethicin concentrations. Conductance fluctuations appear in bursts and each burst has several conductance levels, which are nonintegral multiples of each other and which always appear in a sequential order (Gordon and Haydon, 1972; Eisenberg et al., 1973; Boheim, 1974).

We present here evidence that alamethicin-induced conductance in lipid bilayers undergoes inactivation when quaternary ammonium ions, local anesthetics, or other pharmacologically important quaternary ammonium compounds and tertiary amines are present in the compartment containing alamethicin. The mechanism of this interaction can tell us a good deal about the
architecture of the alamethicin channel and is relevant to the understanding of inactivation in biological membranes. The present data indicates that the inactivation is caused by a change in the transmembrane potential promoted by a flux of the inactivating compound through the membrane, and that this flux is directly associated with the opening of the alamethicin channels.

MATERIALS AND METHODS

Membrane Formation
Except in the case where asymmetric membranes were used (see below) all membranes were formed from a mixture of glycerolmonoooleate (GMO) and phosphatidylserine (PS) in a ratio of GMO:PS of 2:1 (wt/wt). This lipid mixture combines the advantages of having a negative surface potential, and having fairly fast alamethicin kinetics. Lipids were stored at -50°C in CHCl₃ solutions. A portion of this solution was evaporated with a stream of nitrogen, and redissolved in pentane at a concentration of 12.5 mg/ml. Pentane solutions were prepared fresh every day.

Membranes were formed by the method of Montal and Mueller (1972). Two chambers each with a 3-ml capacity were separated by a thin (12-µm) Teflon partition (Fluorofilm, Dielectrix Corp., Farmingdale, N.Y.) in which a small hole (4.5 × 10⁻⁴ cm² area) was punched. The hole was punched by using a hypodermic needle (27 gauge) which was filed down to have a flat tip and then sharpened as a bore. The hole in the partition was treated with a 5% solution of squalene in pentane. The pools of the chamber had a surface area of 4 cm². Monolayers were spread on the surface of the electrolyte solution with 10 µl of the appropriate lipid solution. Membranes were formed by adding solution simultaneously to both compartments, and by raising the liquid until the hole was completely covered. As indicated by their high specific capacitance (0.75 µF/cm²) and their low compressibility, membranes formed by pretreating the hole with squalene are effectively “solvent-free” (see also White, 1978). Membrane conductance was usually ≤10⁻⁸ S/cm². All experiments were performed at 22 ± 1°C.

Nonactin Experiments
Membranes were formed in 20 mM KCl and nonactin was added to both sides of the membrane to a final concentration of 10⁻⁶ M. After a period of 15 min the zero potential conductance was measured. A small portion of a concentrated LiCl solution was then added to both sides of the membrane. After the membrane conductance reached its new steady level, a further addition of LiCl was made, and so forth. In the experiments where the nonactin-induced conductance was measured as a function of the concentration of QA, LA, imipramine, or pancuronium, membranes were formed in 0.1 M KCl. The amphipathic compounds were added to both sides of the membrane in aqueous solutions of various concentrations.

Measurements of Voltage-Dependent Capacitance
The voltage-dependent part of the membrane capacitance can be used to measure the difference in the surface potentials between the two sides of an asymmetric membrane (Alvarez and Latorre, 1978). Asymmetric membranes were formed by spreading at the air-water interface one monolayer consisting of GMO/PS in a 2:1 ratio by weight and the other consisting of GMO/PS in the same ratio. The voltage-dependent part of the capacitance was also measured in the membranes where QA or Ca⁺⁺ was added to only one side of a symmetric GMO/PS membrane.
Alamethicin Experiments

In all of the experiments performed with alamethicin, current measurements were not begun until 1 h after the addition of the alamethicin. In all experiments, alamethicin was added to only one side of the membrane. This side of the membrane will be referred to as the cis side, and the other side, which does not contain alamethicin will be referred to as the trans side.

In order to obtain the dependence of the conductance on alamethicin concentration, membranes were formed in 0.1 M KCl and a given amount of alamethicin was added to one compartment. After 1 h, the conductance was measured and another small addition of alamethicin was made. This procedure was repeated after each new addition of alamethicin. In order to measure the dependence of the conductance on the salt concentration, small additions of 3 M KCl were added symmetrically to the membrane, and the conductance was measured. To observe inactivation, QA or LA were added to the cis side of the membrane. When the pH in these experiments was changed, phosphate or acetate buffers were used. To measure the binding constants for QA or LA, the QA or LA were added to the trans side of the membrane. The conductance-voltage curve in this case was determined by using short (150-ms) pulses. The reason for this was to prevent the QA or LA from flowing down its concentration gradient to the near side of the membrane and changing the surface potential on the cis side.

Direct Measurements of the Membrane Permeability to Nonyltrimethylammonium (C₉) and to Dodecyltrimethylammonium (C₁₂)

Two types of experiments were performed to measure the permeability of QA directly. In the first type of experiment, we measured the current carried by C₉. This was done by measuring the conductance of the single channels of alamethicin at low KCl concentration (30 mM) both in the presence and in the absence of 0.5 mM C₉ on both sides of the membrane. The difference in conductance, after correcting for the change in surface potential due to C₉, was taken as being the conductance due to C₉ ions. The permeability of the C₉ could be calculated from this conductance.

In the second type of experiment we measured the C₁₂ flux using [³H]C₁₂ with one tritium-labeled methyl group. The labeled C₁₂ was added to the cis side of the membrane and a voltage was applied which held the current constant at 20 nA. At intervals of 10 min, the entire trans chamber was perfused three times and the solutions were collected. Scintillation cocktail (Aquasol-2, New England Nuclear, Boston, Mass.) was added to form a gel, and the samples were counted on a scintillation counter. A 50-μl aliquot of the near side of the membrane was collected to determine the specific activity of the hot side.

Electrical Measurements

In all experiments, potentials were applied using a pulse generator built in our laboratory. The cis side is defined as the side containing alamethicin, and positive current is the flow of cations from the cis to the trans side of the membrane. Currents were measured using large (>2 cm²) Ag/AgCl electrodes, which were connected to a wideband, fast-settling operational amplifier (AD41K or AD48K, Analog Devices, Inc., Norwood, Mass.).

In all experiments dealing with the kinetics of inactivation we used a Biomation 810 transient recorder to capture the current pulse and store it in digitized form. The pulse was then transferred to a Nicolet 1070 signal averager (Nicolet Instrument Corp., Madison, Wis.). Several pulses could be averaged in the Nicolet 1070 to improve signal-to-noise ratio. The data was then stored on tape. Calculations of the time-course of the
kinetics were performed on a Hewlett-Packard 9825A calculator (Hewlett-Packard Co., Palo Alto, Calif.). The typical step sizes in the iterations were 1 ms for time and 2 μm for distance.

Materials

The GMO used in these experiments was obtained from Nu-Chek Prep (Elysian, Minn.) and was dissolved in chloroform. Bovine PS was obtained from Supelco, Inc. (Bellefonte, Pa.). Both were used without further purification. Pentane was obtained from Eastman Organic Chemicals (Rochester, N.Y.). The QA compounds were all tri-methyl alkyl bromides or chlorides, and were obtained from Eastman Organic Chemicals. Tetracaine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, Mo.). Lidocaine hydrochloride was purchased from Astra Pharmaceutical Products Inc. (Worcester, Mass.). Imipramine hydrochloride (5-[3-(dimethylamino)propyl]-10, 11 dihydro-5H-di-benz[bf]azepine chloride) was the gift of D. C. Tosteson, Harvard Medical School, Boston. Pancuronium (3α, 17β diacetoxy 2β, 16β-dipiperidino-5α-androstanedimethobromide) was the gift of Jay Yeh, Northwestern University Medical School, Chicago. The tritiated C12 was the gift of Alan Finkelstein, Albert Einstein College of Medicine, Bronx, N.Y. All water used in these experiments was passed through an ion exchanger and double-distilled. Alamethicin was obtained from The Upjohn Co., (Kalamazoo, Mich.) and was used without further purification.

The chemical structures for the long chain quaternary ammonium ions (QA), tetracaine (TC), lidocaine (LC), imipramine (Imp) and pancuronium (PC) are given in Fig. 1. For the quaternary ammonium ions, we will follow Armstrong's (1971) nomenclature, e.g., nonyltrimethylammonium is designated C9.

RESULTS

Characteristics of Alamethicin-Induced Conductance in GMO/PS Bilayers

STEADY-STATE CHARACTERISTICS Because alamethicin has not been well characterized in membranes made by opposition of two monolayers, we report here the main characteristics of the alamethicin-induced conductance in this system. When alamethicin is added on one side only of a GMO/PS bilayer, the conductance increases exponentially with applied potential (see inset of Fig. 2). The conductance increases roughly e-fold with a change in voltage of 5.7 mV. Fig. 2 shows a typical current-voltage curve for a GMO/PS membrane. The curve possesses two branches for positive and negative potentials and is highly asymmetric. This is similar to the results obtained using glycerolmonooleate-cholesterol-hexadecane membranes (Gordon and Haydon, 1975) but is in contrast with the action of alamethicin in phosphatidylethanolamine-decane membranes which exhibit no branch for negative potentials, even at very large negative potentials (Eisenberg et al., 1973).

The alamethicin-induced conductance also depends on alamethicin and salt concentration. Confining ourselves to the branch of the current-voltage curve obtained for positive potentials, we have found that for GMO/PS membranes the parameters α and β of Eq. 1 are respectively 7.0 ± 0.8 and 3 ± 1.2. Thus, in our experimental conditions, the alamethicin-induced steady-state conductance is well described by:

\[ G_{ss} \sim C_{ss}^{2} C_{init}^{exp(4.4eV_{m}/kT)}. \]
FIGURE 1. Chemical structures of the quaternary ammonium ions, local anesthetics, imipramine, and pancuronium.

FIGURE 2. Current-voltage relation for a GMO/PS membrane treated with alamethicin. The membrane was formed in 0.1 M KCl and had an initial conductance of $3 \times 10^{-8}$ S/cm$^2$. Alamethicin was added to the cis side only to a concentration of $6.7 \times 10^{-7}$ g/ml. The sign of the potential is given for the alamethicin-containing compartment. The inset shows the steady-state conductance (logarithmic scale) as a function of applied potential.
KINETICS When the applied voltage across an alamethicin-treated membrane is suddenly changed from zero to a new value, the current increases monotonically to a steady value (Fig. 3) and remains at this value for as long as the voltage is kept constant. In GMO/PS membranes the time-course of the current is well described by a single exponential that is essentially complete in ~200 ms. The time constant for this process depends on the alamethicin concentration and the potential, but in all the range of potentials and alamethicin concentrations used in the present work, the steady-state current is reached in <200 ms. A double exponential time-course has been described by Boheim and Kolb (1978) in dioleoyllecithin membranes. The faster relaxation was identified with a shift in the probability distribution of the conductance states, and the slower relaxation has been ascribed by Boheim and Kolb to a change in the total number of conducting channels. In the experiments described here it is reasonable to assume that the time-course observed in GMO/PS membranes corresponds to the slow relaxation observed by Boheim and Kolb. It is noteworthy that the slow time constant found by Boheim and Kolb in dioleoyllecithin membranes is about 10 times slower than those reported here for GMO/PS membranes at the same temperature, alamethicin concentration, and potential. Our experience indicates that increasing the molar ratio of PS in GMO/PS membranes tends to give much slower time constants.
Determination of the Surface Charge Density of GMO/PS Membranes

Because surface charge density and hence surface potential will be of crucial importance in explaining the effect of QA or LA on the alamethicin-induced conductance, we have measured the surface charge density of GMO/PS membranes with two independent methods. The first method involves the use of the ion carrier nonactin as a probe of membrane surface potential (McLaughlin et al., 1970) in symmetric GMO/PS membranes, and the second involves measurements of the voltage-dependent part of the capacitance in membranes in which one monolayer consists of GMO/PE and the other of GMO/PS (Alvarez and Latorre, 1978).

In the first case the surface charge density, \( \sigma_o \), was found by measuring the nonactin-induced zero voltage conductance \( G(0) \) as a function of ionic strength. The ionic strength was varied by adding LiCl to both sides of the membrane, keeping the KCl concentration constant. \( \sigma_o \) can be obtained by fitting the experimental points with the equation (McLaughlin et al., 1970):

\[
G(0) = KC\left[\alpha + \sqrt{\alpha^2 + 1}\right]^2,
\]

where \( K \) is a constant, \( C \) the potassium concentration, and \( \alpha = 136\sqrt{\sigma_o^3/\sum C_i} \), \( \sum C_i \) being the total molar concentration of all ionic species.

The second method consists in measuring the voltage dependence of the membrane capacitance. In the case of an asymmetric bilayer in which only one side is charged the voltage-dependent part of the capacitance is well described by a parabola which has a minimum at \( V = -\Psi_s \) (Alvarez and Latorre, 1978). \( \sigma_o \) was calculated using the Gouy-Chapman equation for uni-inivalent electrolytes (Aveyard and Haydon, 1973; McLaughlin, 1977):

\[
\sigma_o = \frac{\sqrt{C}}{A} \sinh \frac{\Psi_s}{2kT},
\]

where \( A \) is a constant, \( C \) is the electrolyte concentration, and \( \Psi_s \) the surface potential. With the nonactin method and through Eq. 3 and with the capacitance method and through Eq. 4 we have obtained a \( \sigma_o \) of 0.0040 and 0.0044 charges/\( \text{Å}^2 \), respectively (Table I). Hence at 0.1 M KCl, for example, \( \Psi_s \) will be \(-70 \text{ mV}\). A surface charge density of this magnitude is in very good agreement with the respective areas per molecule, 40 \( \text{Å}^2 \) for GMO (Hladky and Haydon, 1973) and 60 \( \text{Å}^2 \) for PS (McLaughlin, 1977).

Alamethicin Is a Good Probe of Surface Potential

If the KCl concentration is the same at both sides of the membrane and a divalent cation is added to the alamethicin-free (trans) side, the log of the conductance-voltage curve is shifted to the right along the voltage axis (Fig. 4).
We can understand this shift if we consider the voltage dependence of the alamethicin-induced conductance:

$$G_{ss} \sim \exp\left(4.4eV_m/kT\right),$$

where

$$V_m = V_{applied} + \Delta \Psi,$$

and $\Delta \Psi$ is the difference between the surface potentials of the cis and trans sides of the membrane. It is clear from Eqs. 5 and 6 that $G_{ss}$ will decrease if the surface potential at the trans side is made more positive. Addition of Ca$^{2+}$ to the trans side has precisely this effect (Fig. 4). In order to calculate the changes in the surface potential at the trans side promoted by the successive additions of Ca$^{2+}$, we have used the Gouy-Chapman equation for mixed electrolytes (Aveyard and Haydon, 1973):

$$\sigma_0 = \left\{2\varepsilon_r\varepsilon_0 kT \sum_i N_i(\infty)[\exp(-z_i e\Psi_0/kT) - 1]\right\}^{1/2},$$

TABLE I

<table>
<thead>
<tr>
<th>Method</th>
<th>$\sigma_0$ (charge/Å²)</th>
</tr>
</thead>
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<tr>
<td>Voltage-dependent capacitance*</td>
<td>0.0044</td>
</tr>
<tr>
<td>Nonactin-K⁺</td>
<td>0.0040</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>0.0047</td>
</tr>
</tbody>
</table>

*$\sigma_0$ was obtained by measuring the voltage-dependent capacitance of an asymmetric GMO/PS membrane in 0.1 M KCl. The voltage dependence of the capacitance of a symmetric bilayer is well described by a parabola with the minimum at the origin. The voltage-dependent capacitance of an asymmetric bilayer with one monolayer made of GMO/PE in a ratio 2:1 by weight and the other made of GMO/PS in the same ratio was measured. In this case the parabola is shifted and the minimum occurs at -70 mV. $\sigma_0$ was calculated by means of Eq. 4 given in the text. Descriptions of how $\sigma_0$ was obtained when nonactin - K⁺ complex or alamethicin was used as probes of the diffuse double layer potential are given in the text.

where $\varepsilon_r$ is the dielectric constant of the solution, $\varepsilon_0$ the permittivity of free space, $N_i(\infty)$ the bulk concentration of the ionic species $i$, and $z_i$ the valence of the $i$th ion. It is possible to determine the value of $\sigma_0$ from the shift in the conductance-voltage curve shown in Fig. 4. For example, in 0.1 M KCl the shift in potential obtained by changing the Ca$^{2+}$ concentration from 7 mM to 14 mM is 5.2 mV. Solving Eq. 7 numerically with these values we obtain $\sigma_0 = 1$ charge/225 Å² in a good agreement with those obtained using nonactin as a probe or by measurements of the voltage-dependent capacitance (see Table I).

Alamethicin-Induced Conductance Is Modified by QA

ADDITION OF QA TO THE TRANS SIDE  Fig. 5a shows the effect of C$_{12}$ added to the trans side on the alamethicin-induced conductance. Addition of C$_{12}$ to this
compartment shifted the log of the conductance-voltage curve to the right along the voltage axis.

It is well known that ionic surfactants of the type of C\textsubscript{12} adsorb (bind) reversibly to membrane surfaces (Haydon and Myers, 1973; Heyer et al., 1976)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The effect of Ca\textsuperscript{++} added to the trans side. The GMO/PS membrane was formed in 0.3 M KCl. Alamethicin concentration was 6.7 \times 10^{-7} g/ml. The steady-state conductance was measured as a function of applied potential at the Ca\textsuperscript{++} concentration indicated in the figure.}
\end{figure}

b). Inasmuch as C\textsubscript{12} is positively charged, then its binding to the trans surface of a GMO/PS membrane will decrease the surface potential making that side more positive. A shift to the right in the log conductance-voltage curve is obtained for the same reasons that Ca\textsuperscript{++} promotes a shift. C\textsubscript{12}, however, is different from Ca\textsuperscript{++} (see Discussion) in that it binds to the membrane. The change in surface charge density induced by QA, \( \Delta \sigma \), is given by (Heyer et al., 1976 b; see Appendix I):

\[ \Delta \sigma = \beta [QA]^+, \]  

where \( \beta \) is the binding constant of QA to the membrane and \([QA]^+\) is the concentration of the long alkyl chain quaternary ammonium ion in solution at the trans membrane surface.

Since the initial \( \sigma_0 \) of a GMO/PS membrane is known, \( \sigma \) can be obtained for the present experimental conditions by means of Eq. 4 and the shifts of the log of the conductance-voltage curve promoted by the addition of QA in the trans
Figure 5. (a) The effect of C_{12} added to the trans side. The GMO/PS membrane was formed in 0.1 M KCl. Alamethicin was added to the cis side to a concentration of 5 \times 10^{-7} g/ml. The steady-state G_{sc}V characteristics were measured at the various concentrations of C_{12} indicated in the figure. (b) Shift in the trans surface potential as a function of C_{12} concentration. The shifts are those of section a. The curve is drawn according to Eq. 8 with \beta = 100 \text{ charges/Å}^2 \text{M} and \sigma_0 = 0.044 \text{ charges/Å}^2. (c) Plot of C_{12} binding vs. the interfacial concentration of C_{12}. C_{12} binding was calculated from the surface potential shifts given in section a. The interfacial concentration of C_{12} was calculated from Eq. 9. The slope of the straight line is 100 \text{ charges/Å}^2 \text{M}. 
side. The concentration of QA in solution at the membrane surface is given by a Boltzmann distribution:

$$[QA]_t = [QA]_{t bulk} \exp(-e\Psi_t/kT).$$  \hspace{1cm} (9)

Fig. 5c shows a plot of the change in surface charge density promoted by $C_{12}$ against $[QA]_t$. It can be seen that, as predicted from Eq. 8, a straight line is obtained with a slope of 100 charges/Å²M corresponding to the $C_{12}$ binding constant $\beta_{c_{12}}$. Knowing $\sigma_o$ and $\beta$ for a given QA, the surface potential at the trans side can be obtained by means of Eq. 8a given in Appendix I. The solid lines in Fig. 5a were drawn from Eq. 8a. We have also determined $\beta$ independently from the measurements of the variation in the nonactin-induced zero potential conductance by changing the concentration of $C_{12}$ at both sides of the membrane, and from the changes in the voltage-dependent capacitance when $C_{12}$ is added to one side only. In Table II we summarized the value of $\beta$ for the different compounds tested obtained with these three independent methods. The agreement among the methods is good and strengthens the hypothesis that the shifts in the log of the conductance-voltage curve are due to a change in the trans membrane surface potential. Similar results as those shown in Fig. 5a were obtained for the other compounds listed in Table II. As expected from their binding constants, higher concentrations were required to promote the same shift in the conductance-voltage relationship when going from $C_{10}$ to $C_{12}$.

**TABLE II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\beta_{non}$</th>
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<td>charges/Å²M</td>
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</tr>
<tr>
<td>$C_9$</td>
<td>1.79 ± 0.40</td>
<td>1.77 ± 0.59</td>
<td>1.25 ± 0.2</td>
</tr>
<tr>
<td>$C_{10}$</td>
<td>4.7 ± 1.2</td>
<td>3.3</td>
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</tr>
<tr>
<td>$C_{12}$</td>
<td>91 ± 22</td>
<td>69 ± 20</td>
<td>91 ± 22</td>
</tr>
<tr>
<td>$C_{16}$</td>
<td>10,000</td>
<td></td>
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</tr>
<tr>
<td>TC</td>
<td>1.5 ± 0.1</td>
<td></td>
<td>0.95</td>
</tr>
<tr>
<td>LC</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imp</td>
<td>2.5 ± 0.5</td>
<td>2.2 ± 0.4</td>
<td></td>
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</table>

* $\beta_{non}$ was calculated from nonactin experiments.

† $\beta_{non}$ was obtained from measurement of the voltage-dependent capacitance measured in GMO/PS membranes where the compound was added to one side only.

§ $\beta_{non}$ was calculated from alamethicin experiments.

**ADDITION OF QA TO THE CIS SIDE.** If long-chain quaternary ammonium compounds are added to the side of the membrane containing alamethicin, the time-course of the alamethicin-induced current is dramatically changed. As can be seen in Fig. 6, at low conductances (small applied potentials) the time-course of the current is quite similar to the one shown in Fig. 3, but at higher conductances the current rises to a peak and then falls to a new and smaller steady-state value. We obtained the steady-state conductance-voltage curves from current records by taking the value of the current at long times after the voltage pulse has been applied ($t \geq 10$ s). The log $G_{st}V$ curve in the presence of
Ca\textsuperscript{2+} (Fig. 7) is shifted to lower potentials and bends towards the voltage axis at high conductances. This bending is due to the particular shape that the time-course of the current has in the presence of C\textsubscript{12} on the cis side. The experimental points fall on the dotted line for low membrane conductances, but at higher conductances the alamethicin "inactivates" with time and therefore the experimental value of conductance is smaller than the peak conductance given by the dotted lines. The shift to the left along the voltage axis of the log $G_s-V$ curve when C\textsubscript{12} is added to the cis side is due to several factors acting simultaneously. The reduction of the surface potential on the cis side will have three effects. First, a transmembrane potential is established. Second, the interfacial concentration of alamethicin is increased. This is because the main component of natural alamethicin is the so-called F.30 fraction containing one titratable group with a pK of 5.5 (Payne et al., 1970), which most likely corresponds to the free carboxyl group. Therefore, a fraction of the alamethicin is negatively charged and is sensitive to the surface potential. The increase in alamethicin concentration at the membrane surface will depend on the pH of the solution in the cis compartment and on the amount of F.30 fraction present in our sample. Third, the interfacial concentration of K\textsuperscript{+} is reduced.\textsuperscript{1} The two first factors tend to shift the $G_s-V$ curve to the left along the voltage axis, whereas a reduction in the K\textsuperscript{+} concentration at the surface formally shifts the $G_s-V$ curve to the right. In order to make an estimation of the magnitude of the shift promoted by the addition of C\textsubscript{12} to the cis side we need to know the amount of alamethicin charged at pH 5.5. According to Martin and Williams (1976) natural alamethicin contains about 85% of the F.30 fraction and about 12% of the fraction containing an amide group in place of the carboxyl group. It is possible, therefore, on the basis of Martin and Williams' data and the known alamethicin pK to calculate

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure6.png}
\caption{Inactivation of the alamethicin-induced current by addition of C\textsubscript{12} to the cis side of the same membrane used in Fig. 3. Traces a-e show progressively larger potential steps: 54, 58, 62, 66, and 70 mV. C\textsubscript{12} concentration was 8 x 10^{-8} M. The other experimental conditions are the same as those of Fig. 3.}
\end{figure}

\textsuperscript{1} Because our membranes are negatively charged, the interfacial K\textsuperscript{+} concentration is much larger than that of Cl\textsuperscript{-}, therefore the increase in Cl\textsuperscript{-} concentration at the membrane surface due to the decrease in surface potential will have only a minor effect on the overall alamethicin-induced conductance.
the amount of charged alamethicin present in solution at any given pH. Using
the data shown in Fig. 5 b in order to obtain the actual decrease in membrane
surface potential at the cis side and the interfacial concentration of alamethicin
and potassium, the voltage shift of the $G_{sc}V$ curve can be calculated from Eq. 2.
For the experiment shown in Fig. 7 the calculated voltage shifts are 6.7, 11, and
16.5 mV for 2, 6, and 10 μM $C_{12}$, respectively, in good agreement with the
experimental values.\(^2\)

![Figure 7](image)

**Figure 7.** Steady-state conductance-voltage characteristics for a membrane in
the presence of alamethicin (●), and in the presence of alamethicin and various
amounts of $C_{12}$ in the cis compartment (○). Membrane formed in 0.1 M KCl.
Alamethicin was added to a concentration of 2.7 × 10^{-7} g/ml. The curves fitting
the open circles are drawn according to Eq. 9a with $b$ equal to 2 M/S, 6 M/S, and 10
M/S for the $C_{12}$ concentration of 2 μM, 6 μM, and 10 μM, respectively.

\(^2\) Because of the effects of alamethicin and potassium concentration on the conductance, the shift in
the log $G_{sc}V$ curve when QA is added to the cis side is not equal to the actual change in surface
potential. However, changing the trans surface potential by adding QA to the trans side has no effect
on these factors. Therefore, for any given concentration of QA added to the cis side, the actual
change in surface potential can be calculated as if the same amount would have been added to the
trans side.

\(^3\) Even with the largest $C_{12}$ concentration shown in Fig. 10, the total interfacial concentration of
alamethicin is increased by only 10%. On the other hand, the interfacial concentration of $K^+$ is
decreased by 3.3-fold. Thus, in spite of the large power dependence of the conductance on
alamethicin concentration, the shifts to the left of the log $G_{sc}V$ curves observed in Fig. 10 are
mainly determined by the changes in surface potential and interfacial $K^+$ concentration.
C₁₆, C₁₀, and C₉ are also able to promote inactivation (the results for C₁₀ and C₉ are shown in Fig. 8a and b, respectively), but the concentration required to induce the same amount of inactivation at a given conductance follows the order C₁₆ < C₁₂ < C₁₀ < C₉. Heyer et al. (1976b) have proposed that inactivation of the monazomycin channel induced by QA is due to the ability of QA to pass through the monazomycin channel and bind to the opposite surface of the membrane. The same hypothesis can explain the effect of QA on the alamethicin-induced conductance. The support of this conclusion is twofold. First, when QA is added to the trans side of a membrane already containing QA and alamethicin on the cis side, the process of inactivation disappears. Second, the recovery of inactivation is greatly speeded by stirring of the trans side only. As shown in Fig. 9 the recovery from inactivation takes about 1 min when stirring is kept “on” during all the course of the experiment. If stirring is stopped on the trans side the recovery time increases to several minutes.

The results shown in Figs. 6–8 can be described by the known behavior of alamethicin (Eq. 2) and by two properties of the QA compounds, namely, the strength of their binding to the membrane surface and their permeability.
through the alamethicin channel. Basically, in response to a voltage pulse, alamethicin channels open and QA molecules on the cis side of the membrane are able to permeate through the membrane. Once the QA ions reach the trans side of the membrane they bind to that side for a finite period of time. This binding will change the transmembrane potential (the surface potential of the trans side of the membrane becomes less negative). As the trans membrane potential decreases, the alamethicin conductance turns off and the current inactivates. The log $G_{ss} V$ curves shown in Figs. 7 and 8 a and b have been fitted by using Eq. 9 a given in Appendix I. The parameter $\beta$ for the different QA is listed in Table II. According to Eq. 5 a given in Appendix I it is possible to calculate the parameter $b$ if $G_{ss}$ and the [QA]$^t$ are known. As pointed out by Heyer et al. (1976 b) $b$ can be calculated from a single point of, for example, Fig. 8, since the [QA]$^t$ able to generate a given shift in potential can be considered equal to a concentration of QA bulk which, if added directly to the trans compartment, would generate the same shift. For a given point towards the right of the dotted line in Fig. 7, [QA]$^t$ can be then calculated from Fig. 5 b.

**Effect of LA on the Alamethicin-Induced Conductance**

It is implicit in the model presented above and in the Appendix I that an amphipathic tertiary amine able to bind to the membrane surface and to permeate through the alamethicin channel will promote inactivation when present at the cis side if the tertiary nitrogen is in the cationic form. Local anesthetics such as tetracaine (TC) are known to bind to the membrane surface due to their amphipathic characteristics (Muller and Finkelstein, 1972). Table II shows the $\beta$ for tetracaine in GMO/PS membranes as determined by different methods.
Fig. 10 shows the effect of pH on the alamethicin-induced conductance when TC was present at the cis side only. It can be seen in Fig. 10 that TC is able to promote inactivation of the alamethicin conductance when the pH is 5.5. At this pH TC is almost completely charged ($pK_{TC} = 8.5$). At pH 8.0, on the other hand, TC is only partially charged and is able to promote only a slight inactivation. At pH 9 TC is mostly uncharged and shows no inactivation. We think that when the pH of both compartments is raised from 5.5 to 8.0 the uncharged TC permeates through the membrane much faster, decreasing the TC concentration gradient and causing in turn a decrease in the degree of inactivation. To check this point, we performed experiments in which the pH of the trans side only was raised. With the trans side at pH 8 (30 mM phosphate buffer) and the cis side at pH 5.5 (30 mM acetate buffer), the degree of inactivation was intermediate: less than that shown in the left hand record in Fig. 10, but more than that shown in the right hand record of Fig. 10. This suggests that some of the effect of high pH is due to uncharged TC permeating through the membrane, and that some of it is due to the neutralization of the ionized TC on the trans side of the membrane after it permeates through the channel. In either case, we conclude that only the charged form of TC is active, in agreement with the present model for inactivation of alamethicin-induced current.

Lidocaine, another local anesthetic, is also able to promote inactivation, but higher concentrations than TC are required to promote similar inactivation because the binding of lidocaine is much weaker (see Table II).
Other Compounds Able to Promote Inactivation

Our model is applicable to other tertiary amines as well. In Fig. 11 we show that imipramine (Imp), an antidepressant drug, behaves like TC with respect to inducing inactivation of the alamethicin conductance. As we can see in Table II Imp has a binding constant even larger than TC.

Pancuronium (PC), another interesting and important pharmacological agent, is able to inactivate alamethicin-induced conductance at concentrations as low as 200 µM. Pancuronium is a bis quaternary ammonium compound with a rigid steroidal structure. PC selectively blocks the sodium channels in squid axon without any apparent effect on the potassium channels (Yeh and Narahashi, 1977).

DISCUSSION

Alamethicin Is Sensitive to the Intramembrane Potential Difference

The voltage-dependent ion conductance induced by alamethicin has been the subject of several exhaustive studies (Eisenberg et al., 1973; Boheim, 1974; Gordon and Haydon, 1975). On the other hand, the effect on alamethicin-induced conductance of the diffuse double layer potential due to the presence of charged lipids in the membrane has received little attention. In this regard Gordon and Haydon (1976) have reported that symmetrically placed charges on the two membrane surfaces do not have any effect on the movement of alamethicin, and Eisenberg (1972) found that the alamethicin-induced conductance observed in membranes with positive, neutral, or negative surface charge have similar behavior, once corrections for the ion concentration at the surface are made. However, asymmetric surface potentials do affect alamethicin gating. Our experiments where Ca++ was added to the aqueous phase not containing
alamethicin indicate that the conductance induced by this polypeptide is sensitive to the transmembrane potential even when this potential cannot be measured by external electrodes. Although binding of Ca$^{++}$ to the PS molecules cannot be ruled out with the present data, we found that the large shifts of the log $G_{se}V$ curves are accounted for by the diffuse double layer theory. The agreement between the divalent cation effects using the cation carrier nonactin and alamethicin as probes of membrane surface potential further strengthen these conclusions. The effect of QA or LA when added to the trans side of the membrane can then be interpreted in the framework of the diffuse double layer theory if we take into account the binding of these compounds to the membrane. Binding constants for C$_{12}$, C$_{18}$, and C$_{9}$ have been reported previously by Heyer et al. (1976 b). They used phosphatidylglycerol-cholesterol membranes and the binding constants they obtained are consistently smaller than those reported in the present work by a factor of about 4. As discussed by Heyer et al., the values of the $\beta$'s are about 10$^3$-fold smaller than those reported at the oil-water interface by Haydon and Phillips (1958). Further, Heyer et al. suggested that this difference in binding constants was due to the presence of cholesterol in their lipid bilayers. Inasmuch as our bilayers did not contain cholesterol and the binding constants still differed from phosphatidylglycerol-cholesterol membranes by only a factor of four, this explanation seems unlikely.

We have shown that lidocaine and imipramine also bind to the membrane surface but that the binding is weaker than in the case of the QA tested here. This weaker binding may be due to the fact that the hydrophobic domains of these molecules are mainly composed of cyclic hydrocarbons. Cyclic aromatic hydrocarbons are less hydrophobic than other molecules with the same surface area or than alkanes with the same number of carbon atoms (Tanford, 1973; 1978).

Kinetics of Inactivation

All of the amphipathic molecules tested bind reversibly to the membrane. Therefore, the existence of an unstirred layer allows inactivation to occur. As we show in Appendix I (see also Heyer et al., 1976 b) it is possible to treat the steady-state aspects of inactivation quantitatively thanks to the presence of the unstirred layer at the trans side of the membrane. In Appendix II we show that we can calculate the time dependence of the concentration gradient of the inactivating molecule in the unstirred layer. Given this concentration as a function of time, we can calculate the current as a function of time and treat the kinetic aspects of inactivation quantitatively. Fig. 12 shows that the time-course of inactivation is in fact predicted by Eq. 6 b of Appendix II.

Inactivation and Channel Formation

Heyer et al. (1976 b) found that QA are able to induce inactivation of the monazomycin-induced conductance and arrived at the conclusion that QA transport was intimately associated with the monazomycin channels. They also concluded that QA movement across the membrane due to association of monazomycin with QA is unlikely because the QA flux is thousands of times higher than the monazomycin flux across the membrane. The possibility that
QA is transported across the regions disrupted by the monazomycin monomers or aggregates is also unlikely because the QA permeability varies linearly with $G_{ss}$. We think that the transport of all the amphipathic molecules tested in the present work is closely related with the formation of alamethicin channels in the membrane for reasons similar to those given by Heyer et al. First, we consider the possibility of associations of the amphipathic cation to the alamethicin molecule. Pressman (1968) reported that alamethicin is able to bind cations. It is possible, then, that alamethicin binds the charged amphipathic molecule, dragging it across the bilayer. If an actual flux of alamethicin-amphipathic compound complexes across the membrane is taking place when the potential is applied, this would lead to a depletion of alamethicin monomers on the cis side of the membrane. Such a depletion of monomers was used by Heyer et al. (1976

![Graph](image)

**Figure 12.** Experimental time-course of inactivation of the alamethicin-induced current in the presence of $\mathrm{C}_{12}$ in the cis compartment (○). The line was fitted to the data using the model given in Appendix II with a steady-state current of 15.5 nA, $\beta = 80$ and $b = 10 \, \text{M} \, \text{s}$. Ala concentration was $6.7 \times 10^{-7} \, \text{g} / \text{ml}$. $\mathrm{C}_{12}$ concentration was $1 \times 10^{-5} \, \text{M}$. Applied potential was 65 mV.

(a) to explain the inactivation of monazomycin-induced conductance at large conductances. We have not been able to find inactivation of the alamethicin-induced conductance even at conductances as high as $5 \times 10^{-3} \, \text{S/cm}^2$. It seems unlikely, then, that any appreciable depletion of alamethicin from the membrane solution interface at the cis side takes place. Therefore, the possibility that compounds able to promote inactivation of the alamethicin-induced conductance can move across the membrane in association with alamethicin monomers seems unlikely. It can be argued that the amphipathic molecules are being transported by a carrier-like mechanism when alamethicin monomers

---

*Because alamethicin-induced conductance depends on the seventh power of the alamethicin concentration, inactivation is to be expected at large conductances if the membrane permeability to alamethicin is proportional to the alamethicin-induced conductance.*
“flip” through the membrane as a consequence of the applied field, without invoking any depletion of monomers from the cis side. Although this mechanism could explain the high fluxes of amphipathic compounds, it would not explain the proportionality between the permeability for these compounds and the voltage-dependent alamethicin-induced conductance. Second, we consider the possibility that QA or LA cross the membrane via regions disrupted by the alamethicin gating. Since, as in the case of monazomycin $P_{QA}$ or $P_{LA}$ varied linearly with $G_{ss}$, this possibility also seems unlikely.

For the reasons given above we consider it likely that the charged amphipathic compounds are either passing through the lumen of the alamethicin channel or through some membrane domain directly related with it. Heyer et al. (1976 b) proposed a model for the QA permeation through the monazomycin channel. In this model the charged end of the QA ion passes through the lumen of the channel, and the hydrophobic tail slides through the hydrophobic regions formed by the nonpolar domains of the monazomycin molecules. They based their model on two observations. First, not all amphipathic molecules are able to induce inactivation (i.e., they reported that tetracaine is not able to induce inactivation), so there must be some restrictions on the structure of the molecule. If the QA passed through some membrane domain directly related to the channel but not through the channel itself, such geometric restrictions would not be expected. Second, the permeability coefficient, $P_{QA}$, for the different QA’s increases as the alkyl chain length increases, implying that the molecule is not only in the lumen, but in intimate contact with the hydrophobic region of the membrane. We found that tetracaine is able to promote inactivation of the alamethicin-induced conductance. We also found that the permeability for QA ions, although about 40 times that of $K^+$, remains almost constant in going from $C_{12}$ to $C_9$ (Table III). The differences between alamethicin and monazomycin can be ascribed to differences in pore sizes, in that the conductance of the alamethicin channel is several orders of magnitude more than that of the monazomycin channel (Gordon and Haydon, 1972; Eisenberg et al., 1973; Boheim, 1974; Bamberg and Janko, 1976). However, we have found in GMO/PS membranes that tetracaine is able to cause inactivation of the monazomycin channel, in contrast to the results obtained by Heyer et al. (1976 b) in phosphatylglycerol-cholesterol membranes. Furthermore, the model of Heyer et al. does not allow the possibility that a compound like pancuronium with one charge localized at each end of the molecule will promote inactivation (a detailed report on the inactivation promoted by pancuronium will be given elsewhere). Since we have found that pancuronium is able to cause inactivation of the alamethicin channel, the model of Heyer et al. is not completely appropriate for this channel.5

Table III shows that for $C_{12}$, $P_{C_{12}}/P_K$ obtained from electrical measurements and measured directly with tracer flux experiments is about 30 times larger than

5 In GMO/PS membranes, pancuronium is not able to inactivate monazomycin-induced conductance (Donovan and Latorre, unpublished observations). So the model of Heyer et al. may still be appropriate for the monazomycin channel. Because pancuronium is a large $10 \times 18$ Å molecule, it is possible that it can permeate through the alamethicin channel but not through the smaller monazomycin channel.
Furthermore, for C₉, P_{C₉}/P_K also agreed when calculated from macroscopic conductance measurements and from experiments in which we measured the single channel conductance in the presence of C₉. Thus, we feel confident that indeed for all the compounds shown in Table I, their permeability is larger than the permeability of K⁺. If all these amphipathic ions permeate through the lumen of the channel, one would expect, due to their size, that their permeabilities would be much smaller than the permeability of K⁺. On the other hand, if the model of Heyer et al. (1976 b) is correct, one would not expect pancuronium-induced inactivation to occur, although we cannot exclude the possibility that QA ions and pancuronium permeate through different mechanisms. It is possible that QA ions pass through the alamethicin channel in accordance with the model of Heyer et al., and pancuronium crosses the modified region immediately adjacent to the channel.

### Table III

**Permeability Coefficients for QA and TC**

<table>
<thead>
<tr>
<th>Compound</th>
<th>From measurements of single channel conductance</th>
<th>From tracer flux of single channel conductance</th>
<th>From steady-state inactivation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₉</td>
<td>40</td>
<td>-</td>
<td>53</td>
</tr>
<tr>
<td>C₁₀</td>
<td>41</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C₁₂</td>
<td>37</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>TC</td>
<td>19</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* P_{compound}/P_K was calculated using the equation (Heyer et al., 1976 b),

\[
P_{\text{compound}}/P_K = \frac{b}{[\text{compound}]_{\text{cis}}} \frac{[K^+]_{\text{cis}}}{V} \frac{(DA)(1-e^{-\Delta x/RT})}{\Delta x},
\]

where \( b \) was obtained from the steady-state inactivation, \( D \) is the diffusion coefficient and was taken to be \( 6 \times 10^{-6} \text{ cm}^2/\text{s} \) (Blair and Kraus, 1958; Hille, 1977). \( \Delta x \) is the thickness of the unstirred layer and was equal to 100 \( \mu \text{m} \).

The mechanisms of channel formation appear to be very similar for both alamethicin and monazomycin (Bauman and Mueller, 1974; Boheim, 1974; Heyer et al., 1976 a). Thus, it is tempting to suggest that the mechanisms of permeation of large amphipathic molecules are also similar in both types of systems.

### Appendix I

The model we present here is adapted from Heyer et al. (1976 b), and generalized for small surface potentials. The model is that a charged amphipathic compound, C⁺, when added to the cis side of the membrane, binds to the surface and makes the surface potential less negative. This increases the transmembrane potential, increasing in turn the alamethicin-induced conductance. Now if QA or LA passes through the membrane (via the open alamethicin channels), it builds up a small but finite concentration at the

* The model will, of course, apply to pancuronium if the fact that this quaternary ammonium compound is divalent is taken into account.
far surface. This concentration builds up suddenly at first because of the time it takes for the QA and LA to diffuse through the unstirred layer. Then, as the system approaches a steady state, the flux through the unstirred layer equals the flux through the membrane. Therefore at steady state,

$$\Phi_m = \Phi_{ul},$$ \hspace{1cm} (1 a)

where $\Phi_m$ is the flux of QA or LA across the membrane and $\Phi_{ul}$ is the flux across the unstirred layers.

$\Phi_{ul}$ is simply

$$\Phi_{ul} = \frac{D_{C^+}A}{\Delta x} (\Psi_{trans})^\text{bulk} - [C^+]^t, \hspace{1cm} (2 a)$$

where $[C^+]^t$ is the concentration near the *trans* surface of the membrane, but outside the diffuse double layer potential where a linear concentration gradient may be assumed. $D_{C^+}$ is the diffusion constant of $C^+$, $A$ is the membrane area, and $\Delta x$ is the thickness of the unstirred layer. Since $[C^+]^\text{bulk} = 0,$

$$\Phi_{ul} = \frac{D_{C^+}A}{\Delta x} [C^+]^t. \hspace{1cm} (3 a)$$

Now, $\Phi_m$ should be proportional to the number of channels open, so

$$\Phi_m = b' G_{ss}, \hspace{1cm} (4 a)$$

where $b'$ is a constant of proportionality and is a function of the permeability and the concentration of $C^+.$

From Eqs. 1 a, 3 a, and 4 a we see that

$$[C^+]^t = b G_{ss}, \hspace{1cm} (5 a)$$

where $b = b' \Delta x / D_{C^+} A$.

The $[C^+]^t$ determines the change in the *trans* surface potential $\Psi_{st}$. This is derived starting with the Gouy-Chapman equation for a uni-univalent electrolyte at 20°C:

$$\sigma = \frac{\sqrt{C}}{136} \sinh \frac{\Psi_{st}}{50}, \hspace{1cm} (6 a)$$

where $\sigma$ is given in charges/Å² and $C$ is the concentration in moles/liter.

Since the surface charge density, $\sigma_s$, at the *trans* side of the membrane is given by

$$\sigma_s = \sigma_x + \beta [C^+]_x = \sigma_x + \beta [C^+] \exp(-\Psi_{st}/25), \hspace{1cm} (7 a)$$

then combining these equations and using the definition of the sinh function:

$$\sinh \frac{\Psi_{st}}{50} = \frac{e^{\Psi_{st}/50} - e^{-\Psi_{st}/50}}{2} = \frac{136}{\sqrt{C}} (\sigma_x + \beta [C^+] \exp(-\Psi_{st}/25)), \hspace{1cm} (8 a)$$

let $e^{-\Psi_{st}/50} = \chi,$

$$\frac{272}{\sqrt{C}} \beta [C^+] x^2 + A x^2 + \frac{272}{\sqrt{C}} \sigma_x x - 1 = 0, \hspace{1cm} (8 a)$$

$^7$ The parameters $b$ and $b'$ are also functions of voltage. Inasmuch as their dependence on voltage is much weaker than their dependence on $G_{ss}$, we have not taken into account this factor.
combining Eqs. 8a and 5a,

\[
\frac{272}{\sqrt{C}} \beta b G_{ss} x^2 + x^2 + \frac{272}{\sqrt{C}} \sigma_a x - 1 = 0, \tag{9a}
\]

which is an implicit equation relating \(G_{ss}\) and \(\Psi_{ss}\). \(b\) can be found by fitting Eq. 9a to the log \(G_{ss}\) vs. voltage curve.

**APPENDIX II**

**Calculation of Inactivation Kinetics**

Because, in our membranes, the intrinsic alamethicin kinetics are fast compared to the rate of change of the \([C^+]\) at the trans surface, the degree of inactivation at any time is determined by the \(C^+\) concentration at the trans surface of the membrane. Eq. 8a describes the relation between the \([C^+]\)' and inactivation. Calculating the current as a function of time, then, is a matter of calculating \([C^+]\)' as a function of time. This can be done by an iterative method based on the model in Fig. 13. Conceptually, the unstirred layer can be divided up into \(n\) slices, each with a width \(\delta x\). Each slice is thin enough that the concentration gradient across it approaches a constant. That is, the \([C^+]\) decreases linearly across it, so that Fick's first law of diffusion may be used. The concentration change of the \(i\)th slice during a time \(\Delta t\), then, is just the flux into the slice, \(m_{in}\), minus the flux out, \(m_{out}\), divided by the volume:

\[
\Delta C_i(t) = \frac{m_{in} - m_{out}}{V} \Delta t. \tag{1b}
\]

By Fick's first law:

\[
m_{in} = \frac{DA}{\delta x} (C_{i-1} - C_i); \tag{2b}
\]
From Eq. 1 b, 2 b, and 3 b,

$$\Delta C_i(t) = \frac{D}{(\delta x)^2} (C_{i-1} + C_{i+1} - 2C_i) \Delta t. \quad (4 b)$$

After a time $\Delta t$:

$$C_i(t + \Delta t) = C_i(t) + \Delta C_i(t).$$

From Eq. 4 b,

$$C_i(t + \Delta t) = C_i(t) + \frac{D}{(\delta x)^2} (C_{i-1} + C_{i+1} - 2C_i) \Delta t. \quad (5 b)$$

The flux into the first slice is $\Phi_m = b'G_{ss}$ where $b'$ is defined in Eq. 4 a. Then,

$$C_i(t + \Delta t) = C_i(t) + b'G_{ss} - \frac{D}{(\delta x)^2} (C_i - C_2) \Delta t.$$

To a first approximation, these equations can be used to calculate all the $C_i$'s with the boundary conditions: at $t \leq 0$, the current $I$, is 0, and $C_1 = C_2 = C_n = 0$. Then, at $t \geq 0$, $I$ has a finite value and $C^+$ accumulates in slice 1 for a time $\Delta t$. All the $C_n$ are then calculated, allowing some flux to occur. After the next increment of time, all the $C_n$ are recalculated in an iterative fashion. This method approaches the true situation, which is continuous and without slices, as the time increment $\Delta t$ and the thickness of the slice $\delta x$ go to zero. In our calculations, using a time increment of 1 ms and slice thickness of 2 $\mu$M led to insignificant error.

The concentration $C_1$ is equal to $[C^+]$. This concentration determines the degree of inactivation at any instant because a given $C^+$ concentration causes a given $\Delta \Psi$, across the membrane. This $\Delta \Psi$ can be calculated from Eq. 8 a. The conductance at any instant is decreased by the factor

$$\exp[-4.4 \Psi(t)/25].$$

Given this time-course of the inactivation, the overall time-course of the current is calculated by multiplying the normal current at any time by this factor. For times $> 1$ ms the normal time-course can be adequately described by a single exponential (see Fig. 3) described by

$$I(t) = I(\infty)[1 - \exp(-t/\tau)],$$

where $\tau = 25$ ms.

Bringing in the inactivation factors, the net time-course of the current is

$$I(t) = I(\infty)[1 - \exp(-t/\tau)]\exp[-4.4 \Psi(t)/25], \quad (66)$$

which has an ascending phase and a descending phase. Again, the $\Delta \Psi(t)$ is determined by $C_1 ([C^+]_1$) with Eq. 8 a. Because $\Delta \Psi(t)$ increases nonlinearly with time, the expected time-course of inactivation is not described by a single exponential.

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