# ALAMETHICIN ADSORPTION TO A PLANAR LIPID BILAYER

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ABSTRACT The effect of alamethicin and its derivatives on the voltage-dependent capacitance of phosphatidylethanolamine (squalane) membranes was measured using two different methods: lock-in detection and voltage pulse. Alamethicin and its derivatives modulate the voltage-dependent capacitance at voltages lower than the voltage at which alamethicin-induced conductance is detected. The magnitude and sign of this alamethicin-induced capacitance change depends on the aqueous alamethicin concentration and the kind of alamethicin used. Our experimental data can be interpreted as a potential-dependent pseudocapacitance associated with adsorbed alamethicin. Pseudocapacitance is expressed as a function of alamethicin charge, its concentration in the bathing solution and the applied electric field. The theory describes the dependence of the capacitance on applied voltage and alamethicin concentration. When alamethicin is neutral the theory predicts no change of the voltage-dependent capacitance with either sign of applied voltage. Experimental data are consistent with the model in which alamethicin molecules interact with each other while being adsorbed to the membrane surface. The energy of this interaction depends on the alamethicin concentration.

## INTRODUCTION

Ionic channels mediate many vital physiological processes, and it is clearly important to understand in detail at the molecular level how they work. Alamethicin is a small channel-forming peptide only 20 amino acids long (Reusser, 1967; Marshall and Balasubramanian, 1979; Balasubramanian et al., 1981); it is thus simple to modify it in ways which alter its function more or less predictably (Gisin et al., 1977; Hall et al., 1984). The crystal structure of alamethicin is known (Fox and Richards, 1982), and its properties and conformation in organic nonpolar solvents have been extensively investigated (Jung et al., 1975; Schwarz and Savko, 1982; Banerjee et al., 1983). These results indicate that alamethicin has a stable alpha-helical conformation of about 10 residues long beginning at the $NH_2$-terminus and at least in some solvent systems, a region of beta-sheet at the COOH-terminus.

Alamethicin induces a very voltage-dependent conductance in planar bilayers and in the membranes of some living cells (Mueller and Rudin, 1968; Eisenberg et al., 1973; Gordon and Haydon, 1972, 1975; Boheim, 1974; Latorre and Alvarez, 1981; Vodyanoy et al., 1982; Cahalan and Hall, 1982; Hall and Cahalan, 1982; Boheim et al., 1984). Because several alamethicin monomers must aggregate together to form a channel, its study provides direct information about the forces which hold channels together (Eisenberg et al., 1973; Kolb and Boheim, 1978; Hall et al., 1984).

Natural alamethicin is a mixture of peptide components. Several groups have synthesized the major component of this mixture and other derivatives which have different electrical properties (Gisin et al., 1977; Balasubramanian et al., 1981). Especially informative derivatives are Boc 2-20, which has a symmetrical current-voltage (I-V) curve, even when added to only one side of the membrane, and BG, which turns on with the opposite sign of voltage from natural alamethicin (Hall et al., 1984).

One difficulty in the study of alamethicin as a model for formation of channels in biological membranes has been obtaining information about the nonconducting state. We know how many channels form at a given voltage for a given aqueous concentration of alamethicin, but we do not have a very good idea of how many alamethicin molecules are adsorbed to the planar bilayer or of the physical state of the adsorbed molecules. Recent studies of alamethicin interaction with lipid vesicles using circular dichroism support the notion that alamethicin incorporates into the lipid phase to a significant extent (Schwarz et al., 1986).

Rizzo, Stankowski, and Schwarz (1987), using a general thermodynamic approach, have shown theoretically that the concentration dependence of the alamethicin-induced conductance could be explained by a voltage-dependent adsorption and aggregation of alamethicin mediated by interaction of the alamethicin dipole and an applied electrical field. Thus it is very important to understand how alamethicin adsorbs to the lipid bilayer.

The experiments reported here detect an effect of alamethicin adsorbed to the planar bilayer, but not forming channels. This effect is an alteration in the voltage-dependence of the membrane capacitance which depends on the surface concentration of adsorbed alamethicin or alamethicin analogue. The magnitude of the effect and the

sign of the voltage which induces it both depend on the analogue of alamethicin used.

## MATERIALS AND METHODS

The capacitance of lipid bilayers is voltage-dependent and increases with the square of the voltage (Alvarez and Latorre, 1978). We measured the voltage-dependent capacitance of lipid bilayers in the presence and absence of various analogues of alamethicin in two ways: by lock-in amplifier detection of the current moved by a small amplitude sine wave riding on a dc voltage and by taking the difference in the charge moved by a small test pulse riding on a voltage pedestal and by the same test pulse with no pedestal. The second method is that used by Alvarez and Latorre (1978).

Fig. 1 shows a block diagram of the lock-in measurement. A high-purity sine wave generator (General Radio 1309) provides a low amplitude sine wave to a summing amplifier (A1) where it is added to a dc voltage generated by a computer-controlled digital-to-analogue converter (DAC). The signal generator also provides a reference voltage to the lock-in amplifier (G&G Princeton Applied Research PAR 5101). The DAC applies a dc ramp to the summing amplifier, and the output of the summing amplifier drives both the membrane and a linear RC network adjusted to have the same electrical properties as the membrane at low voltages. The current outputs from the membrane and the RC network are subtracted by a differential amplifier, A4, the output of which is fed to the lock-in. The lock-in is tuned in phase with the imaginary part of the membrane impedance and thus detects the difference between the membrane current and the current through the RC network. The RC network is adjusted in each experiment so that this difference is zero at zero dc volts. Because the RC network is linear, the lock-in measures the change in reactive part of the membrane impedance with voltage.

We tested this apparatus using a variety of electrical circuits in the place of the membrane. These were: various RC networks with different series and parallel resistances, several different varactor diodes (whose capacitance depends on voltage), an RC network in series with the bilayer chamber, and its electrodes dipped in the aqueous solution in which the membranes were formed. All of these test circuits gave appropriate results. Most importantly the linear RC networks showed no change in capacitance with voltage. It was also shown that the phase separation was such that three orders of magnitude decrease in conductance produced about a 1% change in apparent capacitance.

We also applied the pulse technique of Alvarez and Latorre (1978) to measure the voltage-dependent capacitance of lipid bilayers. A block diagram of the apparatus used is shown in Fig. 2. The pulse protocol identical to the pulse protocol described by Alvarez and Latorre and a sample data set are shown in Fig. 3. A small test pulse riding on a dc pedestal is applied to the membrane and the transient current response is digitized and stored in a computer. Then the test pulse is applied alone, and the current response is digitized and subtracted from the current data obtained with the test pulse riding on the dc pedestal. The result is the difference in the current required to charge the membrane at zero volts and that required to charge it at the dc voltage of the pedestal. Dividing the integrated charge under the transient by the test pulse voltage gives the capacitance. Capacitance at zero volts (the minimum capacitance value on the capacitance-voltage curve) was always adjusted to the same reference level after each alamethicin addition. Thus our measurements detect only differences in capacitance between the capacitance at zero volts and that at other voltages.

The results obtained by the pulse method on both bare membranes and peptide-doped membranes agree with the results obtained by the lock-in method.

Data collection, pulse, and voltage ramp generation were under control

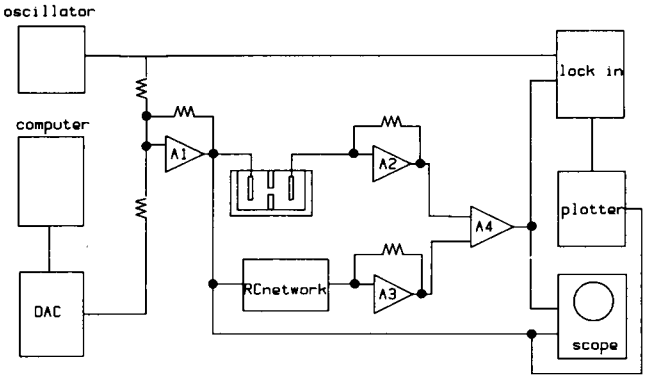


FIGURE 1 Block diagram of the setup used to measure capacitance changes by the sine wave method. The voltages from a high-purity sine wave oscillator and a computer-controlled digital to analogue converter (DAC) are summed and applied to the membrane and a linear RC network. The current through the RC network is subtracted from that through the membrane by differential amplifier (A4) whose output is applied to a lock-in amplifier. The computer-controlled DAC generates a voltage ramp which determines the DC voltage across the membrane and drives the X-axis of the X-Y recorder. The output of the lock-in amplifier drives the Y-axis thus producing a capacitance-voltage curve directly. (Actually the curve shows change in capacitance with voltage.)

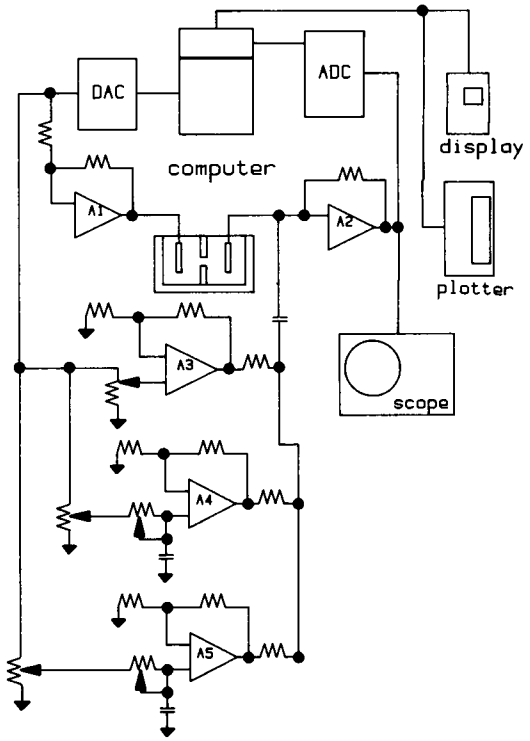


FIGURE 2 Block diagram of the setup used for measurements of membrane capacitance change by pulse method. Appropriate pulse sequences (see Fig. 3) are generated by computer-controlled DAC and applied to the membrane and a three-time constant compensation circuit. The current from the compensation circuit is applied to the summing point of the current amplifier and is thus subtracted from the capacitance transient of the membrane. The voltage output of the current transducer is digitized (12-bit 2-$\mu$s converter) and stored in a special memory cache 12 bit wide and 1,024 words long. Data in this cache is read into the computer by direct bus transfer and subtraction of test and controlled pulses is performed by the computer using floating point arithmetic under fortran control. Results can be displayed on the digital plotter (see Fig. 3).

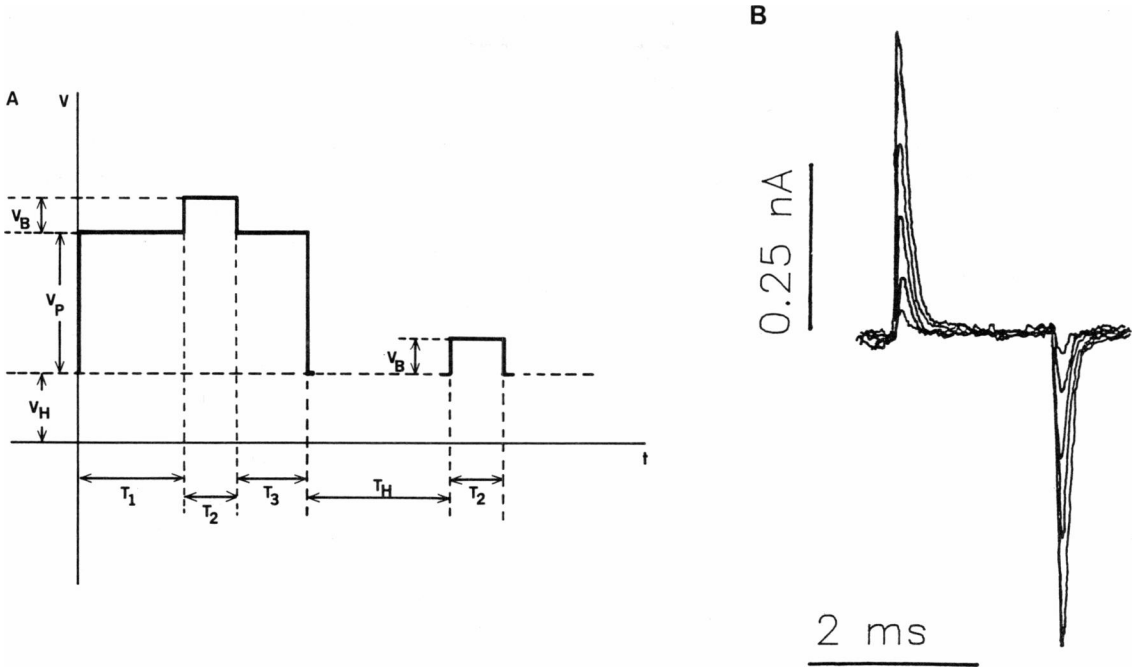


FIGURE 3 Membrane capacitance change pulse protocol and sample of the data. (*A*) A voltage pedestal ($V_P$) is applied to the membrane, and at the time $T_1$ later a small voltage blip $V_B$ is superimposed on the top of the pedestal. The voltage is returned to the holding potential and the blip voltage is reapplied. The current record of blip alone is subtracted from the current record of blip on top of pedestal, and the resulting record reflects the difference in charge necessary to charge the membrane to the blip voltage at zero volts and at the pedestal voltage. (*B*) Typical current record obtained using the pulse protocol of panel *A*. The membrane is phosphatidylethanolamine (squalane) ~500 pF of zero voltage capacitance in 1 M KCl at 20°C. The blip voltage is 20 mV, and the pedestal voltage starts at 50 mV and increases to 250 mV in 50-mV steps. Blip duration was 2 ms, and pedestal duration was 10 ms. Each trace is the average of 150 individual records.

of a Z 80 based computer system (Cromemco Z-2D, Cromemco, Cupertino, CA). Currents were measured and digitized by fast sample and hold (SHM-2, Datel, Inc., Mansfield, MA) driving a 12-bit analogue to digital converter (MAS-1202, Analog Devices, Inc., Norwood, MA). Current-voltage and capacitance-voltage curves were recorded on an X-Y recorder (HP7037A, Hewlett-Packard, Palo Also, CA).

Membranes were formed as described by Montal and Mueller (1972) and slightly modified by Vodyanoy et al. (1983). The membrane forming solution was phosphatidylethanolamine (PE) from *Escherichia coli* (Avanti Polar Lipids Inc., Birmingham, AL) in *n*-pentane (5 mg/ml). Squalane was used for the aperture pretreatment. *n*-pentane and salts were purchased from Mallincrodt Inc., St. Louis, MO). Squalane was purchased from Atomergic Chemicals Corp., Plainview, NY. Fraction 4 is the major component of high-pressure liquid chromatography (HPLC)–purified Upjohn alamethicin. Synthetic derivatives Boc2-20 and BG were prepared as described by Balasubramanian et al. (1981). For their structures see Hall et al. (1984). Alamethicin and its analogues were added to the membrane bathing solutions from methanolic or ethanolic stock solutions.

## RESULTS

We found, in agreement with Alvarez and Latorre (1978), that membranes in the absence of any of the alamethicin analogues have a capacitance which depends on the square of the voltage $V$ according to the formula

$$C(V) = C_0(1 + \beta V^2),$$

where $C_0$ is membrane capacitance at zero volts and $\beta$ is a constant of proportionality which is $\sim 0.021\ V^{-2}$.

When natural alamethicin (or Fraction 4) is added to one side of a phosphatidylethanolamine (squalane) membrane, the voltage dependence of the capacitance is altered so that for positive voltages applied to the *cis* side of the membrane the voltage-dependent increase in capacitance is smaller than in the absence of alamethicin. For negative voltages, the capacitance change is the same as in the absence of alamethicin. This experiment with use of lock-in method (notice the continuous capacitance-voltage [C-V] curve) is illustrated in Fig. 4.

The results obtained on doping the membrane with different alamethicin analogues are quite different and shown in Fig. 5 for different concentrations of antibiotic. Data were obtained by using the pulse method and shown in this figure as data points. Natural alamethicin and its most active component, fraction 4, reduce the amount of capacitance increase with positive voltage. BG, which forms channels when negative voltages are applied, increases the amount of capacitance increase when the voltage is positive and has no effect when the voltage is negative. Boc 2-20, on another hand, always produces a symmetric current voltage curve, and it has no detectable effect on the capacitance at applied voltages of either sign (in used concentration range of alamethicin).

Fig. 5 shows the dependence of capacitance on voltage at increasing concentrations of peptide for alamethicin (Fig. 5 *A*), BG added to the same side as alamethicin (Fig. 5 *B*), and BG added to the opposite side from the additions in

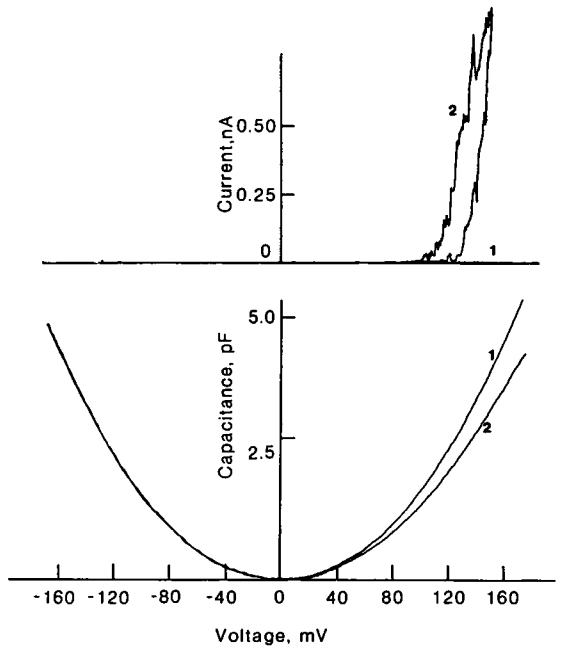


FIGURE 4 Capacitance-voltage curve obtained by the lock-in method. Phosphatidylethanolamine (squalane) membrane in 1 M KCl. (*Upper panel*) current-voltage curves and lower panel shows capacitance-voltage curves in which capacitance was obtained by subtracting total membrane and RC circuit currents and subsequent phase separation by the lock-in amplifier (signal was filtered at 3 ms). Curve 1 shows the capacitance change as a function of voltage in the absence of alamethicin. Curve 2 shows the capacitance change in the presence of 0.2 μg/ml of fraction 4 alamethicin added to the *cis* side of the membrane. Voltage sweep rate was 5 mV/s. Note capacitance change at voltages where there is no significant current change.

Fig. 5, *A* and *B* (Fig. 5 *D*). The numbering convention is the same for all of the curves: curve No. 1 is with no peptide and curves 2 and 3 are for increasing concentrations of peptide (correspondingly the open square, filled triangle, and open triangle on C-V curves). Current-voltage curves obtained at the same peptide concentrations are shown above the capacitance-voltage curves and correspondingly numbered.

Membrane voltage–dependent capacitances at different concentrations of alamethicin (BG) (added to the membrane bathing solution unilaterally) are presented in Table I.

## DISCUSSION

Our results on voltage-dependent capacitance of the bare membrane can be described by an electrostriction model which predicts a symmetric dependence on the square of voltage. The magnitude of this nonlinear capacitance is ~1% (at 250 mV of applied voltage) of the membrane geometrical capacitance and acts as a capacitor in parallel with the membrane geometrical capacitance. In this discussion we will not be concerned with the nature of the voltage-dependent capacitance of the bare bilayer. This has been discussed in detail by many workers elsewhere (Alvarez and Latorre, 1978; Benz et al., 1975; Carius, 1976; Requena et al., 1975; Sargent, 1975; White, 1978, 1981).

When alamethicin is added to the aqueous phase, the voltage-dependent capacitance changes in a very specific way. The change depends on both voltage and alamethicin charge sign. There is no detectable change when alamethicin analogue has no formal charge. Because of the involvement of the alamethicin charge, these effects are difficult to explain simply by a change in membrane compressibility due to alamethicin adsorption. In our first approach we will try to account for the membrane surface charge change due to adsorption of the alamethicin.

$$q_0 = zF\Gamma, \tag{1}$$

where $q_0$ is the membrane charge per unit area (at constant pressure and temperature), $z$ is alamethicin charge, $F$ is the Faraday constant, and $\Gamma$ is the surface concentration of alamethicin.

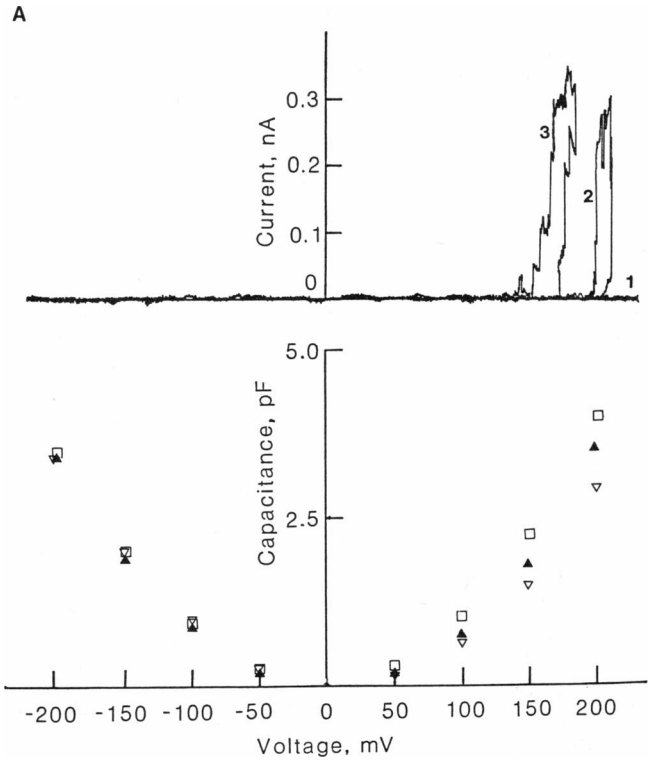


FIGURE 5 Capacitance-voltage curves obtained by the pulse method described in the text. Corresponding I-V curves are shown at the top of each graph. No. 1 is bare membrane measurement (corresponds to open square on C-V curves). Nos. 2 and 3 on I-V curves correspond to solid and open triangles on C-V curves. The membrane is PE in 1 M KCl. (*A*) Alamethicin fraction 4 is added to the *cis* side of the membrane: 2, 20 ng/ml; 3, 80 ng/ml. (*B*) BG is added to the *cis* side of the membrane: 2, 0.83 μg/ml; 3, 6.5 μg/ml. (*C*) Alamethicin fraction 4 is added to the *trans* compartment: 2, 44 ng/ml; 3, 320 ng/ml. (*D*) BG is added to the *trans* side of the membrane: 2, 0.64 μg/ml; 3, 4 μg/ml. (*e*) Boc2-20 is added to the *cis* side of the membrane: 2, 0.27 μg/ml; 3, 1.2 μg/ml. Note capacitance change in panels *B* and *D* in the quadrant where no membrane current change occurs.

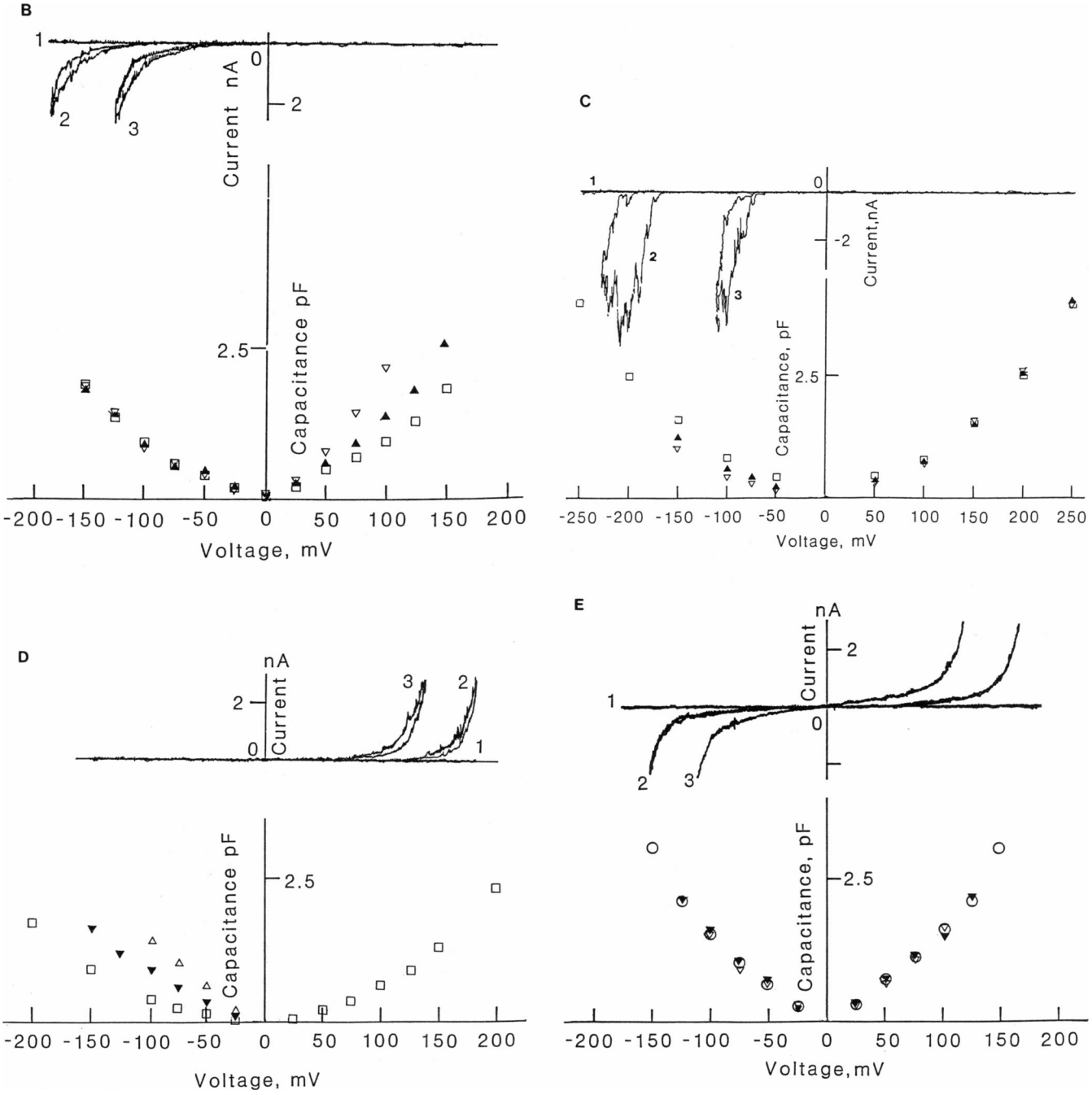


FIGURE 5 Continued

At the given concentration of alamethicin in the aqueous phase, the change in capacitance which we measure due to changes in adsorbed alamethicin can be written in the form

$$\Delta C = \Delta q_0/\alpha\Delta V, \tag{2}$$

where $\Delta q_0$ is the charge change due to applied blip potential $\Delta V$, and $\alpha$ is the proportionality constant between the change in the surface charge due to adsorbed alamethicin and the amount of charge which must be moved in the external circuit to keep the potential constant. The change in the membrane charge due to adsorbed alamethicin at a given applied voltage $V_{app}$ is defined as:

$$q_0 = \int_0^{\alpha V_{app}} \Delta C \, dV. \tag{3}$$

Because the change in the surface alamethicin concentration at the same applied voltage $V_{app}$ is proportional to $q_0$, we can use Eq. 1 to calculate surface concentration of alamethicin, $\Gamma$.

TABLE 1
MEMBRANE VOLTAGE-DEPENDENT CAPACITANCE AT DIFFERENT CONCENTRATIONS OF ALAMETHICIN (BG) ADDED TO THE MEMBRANE BATHING SOLUTION UNILATERALLY

| BG concentration | $2.8 \times 10^{-7}$ M | $6.4 \times 10^{-7}$ M | $1.6 \times 10^{-6}$ M | $3.4 \times 10^{-6}$ M |
|---|---|---|---|---|
| Membrane voltage | Voltage-dependent capacitance | | | |
| *mV* | *F/cm²* | *F/cm²* | *F/cm²* | *F/cm²* |
| 50 | $1.2 \times 10^{-10}$ | $2.4 \times 10^{-10}$ | $3.3 \times 10^{-10}$ | $5.4 \times 10^{-10}$ |
| 100 | $3.8 \times 10^{-10}$ | $2.8 \times 10^{-10}$ | $1.1 \times 10^{-10}$ | $3.3 \times 10^{-10}$ |
| 150 | $4.1 \times 10^{-10}$ | $1.07 \times 10^{-9}$ | $2.79 \times 10^{-9}$ | $7.78 \times 10^{-9}$ |
| 200 | $5.8 \times 10^{-10}$ | $1.27 \times 10^{-9}$ | $2.71 \times 10^{-9}$ | |

Data are the averaged results of 19 experiments in which this capacitance was measured as a difference of the membrane capacitance at a particular alamethicin concentration and the membrane capacitance without the protein for all applied voltages and alamethicin concentrations. Data presented here as specific capacitance. Standard errors of the mean are shown in Fig. 6. Capacitance was calculated from Eq. 2 with $\alpha = 1$.

Our results show that the change in capacitance saturates when capacitance measurements are taken as a function of alamethicin concentration at a given membrane voltage. Therefore an adsorption isotherm in which the free energy of adsorption is independent of the electrical potential can be written.

The isotherm most commonly used to describe adsorption to a surface is that of Langmuir (Langmuir, 1918, 1932) which we will write in the following form.

$$\Theta = Kc/(1 + Kc), \tag{4}$$

where $\Theta = \Gamma/\Gamma_\infty$ is the coverage ($\Gamma_\infty$ is the highest possible alamethicin surface concentration, $K$ is the adsorption coefficient (related to the standard free energy of adsorption $K = \exp(-\Delta G^0)$ (Delahey, 1965; Fridrishsberg, 1984), and $c$ is the alamethicin concentration in the membrane bathing solution.

Eq. 4 can be rewritten in the more convenient form,

$$1/\Gamma = 1/Kc\,\Gamma_\infty + 1/\Gamma_\infty \tag{5}$$

and can be represented graphically where the left part $1/\Gamma$ is a function of $1/c$ with the intercept on the $1/\Gamma$ axis at $1/\Gamma_\infty$ and a slope of $1/\Gamma_\infty K$.

Fig. 6 was generated using Eq. 1 for alamethicin concentration $\Gamma$. Changes in apparent alamethicin concentration $\Gamma$ are plotted as a function of alamethicin concentration in the aqueous phase for the different applied voltages. Statistical Z test of the difference between means of intercepts $1/\Gamma_\infty$ shows that all $1/\Gamma_\infty$ are equal at 5% level of significance and we may hypothesize that $1/\Gamma_\infty$ remain constant within experimental error [$1/\Gamma_\infty = (7.4 \pm 6.0) \times 10^{13}$ cm²/g mol].

This number is in reasonable agreement with the number obtained by Fringeli (Fringeli, 1980) for the adsorption of alamethicin onto the lipid monolayer at the water–air interface. The alamethicin surface concentration was determined by the infrared scanning spectroscopy.

The slopes of Fig. 6 plots seemingly decrease with voltage but the intercepts $1/\Gamma_\infty$ remain constant. Therefore the apparent adsorption coefficient $K$ seems to be voltage dependent. If the natural logarithms of the slopes of the double reciprocal plots in Fig. 6 are themselves plotted against voltage, the adsorption coefficient is seen to increase exponentially with applied voltage (see Fig. 7) as

$$K = K_0 \exp(\alpha V) \tag{6}$$

This implies that the standard free energy of adsorption depends linearly on potential. The proportionality coefficient of this dependence is ~0.71. Therefore the free energy of adsorption can be written as $G_o^0 = G^0 + 0.71(FV_{app}/RT)$ where $G^0$ is the energy due to the chemical potential difference.

The above analysis enables us to estimate the adsorption coefficient $K$ at $\sim 1 \times 10^8$ 1/M and the area which would be occupied by alamethicin on the membrane surface at unity coverage. Such a coverage corresponds approximately to one alamethicin molecule per $1 \times 10^7$ Å².

The potential generated by the adsorbed alamethicin charges can be calculated as $q_{0max}/C$ geometric where $q_{0max} = zF\Gamma_\infty$. This voltage is $\sim 1 \times 10^{-3}$ V. This potential is large enough to account for small change of membrane capacitance but it is small compared with the applied potential. This tells us that alamethicin molecules act as probe charges and do not have an appreciable effect on the existing electrical potential inside the membrane.

The Langmuir isotherm can be derived from the formal treatment of reaction rates (Gileadi and Conway, 1964). The concentration of adsorbed alamethicin on the membrane surface depends on alamethicin electrochemical potential which may be formally written as a sum of a chemical and an electrical term. The latter is $zF\alpha V_{app}/RT$(where $\alpha$ is fraction of the applied voltage between the electrode in the bathing solution and the alamethicin adsorption plane). Thus the standard free energy of adsorption is a linear function of potential, and the adsorption coefficient will depend exponentially on potential. The difference between this approach and that used earlier (Eqs. 1–6) is the assumption that the adsorption coefficient is voltage dependent.

We now consider explicitly the voltage-dependent Langmuir adsorption isotherm.

$$A^\pm + S \rightleftharpoons SA^\pm, \tag{7}$$

where $A^\pm$ denotes positively or negatively charged alamethicin; S is the adsorption site; and $SA^\pm$ is the site with adsorbed alamethicin molecule. The equilibrium constant for this reaction is:

$$K = (k_1/k_{-1}) \exp(\alpha z F V_{app}/RT). \tag{8}$$

Where $k_1$ and $k_{-1}$ are forward and reverse rate constants

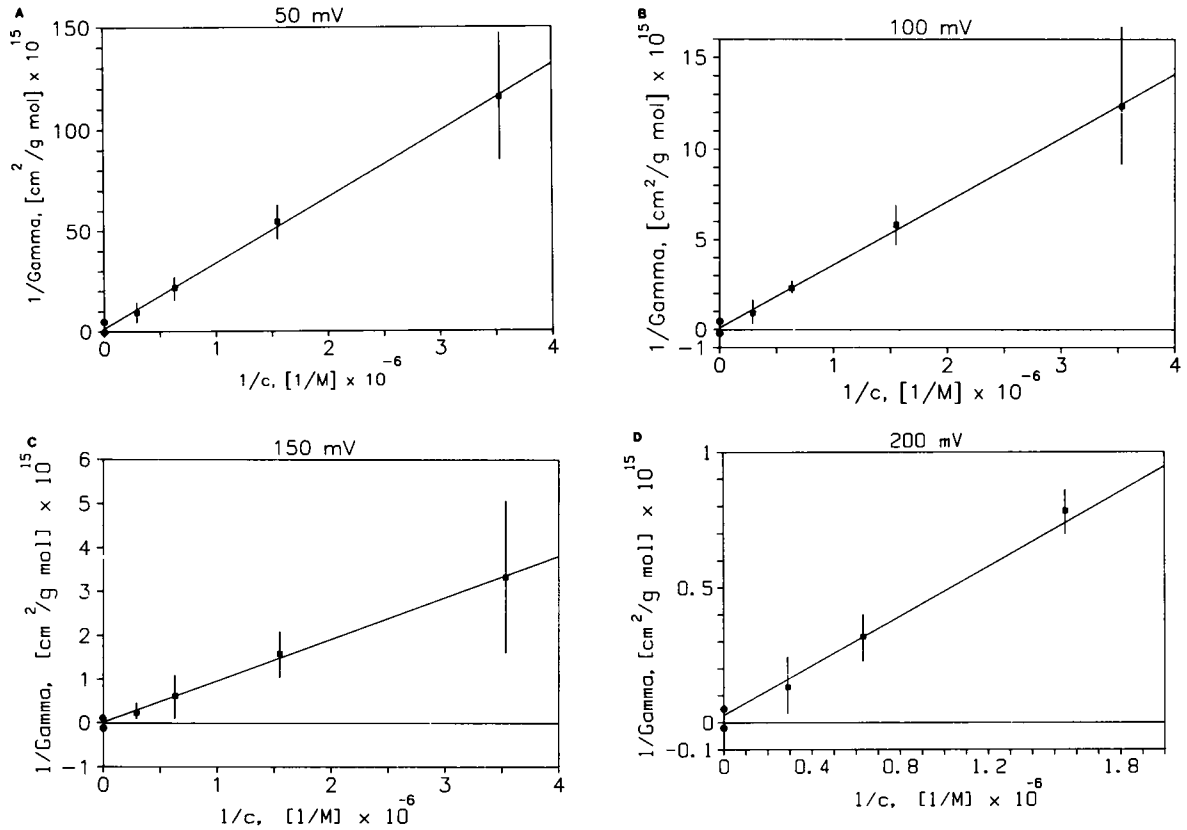


FIGURE 6 Plot of $1/\Gamma$ as a function of $1/c$ for 50, 100, 150, and 200 mV of membrane voltage. BG concentration range of ~ 1.5 orders. The lines are linear regression fits with correlations: $r$ (50 mV) = 0.998; $r$ (100 mV) = 0.998; $r$ (150 mV) = 0.997; and $r$ (200 mV) = 0.997. Filled circles show the standard errors of the regressional fit. Standard statistical Z test (accounting the standard errors of individual points shown as vertical bars) with 5% level of significance ($-1.96 < Z < 1.96$) shows that all the intercepts $1/\Gamma_\infty$ are equal.

of reaction (7), respectively, and $\alpha$ has the same meaning as in the previous treatment. Thus the adsorption isotherm corresponding to Eq. 4 can now be written in an explicitly voltage dependent form.

$$\Theta/(1-\Theta) = (k_1 c/k_{-1}) \exp(zF\alpha V_{app}/RT). \quad (9)$$

The surface charge density due to adsorbed alamethicin can now be written (from Eq. 1 substituting $\Gamma$ for the $\Gamma_\infty\Theta$).

$$q = zF\Gamma_\infty/[1 + (K_0/c)\exp(zF\alpha V_{app}/RT)], \quad (10)$$

where $K_0 = k_{-1}/k_1$. This is essentially the treatment of Stern for the adsorption of ions to an interface.

The change of the coverage $\Theta$ and the consequent change in surface charge density with potential produce an effective capacity given by

$$C = zF\Gamma_\infty d\Theta/dV. \quad (11)$$

This capacitance is called an "adsorption pseudocapacitance" (Conway and Gileadi, 1962).

Electrically the pseudocapacitance ($C$) can be represented as a voltage-dependent capacitor. The charging rate of this capacitor is proportional to the rate of adsorption of alamethicin to the membrane and the discharge rate of the capacitor is proportional to the rate of desorption of alamethicin.

From Eqs. 8, 9, and 11 the pseudocapacitance $C$ is obtained as a function of potential as

$$C = z^2(F^2\Gamma_\infty/RT)(K_0/c)\{\exp(zF\alpha V_{app}/RT)/[1 + (K_0/c)\exp(zF\alpha V_{app}/RT)]^2\}. \quad (12)$$

This function has a maximum at

$$C_m = z^2F^2\Gamma_\infty/4RT \quad (13)$$

Only differential capacitance $\Delta C$ was measured in our

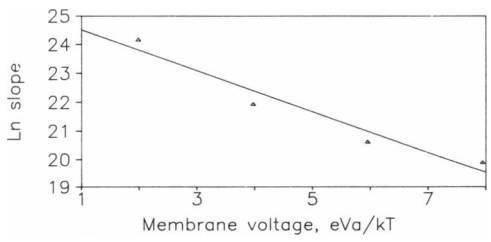


FIGURE 7 Natural logarithm of slopes $1/\Gamma$ vs $1/c$ against membrane voltage. The line is the linear regression fit with correlation $r$ = 0.945 and slope of 0.71. Standard errors of the fits from Fig. 6 are too small to be seen at the logarithmic scale.

experiments. $\Delta C$ is given by

$$\Delta C = C|_{\text{at } V_{app}=\text{const}} - C|_{\text{at } V_{app}=0} \quad (14)$$

From Eqs. 12, 13, and 14,

$$\Delta C/C_m = (4K_o/c)\{\exp(zF\alpha V_{app}/RT)/[1 + (K_o/c)\exp(zF\alpha V_{app}/RT)]^2 - 1/[1 + (K_o/c)]^2\}. \quad (15)$$

This function is symmetrical with respect to the potential at which its rather sharp maximum occurs, $V = \ln(K_o/c)$. The free parameters are $\alpha$ and $K_o$. But no single set of parameters satisfactorily fits to the whole range of our data.

The essential reason for this must be interaction between alamethicin monomers on the surface of the membrane. The Langmuir isotherm is derived under the assumption that the free energy of adsorption is independent of coverage. This condition requires that any interactions between molecules adsorbed to the surface be negligible. It is quite clear however that even at the moderate coverage there is interaction between charged alamethicin molecules on the membrane surface. Adsorbed charges which are associated with the image charges will interact as dipoles. Such an interaction is generally proportional to the coverage and therefore implies a linear dependence of standard free energy of adsorption on the coverage (Gileadi and Conway, 1964). This leads to the following expression for the free energy of adsorption:

$$G_o^0 = G^0 - f\Theta, \quad (16)$$

where $G_o^0$ is the free energy of adsorption in units of $RT$, $G^0$ is the coverage-independent portion of the free energy of adsorption in units of $RT$ and $f$ is the proportionality factor between coverage and the coverage-dependent part of the free energy of adsorption ($f$ has units of $RT$). This isotherm was originally described by Temkin (1941).

The equation for the adsorption coefficient, Eq. 8, can now be written

$$K = K_o \exp(\alpha z F V_{app}/RT)\exp(-f\theta). \quad (17)$$

This equation with $f = 0$ gives an expression for the adsorption pseudocapacitance identical to the Langmuir case (12). For the case where $f$ is nonzero we can write

$$1/C = [-1/(zF\Gamma_\infty)]dV/d\Theta = [1/(zF\Gamma_\infty)] \cdot \{(RT/F)\, d[\ln(\Theta/(1-\Theta)]/d\Theta + (RT/F)f\}, \quad (18)$$

which in turn can be written in the form

$$1/C = 1/C_L + 1/C_T, \quad (19)$$

where $C_L$ is the capacitance produced by the Langmuir type of adsorption and $C_T$ is the additional adsorption pseudocapacitance (Temkin's capacitance) produced by alamethicin surface interactions.

At $V_{app} = 0$; $C_L = 4C_m(K_o/c)/[1 + (K_o/c)]^2$ and $C_T = 4C_m/f$.

To describe our differential measurements we subtract the zero- voltage capacitance to obtain

$$\frac{\Delta C}{4C_m} = \frac{[(K_o/c)\exp(zF\alpha V_{app}/RT)(1/f)]/\{[(1 + (K_o/c)\exp(zF\alpha V_{app}/RT)]^2\}}{\{[(K_o/c)\exp(F\alpha V_{app}/RT)]/[(1 + (K_o/c)\exp(F\alpha V_{app}/RT)]^2\} + (1/f)} - \frac{\{(K_o/c)/[1 + (K_o/c)]^2\}(1/f)}{\{(K_o/c)/[1 + (K_o/c)]^2\} + (1/f)}. \quad (20)$$

Eq. 20 describes the dependence of the adsorption pseudocapacitance on the voltage (see Fig. 8 for the values of the parameters which fit this equation). It accounts for the apparent decrease in adsorption pseudocapacitance with increase in the concentration of negatively charged alamethicin and also the asymmetry of the capacitance-voltage curves. This model also predicts the lack of voltage-dependent capacitance change when the neutral alamethicin analogue, Boc2-20, is used.

Fig. 8 shows the fit of our capacitance data to curves calculated from the model using only three free parameters, $f$, $K_o$, and $\alpha$. The alamethicin charge is taken as positive here because these data are for the positively charged analogue, BG. The value of the adsorption coefficient, estimated as $K_o = (5 \pm 4.5) \times 10^{-8}$ M, is very close to the adsorption coefficient obtained from the first approach. The free energy parameter, $f$, is proportional to the alamethicin–alamethicin interaction at the membrane surface. Dependence of this parameter on the bulk alamethicin concentrations is shown in Fig. 9. Note that as the concentration of alamethicin increases, $f$ decreases. This indicates an appreciable attractive interaction between alamethicin monomers at the surface even at alamethicin concentrations which produce no channel activity at low voltages.

In a previous paper (Hall et al., 1984), we discussed a model in which alamethicin is anchored to the *cis* membrane surface by its COOH end of the molecule which has a negative charge. BG, on another hand, has positive

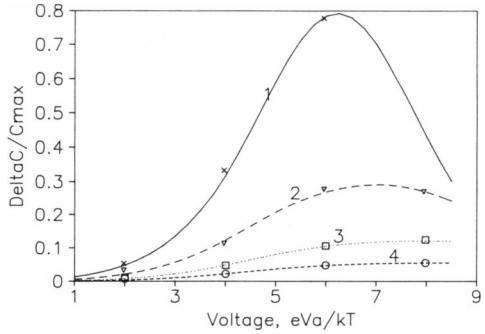


FIGURE 8 $\Delta C/C_{max}$ dependence on applied voltage at different alamethicin concentration $c$ in the bathing solution ($\alpha = 0.9$). No. 1, $c = 3.43\ 10^{-6}$ (M); $k/c = 2.0\ 10^{-3}$ (1/M ); $1/f = 0.95$ ($1/kT$). No. 2, $c = 1.58\ 10^{-6}$ (M); $k/c = 9.21\ 10^{-4}$ (1/M); $1/f = 0.105$ ($1/kT$). 3. $c = 6.45\ 10^{-7}$ (M); $k/c = 3.76\ 10^{-4}$ (1/M); $1/f = 0.035$ ($1/kT$). 4. $c = 2.83\ 10^{-7}$ (M); $k/c = 2.52\ 10^{-4}$ (1/M); $1/f = 0.015$ ($1/kT$).

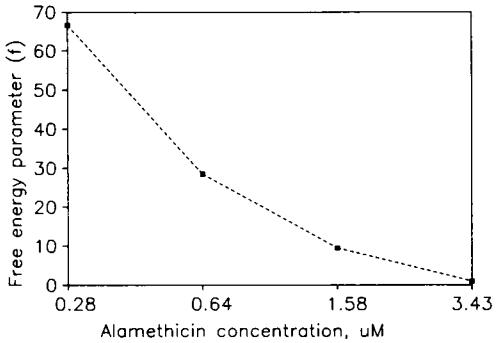


FIGURE 9 Free energy parameter as a function of alamethicin concentration in the membrane bathing solution.

charge at the $NH_2$-terminus which is fixed at the *cis* membrane surface. We also proposed that the neutral alamethicin analogue Boc2-20 can easily cross the membrane interior without interacting strongly with the membrane surface.

Recent studies of $^{31}P$ and $^{2}H$ Nuclear Magnetic Resonance by Banerjee et al., 1985, and also Raman studies by Lis et al., 1976 provide substantial support of the notion that alamethicin interacts with the bilayer near the bilayer-water interface.

An asymmetric surface charge, due to either adsorbed charge (Schoch et al, 1978) or intrinsic to the bilayer (Alvarez and Latorre, 1978), induces asymmetric surface potential in the lipid bilayer. This potential shifts the minimum of the voltage-capacitance curve. Geometric membrane capacitance charge at 100 mV of applied potential is about $5 \times 10^{11}$ elementary charges per $cm^2$. The surface charge induced by alamethicin calculated from $\Gamma_\infty$ is about $1.5 \times 10^9$ elementary charges per $cm^2$ which is ~3% of the geometric capacitance charge. This charge adds a component of membrane voltage whose sign depends on the charge on the alamethicin and the sign of the applied voltage. This small induced voltage is sufficient to generate an apparent change of membrane capacitance. This picture is obviously oversimplified, nonetheless it serves to predict the correct sign of the voltage shift as well as to estimate the magnitude of the effect.

## CONCLUSIONS

We conclude that both the state of alamethicin at the membrane surface and the amount of alamethicin adsorbed are altered by membrane voltage. We also have shown that interaction between alamethicin monomers on the membrane surface appears to occur even at combinations of voltage and concentration at which alamethicin does not form channels. This result suggests alamethicin monomers can aggregate on the membrane surface without forming channels.

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