INNER VOLTAGE CLAMPING

A METHOD FOR STUDYING INTERACTIONS AMONG HYDROPHOBIC IONS IN A LIPID BILAYER

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ABSTRACT Ketterer, et al. (1971) have suggested that a combination of electrostatic and chemical interactions may cause hydrophobic ions absorbed within a bilayer lipid membrane to reside in two potential wells, each close to a membrane surface. The resulting two planes of charges would define three regions of membrane dielectric: two identical outer regions each between a plane of absorbed charges and the plane of closest approach of ions in the aqueous phase; and the inner region between the two planes of adsorbed charges. The theory describing charge translocation across the inner region is based on a simple three-capacitor model. A significant theoretical conclusion is that the difference between the voltage across the inner region, V_i, and the voltage across the entire membrane, V_m, is directly proportional to the amount of charge that has flowed in a voltage clamp experiment. We demonstrate that we can construct an "inner voltage clamp" that can maintain, with positive feedback, a constant inner voltage, V_i. The manifestation of proper feedback is that the clamp current (after a voltage step) will exhibit pure (i.e., single time-constant) exponential decay, because the voltage dependent rate constants governing translocation will be independent of time. The "pureness" of the exponential is maximized when the standard deviation of the least-square fit of the appropriate exponential equation to the experimental data is minimized. The concomitant feedback is directly related to the capacitances of the inner and outer membrane regions, C_i and C_o.

Experimental results with tetraphenylborate ion adsorbed in bacterial phosphatidylethanolamine/*n*-decane bilayers indicate $C_i \sim 5 \cdot 10^{-7} \text{F/cm}^2$ and $C_o \approx 5 \cdot 10^{-5}$ F/cm^2.

INTRODUCTION

Ketterer et al. (1971) have suggested that a combination of electrostatic and chemical forces could cause a hydrophobic ion adsorbed in a bilayer lipid membrane (BLM) to locate in potential wells near the membrane-water interface. Thus, there would be two parallel planes of charge within the bilayer dividing the membrane into three regions: two identical regions on the aqueous side of each plane and a single region between the two planes. Our model (Andersen et al., 1977, 1978), a variation of the model pro-

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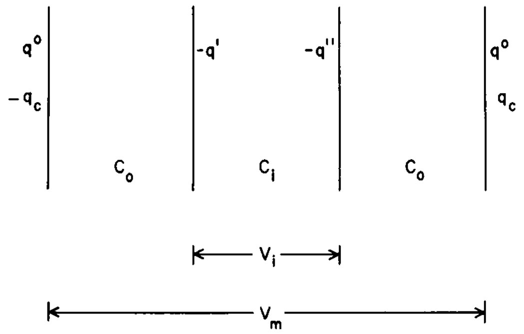

FIGURE 1 Three-capacitor model comprising an inner region with capacitance, C_i, and two outer regions with capacitance C_o. Charges adsorbed in left and right wells are $-q'$ and $-q''$ with nonadsorbed counter charges q^0. Externally applied charges are q_c and $-q_c$.

posed by Markin, et al. (1971), presumes that there are three regions of dielectric (not necessarily homogeneous) delineated by four parallel planes of charges (Fig. 1): two planes, each located in the aqueous phase a Debye length from each membrane surface, and two planes of adsorbed ions within the BLM. Neither the average dielectric constant nor the thickness of each region is directly measurable; however, the ratio of these two parameters is the capacitance and is measurable.

Recently, Andersen, et al. (1977, 1978), pursuing the technique described by Andersen and Fuchs (1975) (see also Bruner, 1975), used this model to analyze the behavior of the tetraphenylborate anion adsorbed in bacterial phosphatidyl ethanolamine (BPE) BLMs, and were able to deduce a capacitance for each of the three regions. They also discuss the limitations of the model as well as present a thorough review of the pertinent literature. Andersen et al. (1977, 1978) also noted that transients obtained in a voltage clamp experiment exhibited nonexponential decay if the applied voltage was high enough and if the density of absorbed charge was large enough. This phenomenon is a direct consequence of the fact that the potential across the inner capacitive region, V_i (see Fig. 1), changes as charge is translocated even if the applied voltage, V_m, is maintained constant.

The essence of Andersen and Fuchs' approach is the measurement of the amount of adsorbed charge translocated (from one potential well to the other) as a function of voltage applied across the BLM. The technique we describe here is based on an analysis of the *rate* of translocation. The rate constants governing that rate are voltage-dependent. If that dependence is nonlinear, the translocation process will exhibit pure (single time-constant) exponential decay only if the potential across the inner capacitive region, V_i, is maintained constant. The mathematical analysis of the three-capacitor model indicates that the difference between the membrane voltage, V_m, and the inner voltage, V_i, is proportional to the amount of charge translocated. The proportionality is directly related to the magnitudes of the inner and outer membrane capacitances, C_i and C_o. By integrating the current passed during a voltage clamp experiment and using positive feedback to modify V_m, it is in principle possible to maintain V_i constant.

GLOSSARY OF SYMBOLS

A (cm^2)	Membrane area.
b (dimensionless)	See Eq. 5.
c (mol/cm^3)	Concentration of inert electrolyte.
C_i, C_o, C_m (F/cm^2)	Capacitance of inner region, outer region, and whole membrane (see Fig. 1).
C_1, C_2 (F)	Capacitances in equivalent circuit (Fig. 3).
e/kT (V^{-1})	38.9 V^{-1} at 25 C°.
i_c (A/cm^2)	External current density moved by voltage clamp: $i_c = \mathrm{d}q_c/\mathrm{d}t$.
i_c^* (A/cm^2)	External current density flowing just after the membrane capacitance has been charged.
i_e (A)	Total current in equivalent circuit.
i'' (A/cm^2)	Internal translocation current density: $i'' = \mathrm{d}q''/\mathrm{d}t$.
k', k'' (s^{-1})	Voltage-dependent rate constants for left to right (′) and right-to-left (″) translocations.
k^* (s^{-1})	Voltage-independent "standard" rate constant for translocation.
q_c(C/cm^2)	External charge moved by voltage clamp.
q', q''(C/cm^2)	Absorbed charge in left (′) and right (″) wells.
q^0(C/cm^2)	Charge of nonadsorbed counter ions (see Eq. 1).
r^0(cm)	Radius of membrane.
R (Ω)	Resistor in equivalent circuit (see Fig. 3).
R_s, R_I (Ω)	Summing resistors in clamp circuit (see Fig. 2).
R_a (Ω)	Access resistance.
s(s^{-1})	Time constant for exponential decay of the current.
t(s)	Time.
t_1(s)	Initial time considered in analysis of current transients.
V_i(V)	Voltage across inner region.
V_I(V)	Output voltage of integrator.
V_m(V)	Voltage across whole membrane.
V_S(V)	Voltage step size.
ΔV_{iR}(V)	Uncompensated voltage drop in across access distance, R_a.
z'(C/cm^2)	Amplitude function. See Eq. 29.
z(V)	Amplitude function combined with integrator-scaling function.
α (dimensionless)	Effective fraction of V_I added to V_S.
α' (dimensionless)	Fraction of V_I fed back to clamp summing resistor.
σ(V)	Standard deviation; see Eq. 31.
Λ^0(Scm^2/mol)	Equivalent conductance.
τ_{RC}(s)	Time constant for charging membrane capacitance through the access resistance, R_a.

THEORY

The basic theory is essentially that discussed by Andersen et al. (1977, 1978). Several assumptions are made: the charge in any given plane is considered uniformly distributed (smeared); during the experiment there is negligible charge translocation between the membrane and the aqueous phases, i.e.:

$$2q^0 = q' + q'', \tag{1}$$

the distribution of the adsorbed charge between the two wells is determined by the

Boltzmann relation; the translocation process across the inner region may be treated like a chemical rate process even though we believe that it is in reality a Nernst-Planck diffusion process. We also assume that the concentrations of the hydrophobic ion in the aqueous phases on each side of the membrane are identical. Thus, with no applied potential the number of charges in each well (within the BLM) are also identical. For simplicity we assume that the charge on the hydrophobic ion is -1.

By using Gauss's theorem, the equations describing the model (Fig. 1) are easily derived:[1]

$$V_i = (q_c + q^0 - q'')/C_i = b(q_c/C_m) + b(q^0 - q'')/C_m, \tag{2}$$

$$V_m = q_c/C_m + b(q^0 - q'')/C_m, \tag{3}$$

where

$$C_m = [1/C_i + 2/C_o]^{-1}, \tag{4}$$

and

$$b = C_m/C_i. \tag{5}$$

The quintessential result, however, is obtained by subtracting Eq. 2 from Eq. 3 to give

$$V_m - V_i = (1 - b)q_c/C_m. \tag{6}$$

Thus we see that the internal voltage, V_i, is rather simply related to the membrane voltage, V_m. The relationship suggests the intriguing possibility that modification of the applied membrane voltage through positive feedback proportional to the charge passed during a clamp experiment might effect a constant V_i. In the sbusequent theoretical discussions we shall describe the difference between the conventional voltage clamp, which maintains V_m constant, and the "inner" voltage clamp, which maintains V_i constant. We shall also show how the translocation kinetics can indicate whether or not V_i has been maintained constant. In our discussion of voltage clamps it is important to remember that we consider only the perfect clamp having an infinitely fast rise time. The current that flows in charging a purely capacitive load is then a delta function. Its integral, of course, is finite.

Conventional Voltage Clamping—V_m Constant

The mathematical description of a constant applied voltage is simply

$$dV_m/dt = 0. \tag{7}$$

Thus we deduce directly from Eq. 3 that

$$dq_c/dt = b\,dq''/dt, \tag{8}$$

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[1] Note that dipole potentials that occur somewhere in the outer region will not change Eqs. 2 and 3 as long as the dipole potential is not a function of the density of adsorbed change, and as long as the dipole potential is the same on each side of the membrane.

or

$$i_c = b\,i''. \tag{9}$$

This states that the external current, i_c, is proportional to the internal translocation current, i''.

The external charge that has flowed, q_c, may be expressed as the sum of two components: a purely capacitive component, and a translocation component:

$$q_c = V_m C_m + b \int_0^t (\mathrm{d}q''/\mathrm{d}t)\,\mathrm{d}t. \tag{10}$$

Combining Eqs. 6 and 10 we obtain

$$V_i = bV_m - [(1 - b)b/C_m] \int_0^t (\mathrm{d}q''/\mathrm{d}t)\,dt. \tag{11}$$

Since one of our assumptions is that the charge is membrane-bound (Eq. 1), the maximum value of the integral is q^0. It is clear from Eq. 11 that V_i will vary with time. The maximum magnitude of that variation will depend upon the value of the term $(1 - b)bq^0/C_m$.

Inner Voltage Clamping—V_i Constant

The operation of the inner voltage clamp[2] is derived from Eq. 6. If the integral, q_c, of external current, i_c, is continuously monitored, and if the voltage initially applied to the membrane is continuously incremented by the quantity $(1 - b)q_c/C_m$, the result will be to maintain V_i constant. Before any translocation, but just after the infinitely fast charging of the membrane capacitance when

$$q_c = V_m C_m, \tag{12}$$

the value of V_m will be

$$V_m = V_i + (1 - b)V_m, \tag{13}$$

or

$$V_m = V_i/b. \tag{14}$$

With subsequent translocation the value of q_c and therefore V_m will increase.

The important question is how does one know the appropriate value of $(1 - b)/C_m$ to be used in modifying the initial voltage? We have already demonstrated that the conventional voltage clamp will not maintain V_i constant, while the inner voltage clamp with proper feedback modification will maintain V_i constant. If the translocation kinetics have a nonlinear voltage dependence, we can demonstrate that the current transient will exhibit pure exponential decay only if V_i is constant.

Kinetics of Translocation

We assume that the equation describing the kinetics of translocation is of the form

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[2] The electronic circuitry of the inner voltage clamp will be discussed in a subsequent section.

$$\mathrm{d}q''/\mathrm{d}t = k'q' - k''q''. \tag{15}$$

Because of the constraint that the ions are membrane-bound (Eq. 1),

$$\mathrm{d}q''/\mathrm{d}t = 2k'q^0 - (k' + k'')q''. \tag{16}$$

If the rate constants k' and k'' are time-independent (implying that the inner voltage, V_i, is constant), we can take the second derivative with respect to time of Eq. 16 and obtain

$$d^2q''/\mathrm{d}t^2 = -(k' + k'')\mathrm{d}q''/\mathrm{d}t. \tag{17}$$

Since V_i is constant, the time derivative of Eq. 2 is

$$\mathrm{d}q_c/\mathrm{d}t = \mathrm{d}q''/\mathrm{d}t. \tag{18}$$

Combining Eqs. 17 and 18 leads directly to

$$\mathrm{d}i_c/\mathrm{d}t = -(k' + k'')i_c. \tag{19}$$

It is obvious from Eq. 19 that the decay of the external current, i_c, will be exponential with a single time constant.

We must now ask whether or not it is possible to obtain pure exponential behavior even during conventional voltage clamping when the internal voltage, V_i, is not constant, and therefore the rate constants, k' and k'', are not constant. If k' and k'' are linearly dependent on V_i then, too, the current will decay exponentially with a single time constant. This will generally be the case when V_i is very small and the exponential terms describing the voltage dependence of k' and k'' can be linearized, i.e.

$$k'' = k^*(1 - eV_i/2kT), \tag{20}$$

$$k' = k^*(1 + eV_i/2kT). \tag{21}$$

Combining with rate Eq. 16 we obtain

$$\mathrm{d}q''/\mathrm{d}t = 2k^*q^0(1 + eV_i/2kT) - 2k^*q''. \tag{22}$$

Solving Eq. 6 for V_i and combining with Eq. 22 gives

$$\mathrm{d}q''/\mathrm{d}t = 2k^*q^0\{1 + (e/2kT)[V_m - (1 - b)q_c/C_m]\} - 2k^*q''. \tag{23}$$

Since V_m is constant in conventional voltage clamping, we differentiate Eq. 23, obtaining

$$\mathrm{d}^2q''/\mathrm{d}t^2 = -k^*\{q^0(e/kT)[(1 - b)/C_m]\mathrm{d}q_c/\mathrm{d}t + 2\mathrm{d}q''/\mathrm{d}t\}. \tag{24}$$

Combining with Eq. 8 we see that

$$\mathrm{d}i_c/\mathrm{d}t = -k^*i_c\{bq^0(e/kT)[1 - b]/C_m + 2\}, \tag{25}$$

and that the current will exhibit pure exponential decay.

In our discussion of the conventional voltage clamp we pointed out that if the term

$(1 - b)bq^0/C_m$ is very small, then V_i will exhibit only a negligible change during translocation. To observe current transients that are not exponential with a single time constant, the internal voltage, V_i, must be high enough to preclude linear voltage dependence of k' and k'', and the term $(1 - b)bq^0/C_m$ must be large enough to effect a significant change in V_i.

Determining When the Current Transient Exhibits the "Purest" Exponential Decay

We have demonstrated that with large V_i and q^0 the current transient during conventional voltage clamping will not be a pure exponential. We have also shown that if we use inner voltage clamping with the proper degree of modification (or feedback), V_i can be maintained constant. The circuitry we shall describe allows us to follow the current integral, $\int_0^t i_c \mathrm{d}t$ or q_c, as a function of time. If the decay of i_c with time is a pure exponential then

$$i_c = i_c^* \exp(-st), \tag{26}$$

where i_c^* is the external current flowing just *after* the membrane capacitance has been charged. This equation is easily integrated to give

$$\Delta q_c = -\left[\vphantom{\frac{i_c^*}{s}}\right._{t_1}^{t} \frac{i_c^*}{s} \exp(-st), \tag{27}$$

where t_1 is a time very close to zero, after completion of capacitive charging. Eq. 27 can be rewritten

$$\Delta q_c = (i_c^*/s) \exp(-st_1)(1 - \exp(-s(t - t_1))). \tag{28}$$

The form of this equation may be further simplified to

$$\Delta q_c = z'(1 - \exp(-s \Delta t)). \tag{29}$$

The experimentally observed transient will be a voltage change proportional to Δq_c; consequently the form of the equation will be the same as Eq. 29:

$$\Delta V_l = z(1 - \exp(-s \Delta t)). \tag{30}$$

The unknowns in the equation are s and z. We assume that the uncertainty in the value of ΔV_l is independent of its magnitude. We fit Eq. 30 to experimental data using a least-square routine. The standard deviation, σ, is an indicator of the "impurity" of the exponential:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n} \{(\Delta V_l)_i - z(1 - \exp(-s \Delta t_i))\}^2}{n - 2}}, \tag{31}$$

where n is the number of data points. The number 2 appears in the denominator because there are two unknowns, z and s. In a series of experiments in which the modification of V_m has been varied, the degree of modification (or feedback) that yields the smallest σ is assumed to be directly related to the correct value of $(1 - b)/C_m$.

We shall not go into details of the least square routine; a qualitative description should suffice. If one guesses at a value of s, Eq. 30 becomes a simple linear equation that can be fit to the data in the standard way. By scanning a range of values of s, the region of the minimum standard deviation is located; we continue to vary s using increasingly finer increments until we have located the value producing the minimum σ. Our criterion is that the next increment of s (plus or minus) change the minimum value of σ by a value at least an order of magnitude smaller than our estimate of the resolution of our data collecting device.

INSTRUMENTATION

Design of Inner Voltage Clamp

The circuit diagram for the inner voltage clamp is shown in Fig. 2. The device consists of two operation amplifiers (field effect transistor input): one acting as the clamp with three summing point resistors for the voltage pulse, the voltage-sensing electrode, and for the positive feedback; the second acting as an integrator with output going to the data-collecting device (in our case, a digital oscilloscope, model 1093, Nicolet Instrument Corp., Madison, Wis.), with some fraction of that output voltage being fed back to the summing point of the first operation amplifier. Because of the inherent instability of an integrating circuit, particularly when there is some form of positive feedback, it is important that the integrating capacitor be shorted out at all times except during the experiment. An electronic switch is opened (thereby activating the integrator) by the same signal triggering the scope sweep and the voltage pulse. An internal "clock" closes the switch after a given time, 0.008–0.1 s, which we can adjust.

The applied voltage, V_m, will be

$$V_m = -(V_S + \alpha V_I). \tag{32}$$

Note that the summing point resistor in the positive feedback loop, R_I, is $1.00 \times 10^5\ \Omega$, while the other two summing point resistors, R_s, are $1.00 \times 10^4\,\Omega$. Thus the fraction of V_I fed back is

$$\alpha = \alpha' R_s/R_I. \tag{33}$$

The integrator voltage is simply

$$V_I = -Aq_c/C_I. \tag{34}$$

The membrane area, A, enters this expression since we are integrating the *actual* charge passed, — — i.e., Aq_c. When the feedback value, α, has been properly selected, the standard deviation of least-square fit will be minimized (see previous discussion) and

$$V_S = -V_i, \tag{35}$$

and

$$\alpha V_I = -(1 - b)q_c/C_m, \tag{36}$$

or

$$\alpha A C_m/C_I = (1 - b). \tag{37}$$

Thus we can directly measure the term $(1 - b)$.

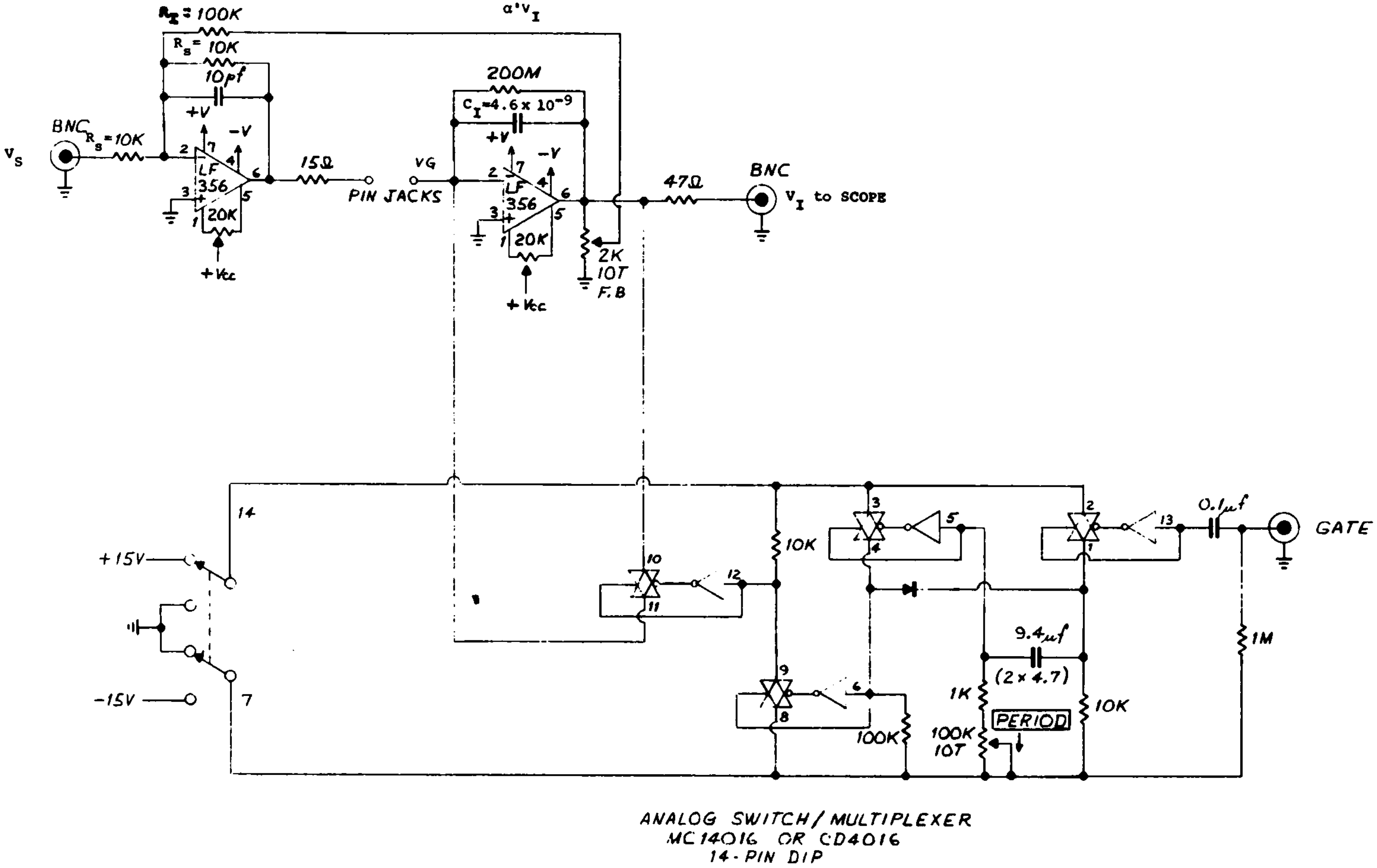

FIGURE 2 Circuit diagram for inner voltage clamp.

It is possible that for different membrane areas, and different values of b, one might select different values for the integrating capacitor, C_f, and the feedback resistor, R_f.

Theory for an Equivalent Circuit to Test the Inner Voltage Clamp

An equivalent circuit that approximately simulates the behavior of a membrane with adsorbed ions is shown in Fig. 3. The analysis of the behavior of this circuit with the inner voltage clamp

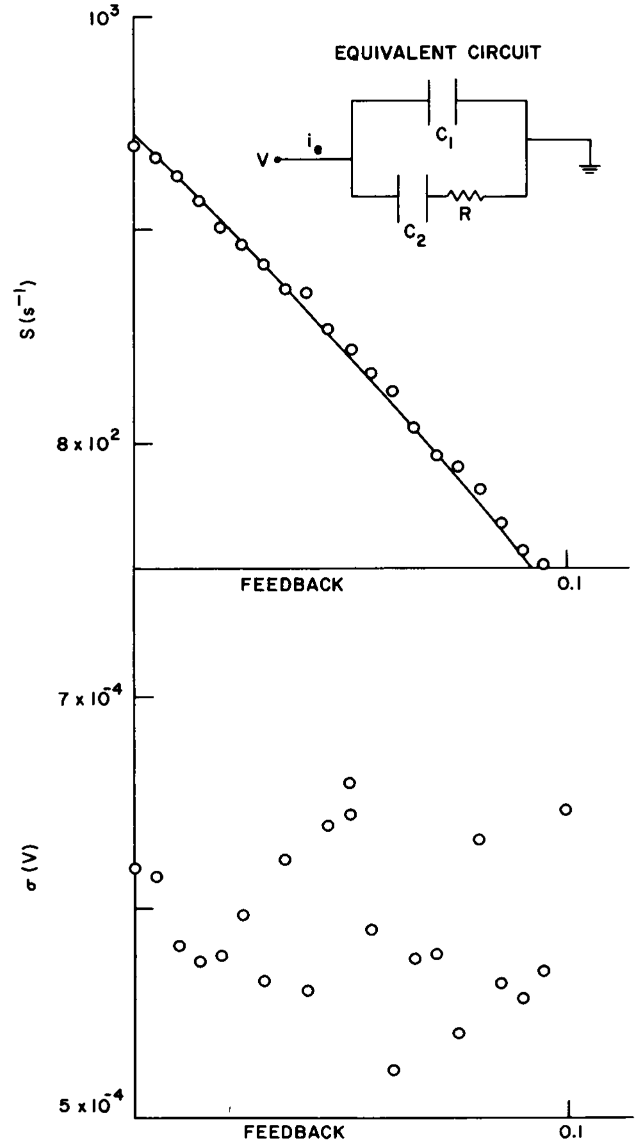

FIGURE 3 Top: Dependence of s (see Eq. 30) on feedback, α. Experimental points are open circles; solid line is drawn according to Eq. 38 where $s = [1 - (C_1 + C_2)\alpha/C_f]/[RC_2(1 - \alpha C_1/C_f)]$. Bottom: σ as a function of feedback, α.

yields

$$\mathrm{d}i_e/\mathrm{d}t = -i_e[1 - (C_1 + C_2)\alpha/C_I]/[RC_2(1 - \alpha C_1/C_I)], \tag{38}$$

where α is the effective fraction of the integrator voltage fed back to the clamp. Eq. 38 indicates that the current transient will exhibit pure exponential decay as long as

$$(C_1 + C_2)\alpha/C_I < 1 \tag{39}$$

It is important to show that this condition will be met when the inner voltage clamp is used on a bilayer with adsorbed charge. Following the method of Andersen et al. (1978, see Appendix B), one can show that a low voltage[3] (when Eqs. 20 and 21 are valid):

$$AC_m^* = C_1 + C_2 = A(C_m + beq^0/2kT)/(1 + (1 - b)beq^0/2kTC_m). \tag{40}$$

Combining Eqs. 37 and 40 and Ineq. 39, we see that the inequality,

$$\frac{(1 - b) + (1 - b)beq^0/2kTC_m}{1 + (1 - b)beq^0/2kTC_m} < 1, \tag{41}$$

must always be true since $0 < b < 1$. This indicates that when the appropriate value of α has been selected, the system will be stable.

We realize that the equivalent circuit does not mimic the important nonlinearities of ion translocation across the inner region of the membrane. (Unfortunately, we cannot think of a circuit that does, which at the same time is not unduly complicated.) With inner voltage clamping, the present circuit, however, approximates the time constants and voltage amplitudes of the experimental transients and allows us to answer two important questions about the clamping method: first, does the feedback of the clamp work properly (i.e. according to Eq. 38); second, is the standard deviation independent of feedback and thus not a function of some electronic idiosyncracies? In addition, it allows us to test the nonlinear least squares program and verify that it is performing properly.

MATERIALS AND METHODS

The membrane-forming material comprised 25 mg of bacterial phosphatidylethanolamine (Supelco, Inc., Bellefonte, Pa.) dissolved in 1 ml of *n*-decane (Matheson Co., Inc., East Rutherford, N.J.). Mueller-Rudin lipid bilayers were formed across an approximately 1-mm orifice in a 10-mil Teflon barrier by the Pasteur pipette technique described by Szabo et al. (1969). The cell design has been described in detail elsewhere (Feldberg and Kissel, 1975). The aqueous 1.00 M NaCl bathing solutions were prepared with doubly distilled water and NaCl (Baker Analyzed, J. I. Baker Chemical Co., Phillipsburg, N.J.). Solutions were filtered to remove particulate matter. Sodium tetraphenylborate (Baker Analyzed) was dissolved in absolute ethanol (Commercial Solvents Corporation, New York) to form stock solutions 1.00×10^{-2} M and 1.00 mM in NaTϕB. All reagents were used without further purification. Using Grunbaum pipettes, appropriate quantities of the ethanolic NaTϕB were added to the aqueous bathing solutions (20 ml on each side of the BLM).

The details of the inner voltage clamp have been discussed previously. Data were acquired

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[3] The value of C_m^* will be greatest at low voltage. This may be deduced directly from the Boltzmann relationship (see Andersen et al., 1978).

with a Nicolet model 1093 digital scope with 12-bit resolution and a maximum acquisition rate of one point per 0.5 μs. Data were recorded on tape and analyzed with a CDC 7600 (Control Data Corp., Minneapolis, Minn.). A Princeton Applied Research Corp. (Princeton, N.J.) model 175 function generator generated the required voltage pulse. A Wavetec pulse generator, model 116 (Wavetek, San Diego, Calif.) provided the pulse triggering the scope, opening the shorting switch of the inner voltage clamp integrator, and triggering the PAR 175 voltage pulse, in that order. The temperature during the experiments was 25 ± 1C°.

RESULTS AND DISCUSSION

Equivalent Circuit Experiment

Our first objective was to test the inner voltage clamp behavior on the equivalent circuit shown in Fig. 3 with $C_1 = 5.03$ nF, $C_2 = 9.71$ nF, and $R = 1.049 \times 10^5\ \Omega$. Transients were recorded for $V_i = 0.200$ V and values of α ranging from 0 to ~ 0.1. The value of s (computed with the least-square-fitting routine previously described) is plotted as a function of α (feedback). The solid line is calculated with Eq. 38 and the known values for C_1 and C_2, $C_f = 4.6$ nF, and adjusting the value of R until we obtained a reasonable fit (solid line, Fig. 3). The adjusted value of $R = 1.09 \times 10^5\ \Omega$ agrees well enough with the measured value of $1.049 \times 10^5\ \Omega$ so that we feel the clamp is working properly.

A plot of σ as a function of the feedback (Fig. 3) shows no trends, with the value hovering around 6×10^{-4} V. Since these data were recorded with 2 V full range, the resolution per bit is $2/2^{12}$ or $\sim 5 \times 10^{-4}$ V. Thus, even with a "perfect" fit one would not expect the value of σ to be less than perhaps 2.5×10^{-4} V.

Experiments with TϕB$^-$

Inner voltage clamp experiments were carried out with two voltages, $V_i = 0.100$ V and 0.200 V, for each concentration of TϕB$^-$ in the aqueous phase. We waited a minimum of about 2 min after formation of the BLM for equilibration (with stirring) with TϕB$^-$. Because a slower transient becomes increasingly evident at high concentrations of TϕB$^-$ in the aqueous phase (Andersen and Fuchs, 1975), we cut off the curve analysis at the point where $i_c/i_c^* < 0.05$. The results for four different levels of adsorbed *Tϕ*B$^-$ are shown in Fig. 4. The feedback is expressed in terms of $(1 - b)$ as calculated from Eq. 37. At the lowest level of absorption, $q^0 \sim 1.0 \times 10^{-7}$ C/cm^2, we see no discernable trends in the value of σ as a function of feedback. This is important because it demonstrates that the membrane itself does not undergo any kind of transformation that might manifest itself as a minimum in σ. The higher absorption levels exhibit a clear minimum in σ at $b \cong 0.980$ (for $V_i = 0.100$ V) and $b \cong 0.984$ (for $V_i = 0.200$ V). The absence of a well-defined minimum at the lowest absorption level is now easily explained. From Eq. 10 we can estimate that the maximum *change* in V_i during conventional voltage clamping would be

$$\Delta V_i = -(1 - b) b q^0 / C_m \sim -4\ \text{mV}. \qquad (42)$$

On an intuitive basis, at least, we are not surprised that a minimum could not be ob-

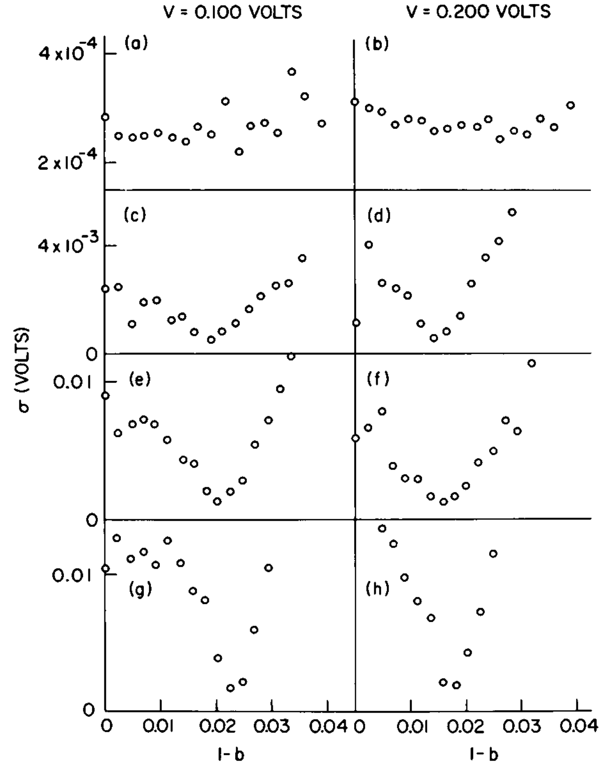

FIGURE 4 σ as a function of $1-b$ for different amounts of adsorbed tetraphenylborate measured at 0.100 and 0.200 V. The quantity adsorbed and the corresponding aqueous concentration of sodium tetraphenyl borate are for *a* and *b*, 1.0×10^{-7} C/cm^2 and 1×10^{-7} M; for *c* and *d*, 6.4×10^{-7} C/cm^2 and 3.5×10^{-7} M; for *e* and *f*, 9.1×10^{-7} C/cm^2 and 6.0×10^{-7} M; and for *g* and *h*, 1.2×10^{-6} C/cm^2 and 1.1×10^{-6} M. Membrane is bacterial phosphatidyl ethanolamine/*n*-decane; impermeant electrolyte is 1.00 M NaCl.

served. At the next highest absorption level, where $q^0 = 6.4 \times 10^{-7}$ C/cm^2, one would estimate that $\Delta V_i \sim 25$ mV and it is quite reasonable that the current transient will be not a pure exponential decay with conventional voltage clamping.

The estimate that $b = 0.98$ is in excellent agreement with the results of Andersen et al. (1977, 1978): $b = 0.97$ at the higher adsorption levels. Because we are observing the kinetics as well as the amount of charge translocation, we feel that model proposed by Andersen et al. is even further substantiated. That the same value of b is found with two significantly different voltages (0.100 and 0.200) diminishes the possibility that the inner voltage clamp feedback is compensating for a two-step process.

The analysis of the data presented in Fig. 4 also gives us the value of the voltage-dependent rate term, $k' + k''$. These data are summarized in Table I. The decrease in the rate constant with an increase in adsorbed charge has been observed by other workers (Andersen and Fuchs, 1975; Andersen et al., 1978; and Ketterer et al., 1971) and is discussed in detail by Andersen et al. (1978). The values of the ratio of the rate

TABLE I

RATE PARAMETER, $k' + k''$, AS A FUNCTION OF THE INNER VOLTAGE AND ADSORBED CHARGE

$[\mathrm{T\phi B^-}]$	$k' + k''$		$(k' + k'')_{0.2}/$ $(k' + k'')_{0.1}$	q^0
	$V_i = 0.100$ V	$V_i = 0.200$ V		
1.1×10^{-6}	1.2×10^2	5.3×10^2	4.4	1.2×10^{-6}
6.0×10^{-7}	2.0×10^2	8.3×10^2	4.2	9.1×10^{-7}
3.5×10^{-7}	3.6×10^2	1.4×10^3	3.9	6.4×10^{-7}
1.0×10^{-7}	8.7×10^2	3.1×10^3	3.6	1.0×10^{-7}

term at 0.200 and 0.100 V, $(k' + k'')_{0.2}/(k' + k'')_{0.1}$, agree well with the values deduced from relaxation data of Andersen and Fuchs, 1975.

Advantages and Disadvantages of the Inner Voltage Clamping Method

Perhaps the single advantage of the method over the relaxation techique first described by Andersen and Fuchs (1975) is that the analysis depends upon a characteristic (i.e., exponentiality) of individual transients and does not rely heavily upon the determination of the maximum charge adsorbed. It serves best, perhaps, as a supplementary approach to a difficult problem.

As our data indicate, the sensitivity of the method diminishes at low charge density. The critical parameter, however, is $(1 - b)bq^0/C_m$, the *change* in V_i which would occur during conventional clamping. If this term is too small, then the change in the values of the voltage-dependent rate constants will be too small to impart a measurable nonexponentiality to the transient. Thus, the precise nature of the voltage dependence of k' and k'' also determines the sensitivity of the method. As we indicated in the Theory section, the translocation process is probably more properly described by a Nernst-Planck diffusion process and the terms analogous to k' and k'' will depend upon the voltage, the diffusion coefficient, and the shape of the energy barrier in the membrane.

Signal averaging techniques could improve the sensitivity of the method, but we have not tried that yet.

R. de Levie (private communication) has pointed out to us that the uncompensated access resistance, R_a, in the aqueous phases could cause nonexponentiality of the transients. Newman (1970) has estimated the access resistance to a disk electrode. Except for the obstruction of the torus, the access resistance to each side of a bilayer membrane would be described by the same equation, and the total access resistance is the sum of the two or

$$R_a = 1/(2r^0\Lambda^0 c). \tag{43}$$

The time constant τ_{RC} for capacitive charging can be estimated:

$$\tau_{RC} = AR_aC_m = \pi r^0 C_m/(2\ \Lambda^0 c). \tag{44}$$

Assuming that $C_m \sim 5 \times 10^{-7}$ F/cm², $\Lambda^0 = 100$ S cm²/mol for NaCl (Harned and Owen, 1950), and $r^0 \sim 0.05$ cm, we find that $\tau_{RC} \simeq 4 \times 10^{-5}, 4 \times 10^{-6}$, and 4×10^{-7} s for $c = 0.01, 0.10$, and 1.0 M, respectively. If we now assume that this capacitive current can be ignored and that a value of $V_i \geq 0.100$ V effectively translocates all the adsorbed charge from one side of the membrane to the other, then (from Eq. 18) when the translocation process is complete,

$$\Delta q_c = q^0, \tag{45}$$

and (from Eq. 28) we deduce the initial (and clearly maximum) translocation current density:

$$i_c^* = q^0 \cdot s. \tag{46}$$

Combining with Eq. 43, we can estimate the maximum value of the voltage drop through the uncompensated resistance:

$$\Delta V_{iR} = A i_c^* R_a = \pi r^0 q^0 s/(2\ \Lambda^0 c). \tag{47}$$

Assigning r^0 and Λ^0 the values previously assumed and using the data in Table I, we can estimate ΔV_{iR} for a 1 M NaCl solution. The maximum value is less than 1 mV, small enough to be ignored. Another system which exhibited faster relaxations (larger s) than TϕB$^-$, produced higher adsorption (facilitated by a larger partition coefficient), or involved a less conductive supporting electrolyte (smaller $\Lambda^0 c$), could easily produce values of ΔV_{iR} too large to be ignored.

An Inner Voltage Clamp with iR Compensation

The present "inner clamp" circuitry could be easily modified to compensate for this ΔV_{iR} simply by placing a resistor, R_{comp},[4] in series with the integrating capacitor, C_I (see Fig. 2). The output of the "integrator," V_I, would then be the sum of two voltages: one voltage across C_I proportional to $A \int_0^{t_1} i_c\, dt$ or Aq_c; the other voltage proportional to $Ai_c R_{\text{comp}}$ and therefore to ΔV_{iR}. Thus, if we presume that the charging of the membrane capacitance, C_m, is finished before t_1, and the feedback adjustments are properly made, we can deduce (see Eqs. 26–30):

$$\Delta V_I = A \cdot [\exp(-st_1)]\{i_c^*/C_I s - i_c^* R_{\text{comp}}\} \cdot [1 - \exp(-s\Delta t)], \tag{48}$$

which is seen to be the same form as Eq. 30. Since the value of R_a can be directly measured or at least reasonably estimated, the appropriate value of R_{comp} could be set. If both C_I and R_{comp} were adjustable components, the optimization procedure would be facilitated. It must be remembered, of course, that the output of integrator no longer represents just the current integral but the combination indicated in Eq. 48. If $R_{\text{comp}} C_I s \geq 1$, the integrator output can describe a square step or even a decaying (rather than ascending) transient.

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[4] This method of *iR* compensation was first suggested by Hodgkin, et al. (1952). One may not be able to effect perfect compensation and maintain stability. For a more explicit discussion see Lauer and Osteryoung (1966).

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