Thermodynamic and Kinetic Studies of the Gating Behavior of a K^+-selective Channel from the Sarcoplasmic Reticulum Membrane

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ABSTRACT A voltage-dependent, K^+-selective ionic channel from sarcoplasmic reticulum of rabbit skeletal muscle has been studied in a planar phospholipid bilayer membrane. The purpose of this work is to study the mechanism by which the channel undergoes transitions between its conducting and nonconducting states. Thermodynamic studies show that the "open" and "closed" states of the channel exist in a voltage-dependent equilibrium, and that the channel displays only a single open state; the channel conductance is 120 pmho in 0.1 M K^+. The channel's gating process follows single exponential kinetics at all voltages tested, and the individual opening and closing rate constants are exponentially dependent on voltage. The individual rate constants may also be determined from a stochastic analysis of channel fluctuations among multiple conductance levels. Neither the thermodynamic nor the kinetic parameters of gating depend on the absolute concentration of channels in the bilayer. The results are taken as evidence that the channel gates by an unusually simple two-state conformational mechanism in which the equivalent of 1.1 net charges are moved across the membrane during the formation of the open channel.

Of the events leading to the contraction of vertebrate skeletal muscle, those involved in the release of Ca^{++} from the sarcoplasmic reticulum (SR) membrane are the least understood. It is clear that the permeability of the SR membrane to Ca^{++} is greatly increased during the release process (Endo, 1977), but it is not known to what extent other ions participate in any voltage and conductance changes that may occur during Ca^{++} release. One obvious question to arise from any consideration of Ca^{++} movements is: what other ions move across the SR membrane to maintain electroneutrality and, hence, to permit the rapid, massive fluxes of Ca^{++} into and out of the SR throughout the contraction-relaxation cycle? Indirect approaches have yielded estimates of the overall SR conductance (Vergara et al., 1978), but the ionic basis of this is entirely unknown. In particular, the role of K^+, the overwhelmingly abundant ion on both sides of the SR membrane in vivo (Somlyo et al., 1977)) is obscure.

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Two lines of study initiated recently point to the existence in the SR membrane of a conductance pathway specific for small monovalent cations. Using a combination of radioactive flux measurements and voltage-sensitive dye methods, McKinley and Meissner (1977 and 1978) have shown that a large fraction of fragmented SR vesicles prepared from rabbit skeletal muscle are highly permeable to K^+ and Na^+, but not to choline$^+$ or Tris$^+$. They inferred that these vesicles carry a specific mechanism mediating this monovalent cation permeability. A second approach, taken in this laboratory, has been to fuse SR vesicles with a planar phospholipid bilayer and to study the conductance properties of the resulting "hybrid" membrane (Miller, 1978). By developing a set of conditions under which massive fusion occurs reproducibly, we have found that the SR-induced conductance is voltage dependent, channel mediated, and selective for small monovalent cations, particularly K^+ (Miller, 1978); Ca^{++} ion shows no measurable permeability through the channel (Coronado et al., 1980). The channel is inhibited by sulfhydryl ligands (Miller and Rosenberg, 1979 *a*), is blocked by Cs^+ (Coronado and Miller, 1979), and is modified by alkaline proteinase *b*, a lysine-arginine endopeptidase derived from pronase (Miller and Rosenberg, 1979 *b*). We consider it likely that this channel is the permeability pathway studied by McKinley and Meissner (1977 and 1978) in native SR membrane vesicles.

Although any proposals concerning the physiological function of this channel would only be speculative, we nevertheless consider the system worthy of thorough study. Previous work has provided a qualitative description of some of the channel's basic properties. We now intend to place its behavior on quantitative grounds. In this and the following paper (Coronado et al., 1980) two fundamental questions are addressed: (*a*) by what mechanism is the conducting state of the channel formed?, and (*b*) in what terms may we understand the process by which the open channel conducts ions? This report deals with the first question, i.e., with the opening and closing of the channel, a process which we will call "gating" throughout. We will show that both equilibrium and kinetic aspects of gating, as well as the voltage dependence of the process, can be understood by a remarkably simple two-state model in which the channel protein exists in only two conformations, "open" and "closed," the former being the conducting state. Transitions between the two conformations involve a change in the protein's dipole moment, a change that confers voltage dependence upon the opening and closing probabilities.

MATERIALS AND METHODS

Biochemical Procedures

SR vesicles were prepared from rabbit back and leg white muscle as described (Miller and Rosenberg, 1979 *a*). In some cases the vesicles were fractionated by density according to Meissner (1975); when this was done, channel activity was found in all three fractions, and it was highest in the "intermediate" fraction, which has the lowest contamination by either mitochondrial or surface membranes (Meissner, 1975; Hidalgo et al., 1979). Channels from back and leg muscle had identical properties, but the SR vesicles formed from back muscle were smaller on the average than those from

the leg muscle; this property made back muscle SR particularly useful for single-channel fluctuation experiments.

Phospholipids used were: phosphatidylethanolamine (PE) purified from beef heart or egg yolk as described below, diphosphatidylglycerol (DPG or "cardiolipin"), and egg phosphatidic acid (PA) from Sigma Chemical Co., St. Louis, Mo., and mixed soy phospholipids ("asolectin") also from Sigma. In some cases, the asolectin was washed free of divalent cations and proteolipids by the following procedure. Asolectin (2 g) was dissolved in 38 ml of chloroform:methanol:0.2 M aqueous EDTA (pH 7.7), 1:2:0.8 (vol/vol/vol). This solution was filtered, and to the filtrate was added 10 ml each of chloroform and EDTA solution. This mixture was centrifuged in glass bottles at 1,000 *g* for 10 min at 4°C. The upper layer was aspirated, and the lower layer dried down under vacuum. The residue was dissolved in ~20 ml of chloroform:methanol, 2:1, and proteolipids were precipitated by the addition of 400 ml of methanol at room temperature. The mixture was centrifuged as described above, and the supernate, which contained most of the phospholipids but an undetectable amount of proteolipid, was dried down. The residue was dissolved in a stock solution of 50 ml chloroform:methanol, 1:1. This lipid mixture, which will be called "washed asolectin," formed bilayers that were more stable than those formed from untreated asolectin.

For the purification of PE, a standard silicic acid preparation (Kagawa et al., 1973) of this lipid was dissolved in chloroform. About 5 mmol of this was applied to a column (2.5 cm i.d.) containing 100 g (dry wt) of Bio-Gel HTP (Bio-Rad Laboratories, Richmond, Calif.), treated as described (Slomiany and Horowitz, 1979) and equilibrated with chloroform. The column was washed with 400 ml chloroform followed by 200 ml of acetone:methanol, 7:3, and then ethanol:water, 9:1, until no more lipid appeared (~300 ml). PE was eluted with diethyl ether:ethanol:water, 10:7:3. The solvent was evaporated and the PE dissolved in chloroform:methanol, 2:1 (50 ml). Occasionally, this lipid was contaminated with small amounts of acidic phospholipids. These were conveniently removed by passing the solution through a 10-ml column of DEAE-acetate in chloroform:methanol, 2:1. This column retained the acidic lipids, allowing the PE to pass through. The final preparation of PE was at least 99% pure, as judged by thin-layer chromatography. All lipid stock solutions were stored under argon at −70°C.

Planar Bilayer System

The planar bilayer and associated electronics have been described (Miller, 1978). Bilayers were formed by either the "painting" method of Mueller and Rudin (1969) or the "monolayer folding" method of Montal and Mueller (1972). In the former case, membranes were cast onto a 0.8-mm diameter hole in a polystyrene partition separating two aqueous solutions, using solutions of 50–70 mM phospholipid in *n*-decane. In the latter case, membranes were formed over a 100–250-μm hole in a Saran Wrap partition; the hole had been previously treated with squalene in *n*-pentane. Bilayer chambers were seated in brass blocks maintained within 0.2°C of the desired temperature by a thermostatted water circulator. Unless otherwise noted, experiments were carried out at 20°C using painted bilayers.

The planar bilayers separated two aqueous solutions composed of 100 mM K^+ (glucuronate or sulfate salt), 5 mM HEPES, and 0.1 mM EDTA, adjusted to pH 7.0–7.5 with Tris base, unless otherwise stated. We refer to the two sides of the bilayer as *cis* and *trans*. SR vesicles were always added to the *cis* side, while the *trans* chamber is defined as zero voltage.

Conductance was measured under voltage-clamp conditions, where the voltage was

supplied by a function generator or a variable battery. The membrane system was connected to the current-to-voltage transducer via small glass salt bridges in series with Ag/AgCl electrodes. Output was recorded on chart paper or a storage oscilloscope and analyzed by hand.

Incorporation of SR Vesicles into the Bilayer

SR vesicles were made to interact with a planar bilayer, and thereby to insert K^+ channels into it, in the presence of anionic lipid, Ca^{++}, and osmotic conditions leading to swelling of the vesicles (Miller and Racker, 1976; Miller, 1978). Typically, SR vesicles (1–50 μg/ml protein) loaded with 0.4 M sucrose were added to the *cis* chamber in the presence of 0.3–1 mM Ca^{++}. The bilayer conductance increased in discrete "fusion events" (Miller and Racker, 1976). When the desired conductance level was attained, excess EDTA was added to stop fusion, and the membrane was allowed to stabilize for several minutes. In some cases the *cis* chamber was perfused with new buffer solution to remove the SR vesicles (Miller and Rosenberg, 1979 *a*).

We will describe two types of experiments here: macroscopic measurements, in which many channels (>1,000) are inserted into the bilayer, resulting in an increase in conductance of several orders of magnitude; and single-channel fluctuation measurements, in which a small number of channels (<10) is inserted. In the macroscopic approach, vesicles were incubated against a bilayer at high protein concentration (50 μg/ml) in the presence of high Ca^{++} (1 mM) for long periods of time (5–10 min) before insertion was stopped. In this case, membrane conductance measured at +50 mV was usually higher than 10 μmho/cm^2. For single-channel experiments, a low concentration of protein (1–5 μg/ml) was added at low Ca^{++} (0.1–0.3 mM), and incorporation was stopped immediately after the first fusion event occurred.

Measurement of Steady-State g-V Curves

Steady-state conductance-voltage (g-V) curves were measured by applying a desired voltage and allowing the current to relax to its steady-state level. The relaxation time depended on bilayer composition, temperature, and voltage, but in all cases at least five half-times were allowed to pass before steady-state conditions were proclaimed. Points were usually taken in a random sequence of voltages, but in some cases we ensured that no hysteresis was occurring by comparing g-V curves with voltages applied in increasing or decreasing sequences.

For certain experiments it was necessary to measure the macroscopic g-V curve at a very low level of mean conductance, with only a small number of channels present in the membrane (<100). Under these conditions the amplitude of conductance noise due to the random opening and closing of channels is a substantial fraction of the mean conductance. We were, therefore, careful to measure the time-averaged conductance over a long period of time for calculating the g-V curve under these low conductance conditions.

It will be shown that the g-V curve is sigmoid, with a low "background conductance" asymptote, g_b, at highly negative voltages and a "maximum conductance" asymptote, g_m, at highly positive voltages. Although the background conductance could be measured directly at voltages more negative than −70 mV, it was usually necessary to estimate the maximum conductance by fitting the data to a theoretical curve. This was done as follows. The data were corrected for background conductance and then graphed according to Eq. 9, using an initially estimated value for g_m; the least-squares regression line was determined and the correlation coefficient was computed. The value of g_m was then incremented and the procedure repeated. The value of g_m yielding the maximum correlation coefficient was taken as the "true"

value of g_m. For all data reported here, correlation coefficients for the best least-squares lines were always >0.990. Experience with the system, with membranes giving data to over 90% saturation but with the data only up to 70% saturation, and with "model data," shows that this method, though not mathematically rigorous, can easily determine g_m to within 10%.

Once g_b and g_m were determined from measurements on a given membrane, the data were normalized to the maximum conductance, thus defining the "relative conductance," g_{rel}:

$$g_{rel} \equiv g/g_m. \tag{1}$$

The relative conductance is a purely empirical parameter useful for comparing data from different membranes. Although values of the background and maximum conductances varied according to the number of channels inserted into the bilayer, the relative conductance was quantitatively reproducible from the membrane to membrane for a given set of conditions. In general, g_b fell in the range 3–8% of g_m.

Gating Kinetics

Measurements of gating kinetics were performed by standard voltage-jump techniques. A "holding voltage" was applied until steady-state current was reached. The applied voltage was then suddenly shifted to a test voltage. The relaxation of current to the new steady state was recorded and analyzed by hand. In some cases it was necessary to signal-average the current responses of membranes containing a very small number of channels (<100) to eliminate the noise arising from individual channel fluctuations. In these cases, the relaxations from 20 repetitive sweeps were averaged by sampling the records at discrete time intervals after the voltage jump and averaging by hand.

The Two-State Model

In previous work we suggested that the SR K^+ channel opens and closes by a simple "two-state" conformational mechanism (Miller and Rosenberg, 1979 *a* and *b*). It is our intention here to describe this model precisely and to test its predictions quantitatively. In this section we wish to set forth the seven explicit postulates upon which the model rests.

We consider a channel protein (or protein complex) that may undergo conformational transitions between only two states, open (conducting) and closed (nonconducting). The gating process can then be described by the following conformational transition scheme:

$$\text{CLOSED} \underset{\mu(V)}{\overset{\lambda(V)}{\rightleftharpoons}} \text{OPEN}, \tag{2}$$

where $\lambda(V)$ and $\mu(V)$ are the voltage-dependent rate constants for the opening and closing reactions, respectively. The postulates of the model are the following.

(1) Channels are incorporated into the bilayer irreversibly. That is, the total number of channels in a given membrane is fixed, and no equilibrium exists among the bilayer, torus, and aqueous phase.

(2) Channels are inserted into the bilayer with a fixed orientation, i.e., the channel protein is not free to reorient by a flip-flop process.

(3) Each channel has a single open and a single closed state.

(4) Channels open and close independently of one another.

(5) The two conformations of the channel carry different dipole moments (normal to the plane of the bilayer).

(6) The single channel conductance is independent of voltage.

(7) The channel is a preformed unit and does not rely upon the aggregation of independently diffusing subunits for its formation.

Throughout the presentation of the results, we will refer to these postulates to show how the model makes quantitative predictions about the channel's gating behavior under the particular conditions under consideration.

RESULTS

(1) *Thermodynamics of Gating*

INCORPORATION OF CHANNELS Under a well-defined set of conditions, SR vesicles added to the aqueous phase of a planar bilayer system cause a massive increase in bilayer conductance. The effect depends on anionic lipid in the bilayer, divalent cation in the medium, and osmotic conditions leading to swelling of the SR vesicles (Miller and Racker, 1976; Miller, 1978). Fig. 1 demonstrates this effect, with K^+ as the major current-carrying ion in the system, at four levels of current and time resolution. With conditions very favorable to fusion (Fig. 1 *A*), the bilayer conductance increases rapidly, reaching levels >10 μmho/cm^2. Under less favorable fusion conditions (Fig. 1 *B*), the conductance increase may be examined at higher gain and is seen to be composed of a sequence of spikelike events of widely varying size. Each such event consists of an abrupt increase in conductance followed by relaxation to a low but nonzero steady level. By reducing the fusion probability further, it is possible to separate the occurrence of the spikes by large time intervals (Fig. 1 *C*), so that an individual spike may be examined at higher gain and time resolution (Fig. 1 *D*). In *D*, the spike relaxation appears to take place via a sequence of discrete and unitary downward conductance transitions of ~120 pmho.

It is known that K^+ conductance induced by SR is mediated by a voltage-dependent channel mechanism, and it has been suggested that each of the spikes described above represents the fusion of a single SR vesicle with a bilayer; it has also been suggested that each of these fusion events results in the insertion of a "package" of K^+ channels (Miller and Racker, 1976; Miller, 1978). This is intimated in Fig. 1 *D* and is shown clearly in Fig. 2, in which single spike events are examined at higher resolution. Here the resolution is obtained by using bilayers containing egg phospholipids, which give slower channel fluctuations than other lipids we have used; this facilitates the accurate recording of the fine structure of the spikes. We see that the spikes do relax in unitary conductance transitions, each having a value of 140–150 pmho. This agrees with the previously determined size of the open channel, which falls in the range 100–150 pmho in 0.1 M K^+, depending upon external conditions such as the bilayer composition, particularly the surface charge (Miller, 1978; Miller and Rosenberg, 1979 *a* and *b*; Coronado and Miller, 1979). We, therefore, conclude that each spike leads to the simultaneous insertion of a package of channels, most of which are open at the moment of insertion, and which then proceed to close as they find their new equilibrium in the planar bilayer, at the prevailing voltage. A typical insertion event

involves the incorporation of 5–50 channels; only occasionally have we observed the insertion of only one channel at a time.

Fig. 2 also provides a comparison between single spikes in bilayers containing large amounts of solvent (painted membranes) and bilayers that are nominally "solvent-free" (folded membranes). Qualitatively, the spikes are similar, with channel fluctuations of equal height. We have consistently

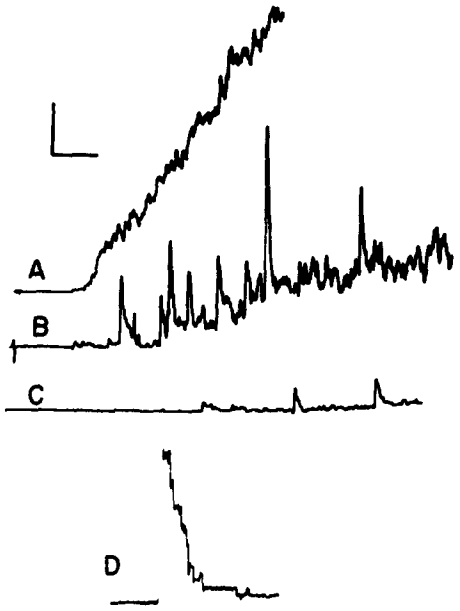

FIGURE 1. Effect of SR vesicles on planar bilayer conductance. SR vesicles were fused with planar bilayers (70% washed asolectin–30% DPG) under conditions variously favorable to the fusion process. Membrane conductance was monitored at −50 mV. In traces *A*, *B*, and *C*, SR vesicles were added at the beginning of the trace. (*A*) Highly favorable fusion conditions. SR (50 μg/ml) were added in the presence of 1 mM Ca^{++} and 200 mM glycerol that had been added to the *cis* chamber. Verticle bar, 10 μmho/cm^2 (0.8 nA); horizontal bar, 20 s. (*B*) "Normal" fusion conditions. SR (10 μg/ml) were added in the presence of 0.5 mM Ca^{++}. Vertical bar, 0.5 μmho/cm^2; horizontal bar, 20 s. (*C*) Reduced fusion conditions. Same conditions and scale as in *B*, except that 1 μg/ml SR was added. (*D*) Single fusion spike. Conditions were as in *C*; a single spike, captured on an oscilloscope trace, is displayed. Vertical bar, 45 pA; horizontal bar, 1 s.

observed that the channel fluctuations are faster in folded membranes than in painted ones, as is evident from the figure.

After channel incorporation has been stopped, the membrane conductance is stable; removal of both Ca^{++} and SR vesicles by extensive perfusion of the *cis* chamber leaves the membrane conductance unchanged for at least an hour (data not shown). We therefore conclude that channels are inserted irreversibly and are not lost to either the torus or the aqueous solution.

VOLTAGE DEPENDENCE OF STEADY-STATE CONDUCTANCE In media containing K^+ as the major current-carrying species, the steady-state macroscopic conductance of bilayers containing many channels depends on the applied voltage, as shown in Fig. 3 *A*. At voltages more negative than −60 mV, the membrane is in a low conductance state representing an ohmic "background conductance" induced by the SR vesicles (Miller, 1978). As applied voltage is varied in the positive direction, the conductance increases and eventually approaches a maximum level.

This type of conductance-voltage relation is predicted by the two-state model of channel gating. The model assumes that the steady-state conductance of many-channel membranes is an expression of a thermodynamic equilibrium

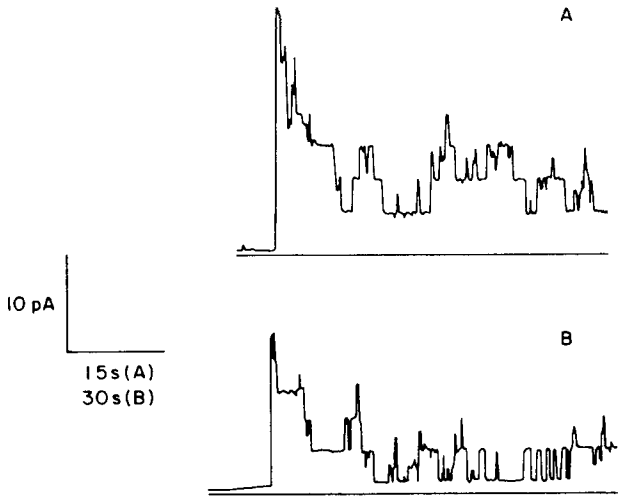

FIGURE 2. Fine structure of fusion spikes. Single fusion spikes are shown, from either folded (*A*) or painted (*B*) bilayers composed of 70% asolectin–30% egg PA. Fusion was monitored at +20 mV, under the conditions of Fig. 1 *B*. Single-channel conductances are 148 ± 4 pmho in folded membranes, and 130 ± 2 pmho in painted membranes.

between the two states of the channel, i.e., that the conductance measures the average number of channels in the open state. The conductance of the open channel is assumed to be independent of voltage. All of the voltage dependence is assumed to be contained in the conformational equilibrium constant of Scheme 2. Because the two states of the protein carry different dipole moments, the free energy change of the conformational transition will have a field-dependent term.

To derive an expression for the g-V curve, we note that the total conductance of a membrane containing n channels is the sum of an ohmic background conductance and a voltage-dependent, channel-mediated conductance:

$$g(V) = g_b + n\gamma p(V), \tag{3}$$

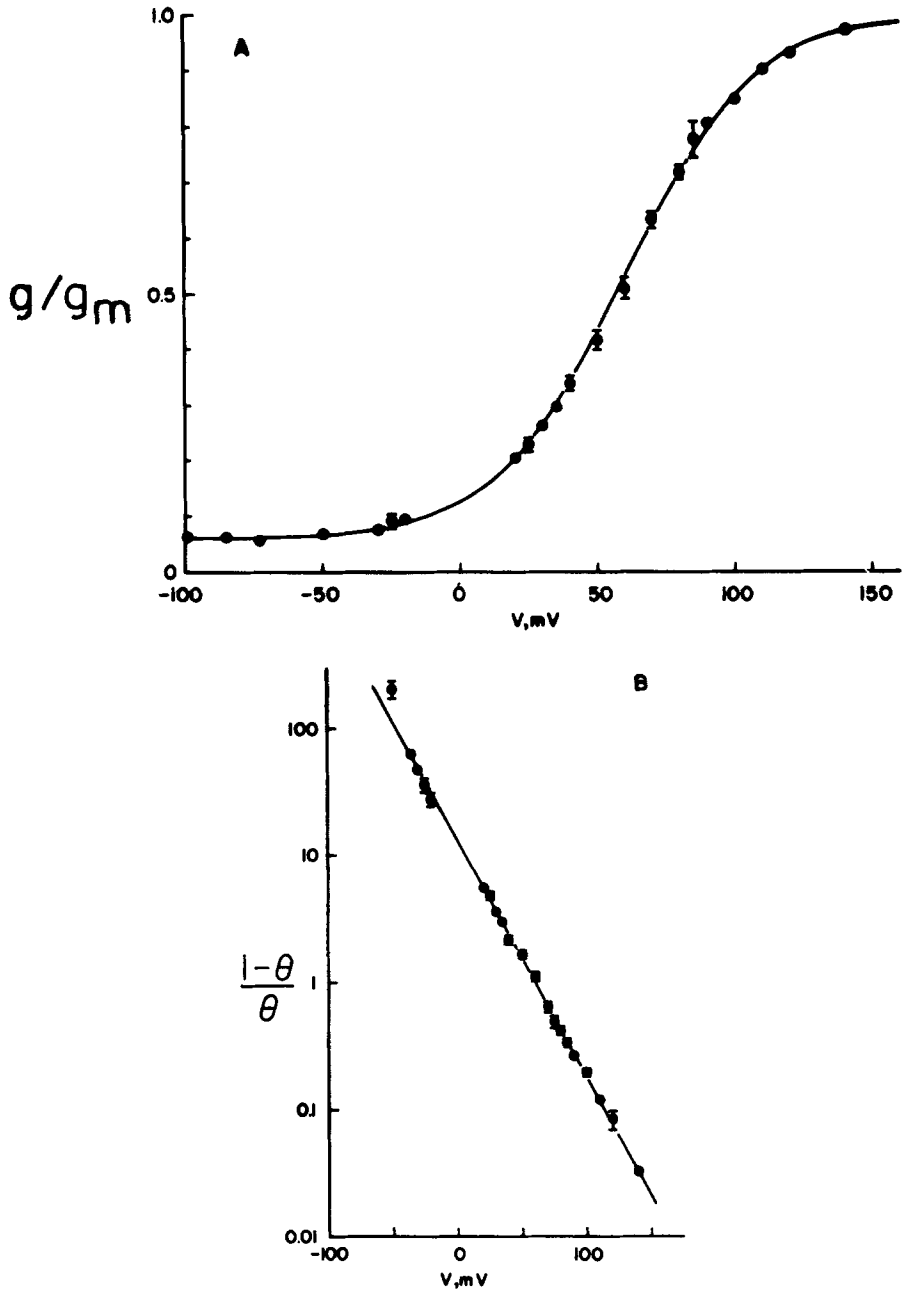

FIGURE 3. Steady-state conductance-voltage relation. SR vesicles were allowed to interact with planar bilayers under the conditions of Fig. 1 *B*. After 5–10 min, fusion was stopped as described in Materials and Methods, and the steady-state conductance was measured at various applied voltages. The aqueous phase was 100 mM K^+-glucuronate buffer. Values of maximum conductance were in the range 13–140 μmho/cm^2. (*A*) Compiled data from four membranes, with each set normalized to its maximum conductance. Points show means ± SEM. Points without error bars are either single determinations or measurements in which the SE is smaller than the width of the point. Solid curve is drawn according to Eq. 6, with $z = -1.08 \pm 0.05$, and $\Delta G_i = +1.48 \pm 0.03$ kcal/mol (SD). (*B*) Same data plotted according to Eq. 9.

where γ is the single-channel conductance and $p(V)$ is the voltage-dependent probability of any one channel existing in the open state. Since we assume that the open and closed states are in equilibrium, $p(V)$ can be expressed in terms of the total Gibbs free energy of the opening reaction:

$$p(V) = (\text{number of open channels})/n \tag{4 a}$$

$$p(V) = (1 + \exp \Delta G/RT)^{-1}. \tag{4 b}$$

We can write the voltage dependence of ΔG explicitly. We assume that the total free energy of opening is the sum of a "chemical" part, ΔG_i, which we shall call the "internal free energy of opening," and an electrical part given by the difference in energy of the dipole moments of the two conformations of the protein in the electric field:

$$\Delta G = \Delta G_i + zFV. \tag{5}$$

Here the parameter z is the "effective gating charge," a fictitious electrical charge introduced as the energy equivalent of the protein's change in dipole moment (Ehrenstein and Lecar, 1977).

We can now write the expression for the macroscopic g-V curve:

$$g(V) = g_b + n\gamma\{1 + \exp([\Delta G_i + zFV]/RT)\}^{-1}. \tag{6}$$

An expression of this type has been derived for a similar case by Ehrenstein et al. (1974), but we prefer to use the alternative form here because it emphasizes the thermodynamic meaning of the conductance at zero voltage, which was presented as a "lumped" parameter in the previous treatment. The graph of Eq. 6 (Fig. 3 *A*) is a sigmoid curve whose slope and position on the voltage axis are determined by the two parameters z and ΔG_i. To apply the equation to experimental results, it is helpful to linearize it in such a way that it may be used on g-V curves taken from different membranes, with different levels of absolute conductance. We note that:

$$n\gamma = g_m - g_b, \tag{7}$$

and so we can define the normalized conductance corrected for background, which we call the "normalized channel activity," θ:

$$\theta(V) = (g(V) - g_b)/n\gamma \tag{8}$$

Thus, Eq. 6 now takes the form:

$$\ln\{[1 - \theta(V)]/\theta(V)\} = \Delta G_i/RT + zFV/RT. \tag{9}$$

A plot of θ vs. V as in Eq. 9 should be linear and will determine both ΔG_i and z. Such a plot (Fig. 3 *B*) shows that the two-state model is adequate to describe the steady-state g-V curve of a membrane containing many channels. For the conditions used in this figure, $\Delta G_i = +1.5$ kcal/mol, and $z = -1.1$. The positive sign of ΔG_i indicates that the closed state is favored thermodynamically at zero voltage, and the negative sign of z indicates that net negative charge moves towards the *cis* side of the membrane when the channel opens.

We have found that, whereas z is independent of external variables such as

lipid composition and temperature, ΔG_i is quite sensitive to these and other variables. In Fig. 4, for instance, the effect of changing the pH of the aqueous phase is shown. It has been found that lowering the pH inhibits the macroscopic conductance at a fixed voltage (Miller, 1978), but this effect was not explained. We now see that this inhibition occurs because the *g-V* curve shifts to the right along the voltage axis as pH is lowered; in other words, ΔG_i becomes increasingly positive with increased acidity, whereas the gating charge varies little with pH in this range.

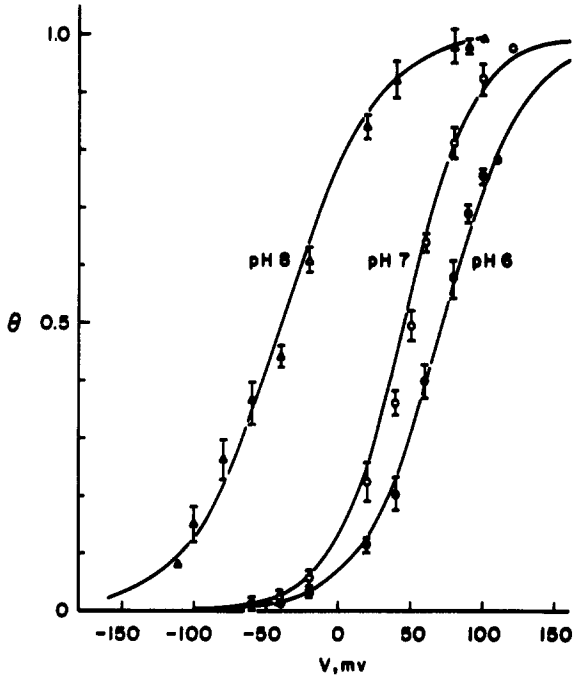

FIGURE 4. Effect of pH on *g-V* curve. Conductance-voltage curves were determined as in Fig. 3, with bilayers of 70% asolectin–30% DPG, as a function of pH of the aqueous solution (100 mM K-glucuronate medium with 5 mM HEPES or MES, adjusted to the indicated pH with Tris base). Data were corrected for background and normalized, and the channel activity, θ, is plotted. Solid curves are drawn according to Eqs. 6 and 8, with parameters as follows:

pH 6: $z = -0.92$; $\Delta G_i = +1.56$ kcal/mol.

pH 7: $z = -1.1$; $\Delta G_i = +1.16$ kcal/mol.

pH 8: $z = -0.8$; $\Delta G_i = -0.73$ kcal/mol.

By studying the *g-V* curve as a function of temperature, we can ask to what extent the internal free energy of opening is controlled by enthalpy and entropy, since for this equilibrium,

$$\Delta H_i = \Delta G_i + T\mathrm{d}\Delta G_i/\mathrm{d}T \qquad (10\,a)$$

$$\Delta S_i = -\mathrm{d}\Delta g_i/\mathrm{d}T. \qquad (10\,b)$$

In Fig. 5, we show that ΔG_i varies with temperature in asolectin membranes in a way indicating that the opening reaction is enthalpically favored (ΔH_i = −9.7 kcal/mol) and entropically disfavored (ΔS_i = −36 cal/mol-K). We have made careful measurements of these thermodynamic functions only in asolectin membranes, but preliminary results suggest that the magnitudes and even the signs of ΔH_i and ΔS_i change with the lipids used to form the bilayer. We are therefore unwilling to claim that the state functions of opening are determined solely by the protein structure; lipid-protein interactions may also be involved.

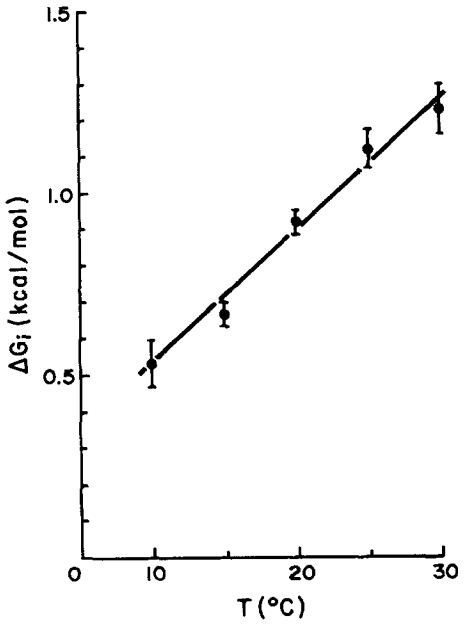

FIGURE 5. Effect of temperature on free energy of channel opening. SR vesicles were fused with asolectin membranes, under the conditions of Fig. 3, and g-V curves were determined at various temperatures. The effective gating charge, z, and the internal free energy of opening, ΔG_i, were calculated for each membrane. The effective gating charge did not vary with temperature (z = −1.0 ± 0.1), and the figure displays the variation of ΔG_i. Each point represents the mean and SE of three to four determinations, each on a separate membrane. Results did not depend upon the temperature at which fusion occured, only upon the temperature at which the g-V curves were measured.

The presence of solvent in the planar bilayers is a serious concern in the study of the function of the channel. We therefore compared gating properties in painted bilayers containing massive amounts of decane with those in folded membranes, which are nominally solvent-free. Table I reports the values of the g-V curve parameters, as well as the single-channel conductance in painted and folded membranes. In both types of membrane the g-V curve follows Eq. 6, and the effective gating charge is 1.1; a small difference in ΔG_i is seen, with

the open state being slightly more favored (0.4 kcal/mol) in folded membranes. The single-channel conductances in the two types of membrane are within 10% of each other. Even folded membranes probably contain some solvent (squalene in this case), although the amount is low (Montal and Mueller, 1972; White et al., 1976; White, 1978; Alvarez and Latorre, 1978; Fahey and Webb, 1978). Therefore, we cannot say rigorously that the channel is insensitive to the bilayer solvent, but its does seem unlikely that its gross features are destroyed by painted membranes.

SINGLE-CHANNEL GATING STATISTICS The results described above show that the voltage-dependent conductance of many-channel membranes can be explained by the two-state model and that variations in the g-V curves can be understood naturally in terms of the model. But this agreement hardly verifies the assumptions underlying Eq. 6. To do this, we need to study the gating properties of individual channels, and the planar bilayer system does, in fact, provide this capability. By allowing only a single SR vesicle to fuse with the

TABLE I

COMPARISON OF THERMODYNAMIC GATING CONSTANTS IN PAINTED AND FOLDED BILAYERS

Type of bilayer	z	ΔG_i	Voltage
		kcal/mol	*mV*
Painted	1.05±0.03	0.87±0.07	36±2
Folded	0.93±0.06	0.60±0.02	28±3

Conductance-voltage curves were determined as in Fig. 3, using 70% asolectin–30% PA bilayers formed by either the painting method or the folding method. Capacitances were 0.4–0.5 $\mu F/cm^2$ for painted membranes and 0.7–0.8 $\mu F/cm^2$ for folded membranes. The means and SE of three to four determinations, each on a different bilayer, are reported. The voltage (V) at which the macroscopic conductance is half saturated is also reported, and is given by $V = \Delta G_i/zF$.

bilayer, we can examine the low-level conductance fluctuations due to the random opening and closing of single channels. In particular we can discover in what way the individual channels are controlled by the applied voltage.

Fig. 6 displays single-channel fluctuation traces at −50 and +50 mV in a membrane containing only one channel. We see that at −50 mV most of the time the conductance is at a low level representing the channel's closed state. Only occasionally does the channel open. At +50 mV, the reverse situation holds. Now the channel is open most of the time. Thus, changing the voltage from −50 to +50 mV has had the effect of increasing the probability of forming the open channel; the channel conductances at the two voltages are identical. The channel formation probability can be directly measured from these traces as the fraction of time the channel spends in the open state.

A membrane with only one channel incorporated into it is rarely obtained. The usual result of a single vesicle fusion is the insertion of several channels, typically 5–10, as in Fig. 2. Here, the analysis is more complicated because the membrane conductance can now fluctuate among several levels representing different numbers of channels simultaneously open. It is possible to analyze these multiple-level fluctuations quantitatively by constructing frequency

histograms of the membrane conductance. These histograms, such as those shown in Fig. 7, demonstrate that only well-defined conductance levels appear, and that applied voltage controls the probabilities of the appearance of the various levels. The spacing between the levels is independent of voltage and has a value of 110 pmho under these conditions.

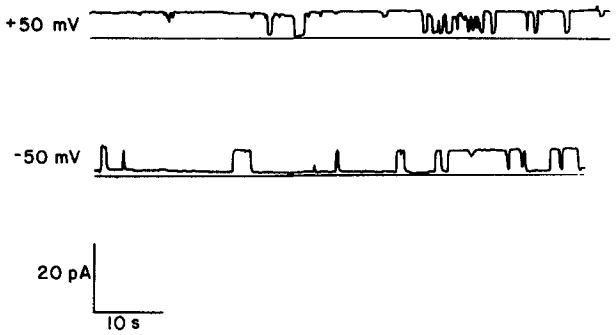

FIGURE 6. Channel fluctuations in a single-channel membrane. Current records were taken at the indicated voltages in a bilayer (asolectin) after fusion with a vesicle containing only a single channel. Aqueous phase was 50 mM K_2SO_4 buffer.

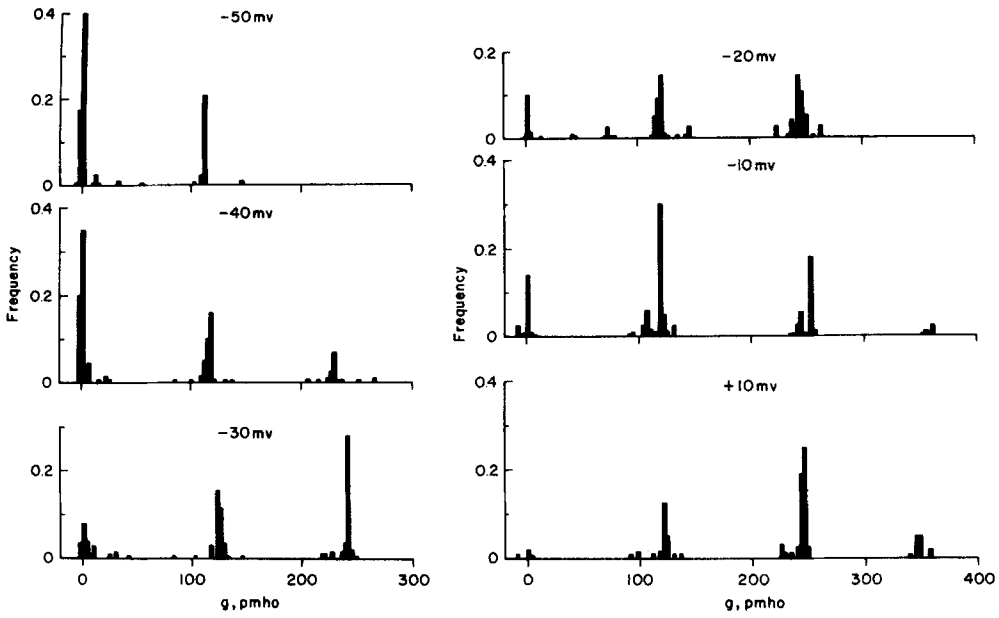

FIGURE 7. Channel conductance histograms as a function of voltage. A single SR vesicle was fused with a bilayer composed of 90% egg PE–10% egg PA. Fusion was then stopped, and current records were taken at the various applied voltages indicated on the figure. Data were taken for 2–3 min for each voltage. Current traces were sampled at 0.2-s intervals, and frequency histograms were calculated by normalizing to the total number of data points (600–1,000 points at each voltage).

This type of behavior would be expected qualitatively for a membrane containing a small number of identical channels, each having only a single open state. The higher conductance levels would represent the simultaneous appearance of several open channels rather than a single channel with several equally spaced open states. As voltage is made more positive, the probability of opening would increase, according to the interpretation of the macroscopic g-V curve, and so the higher levels would be observed more frequently.

The above interpretation of the multiple-level fluctuations needs to be verified quantitatively before it can be accepted. First, we must ask whether the voltage dependence of the time-averaged microscopic conductance matches that of the macroscopic g-V curve. If a membrane contains a small number of n channels, then the conductance will fluctuate among $n + 1$ levels, and the time-averaged, normalized channel activity, $\langle\theta\rangle$ can be calculated:

$$\langle\theta\rangle = \sum_{i=0}^{n} if_i/n, \tag{11}$$

where f_i is the frequency of appearance of the ith conductance level, or the "i-level frequency." It is easily measured from single-channel histograms, as in Fig. 7. In Fig. 8 we compare the voltage dependence of the macroscopic channel activity measured in a membrane containing ~5,000 channels with that of the time-averaged channel activity measured in a membrane with five channels. It is apparent that the macroscopic (ensemble-averaged) g-V curve can be fully described by the microscopic (time-averaged) channel formation probability.

Another important conclusion from Fig. 8 is that the channel appears to operate as a preformed unit rather than by a subunit aggregation mechanism. If the open channel were formed by the aggregation of independently diffusing subunits, as in the case of alamethicin, for example (Eisenberg et al., 1973; Boheim and Kolb, 1978), then we would expect the position of the g-V curve along the voltage axis to depend upon the absolute concentration of channels in the bilayer. This is not the case, and so the simplest explanation is that independently diffusing subunits are not involved in channel gating.

The result illustrated in Fig. 8 shows only that the macroscopic and microscopic methods are assaying the same phenomenon, i.e., voltage-controlled channel gating. But what evidence is there that the gating operates on the single-channel level as required by the particular model under consideration here? First, we should note that there is already strong evidence that the multiple conductance levels represent a number of open channels rather than a single channel fluctuating among several open states (Fig. 6). This conclusion can be further strengthened and the two-state model tested crucially by attempting to predict the values of the i-level frequencies from an *a priori* calculation. If we assume that the channels gate independently, then we can state that the i-level frequencies must follow a binomial distribution:

$$f_i = \binom{n}{i} p^i (1 - p)^{n-i}, \tag{12}$$

where p is the voltage-dependent probability that any one channel exists in the open state, and n is the member of channels in the membrane. The value

of p is given by Eq. (4 *b*), and is measured directly from the macroscopic g-V curve. The number of channels in a given membrane is also directly measurable using Eq. 7. Therefore, the theoretically expected values of all the f_i can be calculated *a priori* using only the independently determined values of p and n. These calculated i-level frequencies can then be compared with those measured from conductance histograms. Fig. 9 shows such a comparison made in a membrane containing four channels. The measured frequencies follow

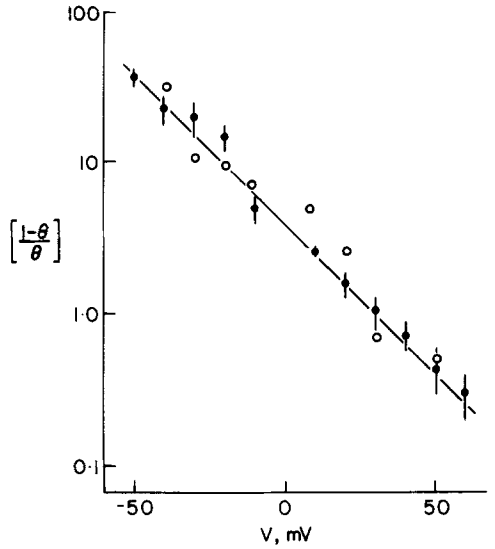

FIGURE 8. Macroscopic and microscopic channel activities. Conductance-voltage relations were measured as in Fig. 3 for membranes containing either a large number (1,500–5,000) of channels (filled circles) or a small number (5–6) of channels (open circles). For each case, the normalized channel activity, θ, was calculated, and a linearized plot of this parameter is plotted, as in Fig. 3 *B*. For the membranes containing a small number of channels, the g-V curve was calculated by time-averaging the current trace, as explained in Materials and Methods. For the high-conductance membranes, each point represents the mean ± SEM of five determinations, each on a separate bilayer. Membrane and aqueous phase compositions were as in Fig. 7. Line is drawn according to Eq. 9, with $z = -1.14$ and $\Delta G_i = +0.87$ kcal/mol.

the required binomial distribution well at all voltages tested, and this has been our experience using measurements with varying external conditions, such as pH and lipid composition.

We consider that the success of this test establishes: (*a*) that each channel has only a single open state, (*b*) that channels gate independently, and (*c*) that the probability of channel formation varies with voltage as required by the two-state model.

(2) *Kinetics*

MACROSCOPIC GATING KINETICS The results presented in the previous section show that the two-state conformational model adequately describes the equilibrium aspects of the channel's gating. But this agreement could also be

expected with a model postulating the existence of a single open state in equilibrium with several closed states, such as the squid axon Na^+ channel model of Armstrong and Bezanilla (1977). To test the possibility of multiple closed states, we must resort to kinetic experiments. The simple two-state scheme (Eq. 2), makes several strong predictions for the kinetic response to a sudden change in voltage across a membrane containing many channels.

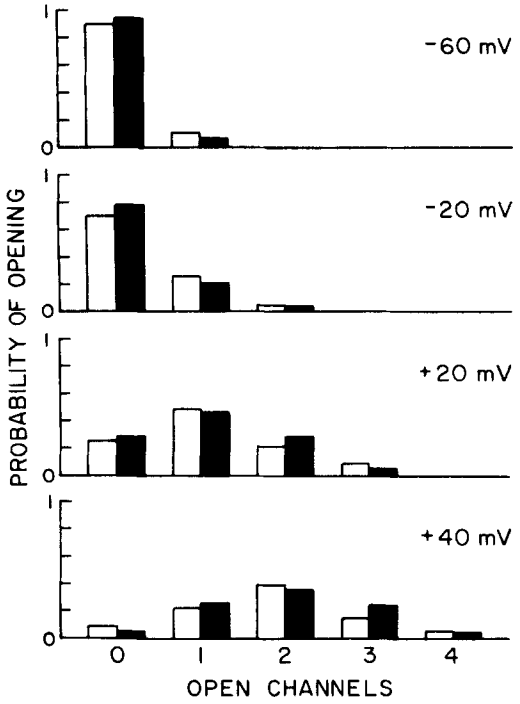

FIGURE 9. Multiple-level frequencies follow binomial statistics. Single-channel recordings were made on a single membrane at various voltages (indicated on figure), as in Fig. 7. This membrane contained four channels, a number determined by measuring the time-averaged conductance at various voltages and applying Eq. 7. Data were taken for 4–5 min at each voltage. Open bars represent the measured fraction of time spent with 0, 1, 2, 3, or four channels open simultaneously. Filled bars were calculated for a binomial distribution, Eq. 12, using $n = 4$, and voltage-dependent values of p given by equation 4 *b*, with $z = -1.2$ and $\Delta G_i = +1.03$ kcal/mol. These parameters were determined independently from g-V curves in many-channel membranes, as in Fig. 3.

First, the scheme predicts that the current after such a voltage jump must always relax in a single-exponential fashion from an initial value representative of the conductance at the "holding voltage" before the jump. The relaxation must follow:

$$[g(V, t) - g(\infty)]/[g(0) - g(\infty)] = \exp[-t/\tau(V)]. \quad (13)$$

The measured time constant $\tau(V)$ is given in terms of the opening and closing

rate constants in Scheme 2:

$$1/\tau(V) = \lambda(V) + \mu(V). \tag{14}$$

In a model with multiple closed states, more complicated kinetics are generally expected. For instance, a delay in channel opening would be anticipated in jumps for highly negative to highly positive voltages.

Fig. 10 offers a current response to a voltage jump from zero voltage to −50 mV. A net closing of channels is seen here, and the relaxation is single exponential. This is our experience in general; we have never observed multiple exponential kinetics or delays in any voltage-jump conditions employed, down to 1-ms time resolution.

Scheme 2 also predicts that although the relaxation time constant may depend upon the test voltage after the jump, it must be independent of the holding voltage from which the jump is made. This prediction is verified, as shown in Fig. 11 *A*. Here we observed relaxations at −50 mV from various holding voltages; the time constant is independent of the holding voltage, regardless of whether net opening or net closing occurs during the relaxation.

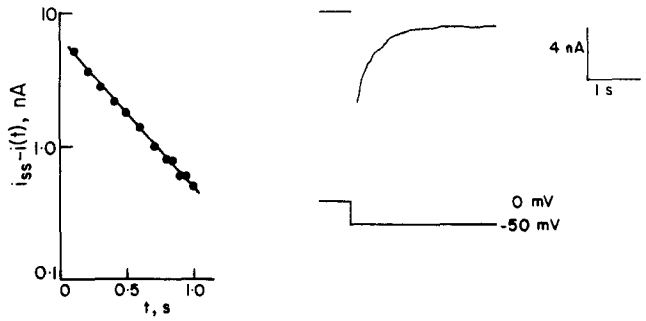

FIGURE 10. Macroscopic gating kinetics: voltage-jump relaxation. Relaxation of current was followed after a sudden change in voltage from zero to −50 mV, in a many-channel membrane (70% asolectin–30% DPG), at 30°C. *Inset* shows a semilogarithmic plot of the trace, sampled at 0.1-s intervals.

This behavior would not be expected of a model with multiple closed states, which would in general possess "memory" of the voltage from which a jump is made.

A further requirement of any "conformational" model is that the relaxation time constant be independent of the total concentration of channels in the bilayer; this would not be the case for a subunit aggregation mechanism, as, for instance, with gramicidin (Bamberg and Läuger, 1973). In Fig. 11 *B* we see that the kinetic constant is in fact independent of the absolute steady-state conductance of the membrane, over at least two orders of magnitude.

A final strong prediction of Scheme 2 concerns the voltage dependence of the individual opening and closing rate constants λ and μ. A macroscopic relaxation experiment does not measure these constants directly, only their sum (Eq. 14). However, we can use the model to extract the individual rate

constants, because the steady-state conductance is an expression of the equilibrium constant between open and closed states, and this equilibrium constant is given by the ratio λ/μ. Thus, the rate constants are given in terms of normalized channel activity θ and the relaxation time constant τ, both of

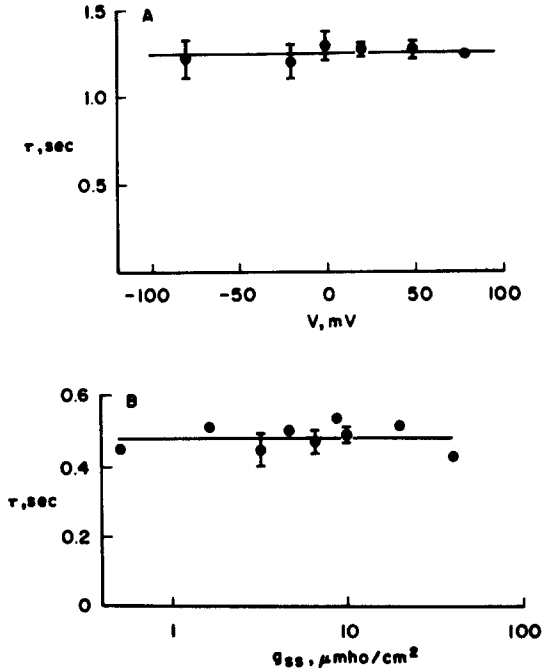

FIGURE 11. Independence of relaxation time of holding voltage and absolute conductance. The relaxation times, τ, of voltage-jump relaxations were measured under the conditions of Fig. 10, except that the holding voltage or the absolute membrane conductance was varied. Points with error bars represent three to four determinations, each on a separate membrane; points without error bars are single determinations. (*A*) Variation in holding voltage. The holding voltage indicated on the graph was applied until steady-state conductance was reached, and then voltage was jumped to −50 mV in all cases. Relaxation of current was followed, and the relaxation time was determined. (*B*) Variation in conductance. Voltage jumps from zero to +40 mV were applied to membranes that had interacted with SR vesicles to various extents, yielding the steady-state conductances, g_{ss}, shown on the figure. Estimated number of channels in the bilayer was 25 for the lowest conductance point and 2,700 for the highest conductance point. Temperature was 30°C.

which are measurable:

$$\lambda = \theta/\tau \tag{15 a}$$

$$\mu = (1 - \theta)/\tau \tag{15 b}$$

In general, the voltage-dependence of λ and μ will reflect the electrical energy profile experienced by the channel protein during the entire gating

process. However, the simplest kinetic model makes a simple prediction. If we assume that there is a single Eyring-type transition state between the open and closed states, then it follows that the opening and closing rate constants must vary exponentially with voltage (Ehrenstein et al., 1974). This prediction is a consequence of the fact that voltage appears as a linear term in the activation enthalpy between the open state, the "gating transition state," and the closed state. Fig. 12 shows that the rate constants λ and μ do in fact vary with voltage as anticipated. The figure shows that most of the voltage

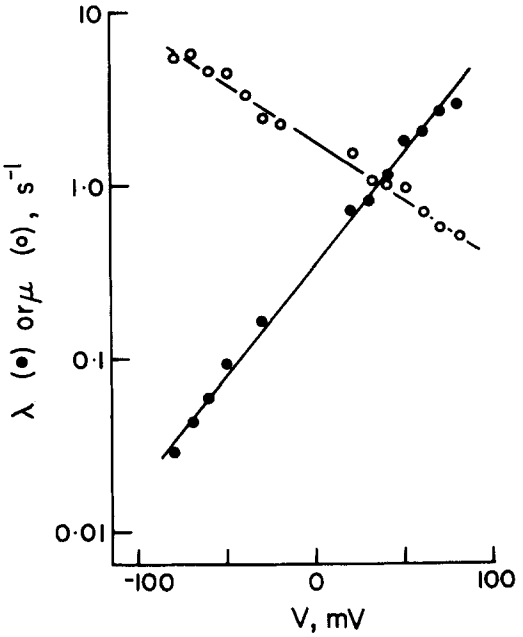

FIGURE 12. Voltage dependence of individual rate constants of gating. Voltage-jump experiments were performed under the conditions of Fig. 10, from a holding voltage of zero to the test voltages indicated on the abscissa. Individual rate constants of opening (λ) and closing (μ) were calculated according to Eq. 15, using values of normalized channel activity measured from steady-state g-V curves under identical conditions. Each point represents the mean of five determinations; the standard errors are about equal to the width of the points.

dependence of the channel gating resides in the rate constant of opening; the closing constant is relatively insensitive to voltage.

SINGLE-CHANNEL FLUCTUATION KINETICS To calculate the individual opening and closing rate constants using macroscopic kinetics, it was necessary to assume the two-state model. This method gave self-consistent results, but it would be desirable to have an alternative way of measuring kinetic constants. This can be done by studying the kinetics of single-channel fluctuations, i.e., by analyzing the statistical distribution of dwell times in the various discrete conductance levels. A complication is that we must deal with fluctuations in

membranes containing more than one channel. This does complicate the analysis, but it also enriches it, since the model makes a number of strong predictions about these multiple-level fluctuations.

Let us consider the following scheme describing the transitions among multiple levels, L_i, of membrane conductance in a membrane containing n channels.

$$L_0 \underset{\mu}{\overset{n\lambda}{\rightleftharpoons}} L_1 \underset{2\mu}{\overset{(n-1)\lambda}{\rightleftharpoons}} L_2 \cdots L_i \cdots L_{n-1} \underset{n\mu}{\overset{\lambda}{\rightleftharpoons}} L_n. \tag{16}$$

Notice that the rate constants of the discrete transitions between adjacent levels are not given by λ and μ, but rather by these constants weighted according to the number of open or closed channels in each level. Let us imagine that the membrane has just entered the ith level. The process of now leaving this level (by entering the $(1 + 1)$th or the $(i - 1)$th level) is, under the assumptions of the model, a simple Poisson process. Therefore, the dwell times in the ith level will be exponentially distributed; if $P_i(t)$ is the probability that the dwell time t_i in the ith level is greater than time t, then:

$$P_i(t) \equiv P(t_i > t) = \exp(-t/\tau_i). \tag{17}$$

Furthermore, the time constant of this i-level relaxation, τ_i, will be given in terms of the total rate constant of leaving the ith level:

$$1/\tau_i = n\lambda + i(\mu - \lambda). \tag{18}$$

Therefore, this scheme requires that the distribution of dwell times in each level be exponential and that the reciprocal time constants of these exponentials vary linearly with the level number. The slope and intercept of the graph of Eq. 18 will determine both λ and μ, since n can be measured independently.

When we examine a large number of fluctuations among several levels at a given voltage, we can experimentally determine the statistical distribution of dwell times. Fig. 13 shows the result of such a determination at -50 mV on a membrane containing seven channels. The predictions of Scheme 16 are borne out: each level is exponentially distributed in time, and reciprocal time constants vary lineary with the level number (*inset*, Fig. 13). This is also true for similar determinations at $+20$ mV (*inset*, Fig. 13).

The individual opening and closing rate constants measured by this stochastic analysis of multiple-level fluctuations are in reasonable agreement with the same rate constants measured by the macroscopic voltage-jump method under comparable conditions (Table II). This gives us confidence that the calculation of the individual rate constants by macroscopic relaxations is a valid one, i.e., that the calculations of Eq. 15 *a* and *b* are kinetically meaningful.

The *inset* of Fig. 13 also shows a plot of reciprocal time constant against level number at $+20$ mV. The slope of this plot is smaller than that measured at -50 mV in the same membrane, and this is also to be expected from the ideas expressed in Scheme 16. It is easy to show that the reciprocal i-level time constant, $1/\tau_i$, normalized to the reciprocal time constant for the zero level, $1/\tau_0$, can be expressed in terms of the normalized channel activity θ, measured

from the macroscopic *g-V curve:*

$$\tau_0/\tau_i = 1 + (i/n)\,[(1 - 2\theta)/\theta] \tag{19}$$

Thus, when $\theta < 0.5$, i.e., at voltages more negative than the half-saturation voltage of the *g-V* curve, a plot such as that in Fig. 13 (*inset*) will have a positive slope; as the half-saturation voltage is approached, the slope of the plot approaches zero, becoming negative above this voltage. This explains why the slope of the plot at +20 mV is lower than that at −50 mV, and it

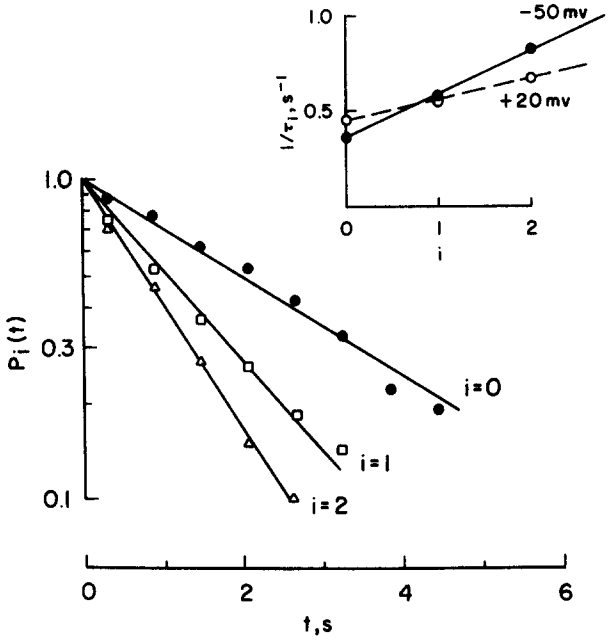

FIGURE 13. Stochastic analysis of multiple-level channel fluctuations. Single-channel recordings were taken from a folded membrane (70% asolectin–30% egg PA) containing five to six channels. The cumulative distribution of dwell times in the ith conductance level, $P_i(t)$, was calculated by tabulating the measured dwell times and then calculating $P_i(t)$ according to the definition of Eq. 17. The main graph shows this distribution for levels 0, 1, and 2 at an applied voltage of −50 mV. For clarity, dwell times in levels higher than $i = 2$ are not shown. (*Inset*) Variation in time constant, τ_i, of the decay of probability with level number. The *inset* shows the reciprocal time constants at −50 mV, measured from the data of the main figure, and at +20 mV, measured in an identical manner from data (not shown) taken from the same membrane. Individual opening and closing rate constants are calculated from this plot according to Eq. 18 and are reported in Table II. Total number of dwell times for generating these data was 638.

shows that, qualitatively at least, the predictions of the stochastic model described above hold; in future work, we shall extend these measurements to a voltage wider range.

The general conclusion we draw from these kinetic experiments is that we have failed to uncover any gating behavior that is inconsistent with the two-state conformational model.

DISCUSSION

During the past few years we have been investigating a K^+-selective ionic channel from the sarcoplasmic reticulum in a planar bilayer system. Our results suggested that the channel operates by a two-state gating mechanism (Miller, 1978; Miller and Rosenberg, 1979 *a* and *b*), but we had not subjected this hypothesis to serious quantitative challenges. It has been the purpose of this report to do this, and we conclude that the proposal is tenable. We have studied both thermodynamic and kinetic aspects of the gating process, using both macroscopic and microscopic approaches, and all the results have been in harmony with the model. In Results, we discussed the cogency of each type of experimental test to the validation of the model, and we will not review those arguments here. It is worthwhile, though, to review the seven postulates on which the model is based, to learn which of these have been verified

TABLE II

MACROSCOPIC AND MICROSCOPIC DETERMINATION OF GATING RATE CONSTANTS

Voltage	Rate constant	Macroscopic	Microscopic
mV		s^{-1}	s^{-1}
−50	λ	0.020±0.003	0.050±0.005
	μ	0.33±0.04	0.33±0.01
+20	λ	0.12±0.02	0.080±0.005
	μ	0.14±0.03	0.171±0.006

SR vesicles were fused with folded bilayers (70% asolectin–30% PA) under either macroscopic fusion conditions (Fig. 3) or single-channel conditions (Fig. 13). Macroscopic rate constants were determined as in Fig. 12, using membranes containing 200–10,000 channels. Microscopic rate constants were determined using the data of Fig. 13 and applying Eq. 18. Note that the microscopic data were all obtained on a single planar bilayer.

directly, and which have only been inferred to be true.

The first two assumptions have been directly confirmed. We know that channels are inserted irreversibly (on the time scale of the experiments) from the fact that, after the insertion process has been stopped and the SR vesicles removed from the medium, there is no decrease in bilayer conductance. There is also ample evidence that once inserted into the membrane the channels are nearly 100% oriented. The asymmetry of the g-V curve demonstrates this, as does the fact that the channel may be chemically modified asymmetrically in several ways. The channel is blocked by Cs^+ from the *cis* side of the bilayer only (Coronado and Miller, 1979), is attacked by alkaline proteinase b from the *trans* side only (Miller and Rosenberg, 1979 *b*) and is modified by certain transition metal ions only from the *trans* side (Miller and Rosenberg, 1979 *a*). A conservative estimate of the degree of orientation is at least 98% (Miller and Rosenberg, 1979 *a*). Obviously, the asymmetry of the system is an enormous benefit to the analysis of experiments done on this system.

Only half of the third postulate, that each channel has a single open and a single closed state, has been directly validated. Single-channel analysis demonstrates that each channel has only one open state, but it is necessary to infer the existence of a single closed state by means of kinetic tests. We should

mention that these tests are not foolproof. It is possible to imagine a channel with several closed states that would give the same behavior seen here (lack of memory, lack of kinetic delay, apparent rate constants exponentially dependent on voltage). For this to be the case, however, the closed states would have to be in voltage-independent equilibrium with each other, and all the rate constants of these "silent" equilibria would have to be very large compared with those of the opening process.

One question to arise from the postulate of a two-state channel is, is the closed state truly nonconducting or does it have some finite conductance? An analysis of the g-V curve allows us to place an upper limit upon the closed-state conductance.The background conductance (at highly negative voltages) of this curve represents the conductance of all channels in the closed state plus any "leak" conductance in the system. Typically, we find that the background conductance is 4–8% of the maximum conductance with all channels open. Occasionally, on particularly blessed days, we have observed membranes with background conductance <3% of maximum conductance. Therefore, the conductance of the closed state is certainly <5 and probably <3% of that of the open state.

The fourth assumption, that the channels gate independently, is not possible to verify unambiguously, but we have found that all gating properties examined are consistent with the idea. The closest thing to a direct test presented here is the demonstration that the i-level frequencies in the conductance histogram follow a binomial distribution (Fig. 9), with values predicted from *a priori* calculations. Furthermore, results to be reported elsewhere[1] show clearly that channel transitions follow Markov chain statistics, which would not be the case if there were interactions among channels.

The assumption of a difference in dipole moment is strongly indicated (though not proven) by the easily understood voltage dependence of the g-V curve and of the channel fluctuation probabilities. An alternative possibility is that the protein does not sense the intramembrane electric field, but rather that it responds to an electrostrictive effect of the field on the artificial membrane. This is an unlikely possibility, however, for several reasons. First, an electrostrictive effect would not display the polarity shown in the channel's voltage dependence. Second, electrostrictive effects would be less important in "solvent-free" folded membranes than in decane-containing painted membranes. The channel's voltage dependence, however, is the same in the two types of membrane. A final argument against an electrostrictive effect is that it is possible to uncouple the channel's gating probabilities from applied voltage by a specific enzymatic modification of the channel after it is incorporated into the bilayer (Miller and Rosenberg, 1979 *b*). We therefore argue that it is the channel protein itself which senses the electric field within the bilayer, and this necessarily implies that net charge movement occurs when the channel opens and closes.

The sixth postulate, that the channel conductance is not dependent on

[1] Coronado, R., and C. Miller. Unpublished observations.

voltage, has been directly shown here and elsewhere in the range −100 to +100 mV (Miller, 1978; Coronado and Miller, 1979; Coronado et al., 1980).

The final assumption, that the channel is a preformed unit and does not operate by a subunit aggregation mechanism, has not been established. However, its validity is suggested by the independence of the gating parameters of the absolute concentration of channels in the membrane. This is not a rigorous argument against aggregation, however, since it is possible that upon fusion of SR vesicles, the channels remain in SR-like "patches" in the bilayer. In this case, an increase in the number of channels would not be equivalent to an increase in the local concentration of channels, and any mechanism of channel gating would be independent of the number of channels in the bilayer. However, the channel's gating properties, particularly the kinetics, are extremely sensitive to the phospholipid composition of the bilayer,[2] and one would have to argue that if patches do exist, lipids but not proteins are free to exchange between the patch and the bilayer. In other words, one would have to postulate a model in which the proteins are somehow "corralled" into a patch, with the SR lipids free to dilute into the vast excess of bilayer phospholipid. It is not difficult to imagine such a situation, but it is difficult to imagine how such a situation could exist. We therefore tentatively accept the idea that the channel gates by a conformational mechanism rather than by an aggregation process.

The overall conclusion to arise from this report is that a simple two-state conformational model is reasonable and adequate to explain the gating behavior of the SR K^+-selective channel. This is a surprising conclusion. At the beginning of the work, we certainly did not expect that a conductance channel whose origin is an animal cell membrane would behave in such a simple manner. Electrophysiological experience with those few channels that can be studied in voltage-clamped cell membranes has been that gating is not a simple process. Even channels without inactivation, such as the K^+ channel of axons, must be assumed to have multiple closed states.

In spite of the fundamental simplicity of the gating process described for the SR channel, we should not lose sight of the fact that the channel protein must possess considerable structural complexity. This is indicated by the rather specific modifications that can be made in the channel's gating and conduction properties, for instance by sulfhydryl liganding and alkylating reagents (Miller and Rosenberg, 1979 *a* and footnote 3), by enzymatic treatments (Miller and Rosenberg, 1979 *b*), and by Cs^+ ion (Coronado and Miller, 1979). A more striking indication of the complexity of the channel protein is the evidence previously offered pointing to the reality of a fairly extensive structural change in the channel protein upon gating. It has been shown that only the open state of the channel is able to react with certain organomercurial reagents, and that an arginine or lysine residue, buried in the closed conformation of the channel, becomes exposed to the *trans* side of the membrane when the channel opens (Miller and Rosenberg, 1979 *a* and *b*).

[2] Miller, C. Unpublished observations.

[3] Miller, C., and B. Breit. Unpublished observations.

In addition, it is likely that Cs^+ is unable to enter the channel from the *cis* side of the membrane when the channel is closed (Coronado and Miller, 1979); therefore, when the conformational rearrangement occurs, it makes itself felt on both sides of the membrane. Although this channel is simpler in its gating mechanism than the familiar channels of axons, it is clearly of a higher order of complexity than the equally familiar "model channels" such as gramicidin, alamethicin, etc. (Ehrenstein and Lecar, 1977).

It may not be an entirely idle exercise to speculate upon the physiological role of this channel. It is clear from purification studies[4] that the vesicles carrying the channel are from the SR and are not derived from a contaminating membrane fraction in the muscle homogenate. But of what use would a K^+-selective channel be to SR function? As was mentioned in the Introduction, there must be counterion flow into and out of the SR membrane during the massive Ca^{++} movements accompanying contraction and relaxation. One way for this to occur would be that the SR membrane becomes leaky to the major small ion in the muscle, namely K^+. Thus, the channel may serve as an electrical shunt to prevent the development of a large voltage across the SR membrane as a result of the flow of Ca^{++} during release or re-uptake. It is important to ask, therefore, whether the K^+ transport rates mediated by the channel would be sufficient to balance the Ca^{++} release and re-uptake rates in the muscle. We know that the number of channels per SR vesicle is only ~1% of the number of Ca^{++}-ATPase complexes per vesicle, but typical transport rates of the channel are about four orders of magnitude higher than the maximum turnover rates of the pump enzyme. Therefore, the channel could easily act as an electrical shunt across the SR membrane. These ideas assume that Ca^{++} release and re-uptake are themselves electrogenic processes; while little is known about the Ca^{++} release mechanism, biochemical reconstitution of the Ca^{++} ATPase of SR has shown quite convincingly that the Ca^{++} pump is electrogenic (Zimniak and Racker, 1978).

Throughout this paper, we used the K^+ conductance of the channel as an assay of its activity in the bilayer. We have not addressed questions of its ion selectivity or other conductance properties. The reason for this is that here we are concerned only with the gating of the channel. In the following paper, we discuss the conduction process of the open channel.

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[4] Hidalgo, C., and C. Miller. Unpublished observations.

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