Asymmetric electrostatic effects on the gating of rat brain sodium channels in planar lipid membranes

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ABSTRACT The effects of ionic strength (10–1,000 mM) on the gating of batrachotoxin-activated rat brain sodium channels were studied in neutral and in negatively charged lipid bilayers. In neutral bilayers, increasing the ionic strength of the extracellular solution, shifted the voltage dependence of the open probability (gating curve) of the sodium channel to more positive membrane potentials. On the other hand, increasing the intracellular ionic strength shifted the gating curve to more negative membrane potentials. Ionic strength shifted the voltage dependence of both opening and closing rate constants of the channel in analogous ways to its effects on gating curves. The voltage sensitivities of the rate constants were not affected by ionic strength. The effects of ionic strength on the gating of sodium channels reconstituted in negatively charged bilayers were qualitatively the same as in neutral bilayers. However, important quantitative differences were noticed: in low ionic strength conditions (10–150 mM), the presence of negative charges on the membrane surface induced an extra voltage shift on the gating curve of sodium channels in relation to neutral bilayers. It is concluded that: (*a*) asymmetric negative surface charge densities in the extracellular ($1e^-/533Å^2$) and intracellular ($1e^-/1{,}231Å^2$) sides of the sodium channel could explain the voltage shifts caused by ionic strength on the gating curve of the channel in neutral bilayers. These surface charges create negative electric fields in both the extracellular and intracellular sides of the channel. Said electric fields interfere with gating charge movements that occur during the opening and closing of sodium channels; (*b*) the voltage shifts caused by ionic strength on the gating curve of sodium channels can be accounted by voltage shifts in both the opening and closing rate constants; (c) net negative surface charges on the channel's molecule do not affect the intrinsic gating properties of sodium channels but are essential in determining the relative position of the channel's gating curve; (*d*) provided the ionic strength is below 150 mM, the gating machinery of the sodium channel molecule is able to sense the electric field created by surface changes on the lipid membrane. I propose that during the opening and closing of sodium channels, the gating charges involved in this process are asymmetrically displaced in relation to the plane of the bilayer. Simple electrostatic calculations suggest that gating charge movements are influenced by membrane electrostatic potentials at distances of 48 and 28 Å away from the plane of the membrane in the extracellular and intracellular sides of the channel, respectively.

INTRODUCTION

The study of the interaction between divalent cations and ion channels is a classical chapter in membrane biophysics. Frankenhaeuser and Hodgkin (1957) provided the first quantitative description for the effects of Ca^{2+} on Na^+ currents. They proposed that Ca^{2+} could adsorb to the outer surface of the cell membrane, thus modifying the external field caused by fixed negative surface charges on the membrane. It was assumed implicitly that the gating of Na channels would not only respond to changes in membrane potential but also to changes in electric field caused by surface charges on the membrane.

Following that initial suggestion, several groups have applied the Gouy-Chapman or Gouy-Chapman-Stern theories to explain the effects of divalent cations on different ionic currents (Chandler et al., 1965; Gilbert and Ehrenstein, 1969; Mozhayeva and Naumov, 1970; Hille et al., 1975; Dani et al., 1983). There were several uncertainties and limitations in those experiments: (*a*) it was not possible to discriminate between the effects of divalent cations on membrane surface charges from their effects on surface charges present on the channel molecule itself; (*b*) recorded transmembrane currents were the result of the activation of more than one type of ionic current and divalent cations could have affected more than a single ionic current thus hampering the analysis; (*c*) there are minimum requirements for keeping the experimental preparation alive and responsive to stimuli. These requirements reduce the number of experimental manipulations necessary to carry out an extensive investigation on the effects of surface charges on the gating of ionic currents.

There are indications suggesting that divalent cations do not interact with Na^+ channels by causing only a simple change in the electric field across the channel's voltage sensor. Using pronase-treated squid axons, Gilly and Armstrong (1982*a*, *b*) showed that divalent cations applied to the extracellular side of the membrane slowed

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the activation kinetics of sodium currents, leaving their closing kinetics essentially unaltered. While the effects of calcium on all parameters that describe the gating of sodium currents in frog skeletal muscle were in agreement with a simple change in the electric field across the channel's voltage sensor, the effect of protons were not (Hahin and Campbell, 1983; Campbell and Hahin, 1984). We have found that external divalent cations caused a larger voltage shift of the voltage dependence of the opening rate than of the closing rate constant. An opposite situation was found with internal divalent cations: the closing rate constant was shifted along the transmembrane voltage axis by a larger amount than the opening rate constant (Cukierman et al., 1988; Cukierman and Krueger, 1990; Cukierman and Krueger, 1991). Overall, those experimental observations on the effects of divalent cations on Na^+ channel gating cannot be reconciled with a simple mechanism involving only an alteration in the transmembrane electric field.

To fully understand the influence of surface charges on the gating of Na^+ channels, it is imperative to study the effects of ionic strength. Surprisingly, and to the best of our knowledge, there are only two references to the effects of ionic strength on sodium channel gating. The first one by Chandler et al. (1965) showed that the position of the inactivation curve of sodium currents is affected by intracellular ionic strength. They proposed that the inactivation mechanism senses an intracellular electric field caused by a negative surface charge density of $1e^-/714Å^2$. The second reference is the work by Hille et al. (1975) where it was shown that the position of the Na^+ current-voltage relationship is a function of ionic strength.

To understand how surface charges modulate the gating behavior of Na^+ channels, it is essential to discriminate between the effects of surface charges present on the membrane from those charges located on the channel molecule. By reconstituting Na^+ channels in neutral or in negatively charged bilayers it is possible to investigate the relative influence of surface charges of different origins on Na^+ channel gating. In recording single channel currents, it is possible to separate electrostatic effects on channel gating from those effects related to permeation. In this study, I evaluate the effects of intracellular and extracellular ionic strengths on the gating of sodium channels reconstituted in two different types of planar lipid bilayers: neutral and negatively charged. I show that a simple electrostatic mechanism is consistent with the effects of ionic strength on Na^+-channel gating, and it is proposed that two different and important asymmetric electrostatic effects modulate the opening and closing of Na^+ channels.

MATERIALS AND METHODS

Preparation

Membrane vesicles containing Na^+ channels were prepared by conventional methods from rat brains (Gray and Whittaker, 1962; Krueger et al., 1979). These vesicles were pretreated with batrachotoxin (BTX, 0.6 μM), an alkaloid that removes Na^+ channel inactivation, before being added to the experimental chamber.

Lipids

Two different membranes were used in this study. Neutral bilayers contained 80% 1-polmitoyl 2-oleoyl phosphatidylethanolamine (PE) and 20% 1-palmitoyl 2-oleoyl phosphatitylcholine (PC) and charged bilayers were made of 70% 1-palmitoyl 2-oleoyl phosphatidyl serine (PS) and 30% PE. Those synthetic lipids were purchased from Avanti Lipids (Alabaster, AL). The lipid mixture was dissolved in decane at a concentration of 60 mg/ml. The choice of that lipid composition for charged membranes was a consequence of several different factors. First, charged membranes containing more than 70% PS became a gel at room temperature in a short period of time. The thinning and stability of membranes with higher PS concentration were not good. Also, the rate of fusion of membrane vesicles with bilayers with higher PS concentration was very low.

The density of negative surface charges on bilayers was measured by the nonactin method (McLaughlin et al., 1970). Briefly, $\sim 10^{-6}$ M nonactin was added to bilayers in presence of symmetrical KCl solutions (0.015 M, pH = 7.00). The membrane conductance at 5 mV was measured (G') and the ionic strength was increased by adding LiCl from a concentrated stock solution until a steady, low level of membrane conductance was obtained (G''). Under these experimental conditions, it can be shown (see McLaughlin, 1977) that:

$$G''/G' = \exp(-F\Delta\psi_o/RT), \qquad (1)$$

where $\Delta\psi_o$ is the change in membrane surface potential after addition of an inert electrolyte (LiCl). This value for $\Delta\psi_o$ together with the appropriate association binding constant of monovalent cation to phospholipids (Eisenberg et al., 1979) were introduced into the Stern equation (see below) and a value for the surface charge density (σ) of the bilayer was obtained. σ had values of $1e^-/107$ $Å^2$ and less than $1e^-/2{,}000$ $Å^2$ for charged and neutral bilayers, respectively. My results are in good agreement with previous determinations under similar experimental conditions (McLaughlin et al., 1970; Bell and Miller, 1984). It is interesting to note that the determination of σ in charged bilayers is in good agreement with the value expected if a phospholipid headgroup surface area of 70 $Å^2$ is assumed ($1e^-/100$ $Å^2$).

Experimental procedure

Bilayers were formed by painting with the appropriate lipid solution a 0.1–0.2 mm hole in a plastic partition separating two different compartments. Bilayer thinning was followed by measuring its capacitance increase. Once the bilayer thinned, membrane vesicles were added to one side of the partition (*cis*-side) under continuous stirring. Transmembrane voltages were applied and the membrane conductance was continuously monitored on the oscilloscope screen. The fusion of membrane vesicles containing Na^+ channels with bilayers in solutions with 0.08 M ionic strength or higher could usually be obtained within the first hour of experiment. If not, the experimental chamber was washed and the process repeated again. In low ionic strength (below 0.08 M), fusion events became rare (see also Green et al., 1987). To overcome this difficulty, the following experimental procedure was adopted. 30 min after addition of vesicles to the *cis*-side, the membrane was repainted without breaking the original bilayer. It was not unusual to see a Na^+ channel after the new repainted membrane had thinned. This procedure usually resulted in the incorporation of a channel with an orientation opposite to the most common one which is, *cis*-side = extracellular side. Because this manipulation does not hurt the normal course of an experiment, I decided to adopt it as an experimental routine even in higher ionic strength conditions.

The basic solution employed in this study contained NaCl and 10mM Hepes (pH = 7.00 with NaOH). In this study, the salt concentration (corrected for the amount of base necessary to buffer the pH of the solution to 7.00) will be expressed by the convention *x*//*y*, where *x* and *y* represent the extracellular or intracellular salt concentration (in milliMolars), respectively. While in the majority of the experiments the ionic strength was varied by changing the NaCl concentration, in several control experiments in neutral bilayers, the ionic strength was modified using KCl (or Na_2SO_4). The results were not different from those where NaCl concentration was altered (Fig. 6). After obtaining information on the effects of a given ionic strength condition on Na^+-channel gating, the ionic strength of the extracellular or intracellular solution was changed. Typically, only one solution change was possible for each bilayer. The change in ionic strength was performed either by adding a small volume of a concentrated salt solution to a specific side of the membrane, or alternatively, by extensively perfusing one side of the channel with a different ionic strength solution.

To ascertain that ionic strength is the true variable in our experimental conditions, two different groups of control experiments were done. In the first group, the tonicity of the solution was increased by adding sucrose instead of salt. No measurable effects of sucrose as the osmoticant were identified on the gating of Na^+ channels suggesting that the described effects in this study cannot be attributed to changes in osmotic strength. A second set of control experiments was done to examine potential problems with contamination of solutions with divalent cations, notably Ca^{2+}. It was found that the addition of 5 mM EGTA to different NaCl solutions (50 or 500 mM) did not by itself shift the gating curve of sodium channels in relation to control conditions. It is important to mention that with a 150-mM NaCl solution, the concentration of external divalent cations necessary to produce the same voltage shift in the gating curve of Na^+ channels as increasing the extracellular NaCl concentration from 150 to 300 mM, is 3–5 mM (see Fig. 6, and Cukierman et al., 1988; Cukierman and Krueger, 1990). This level of Ca^{2+} contamination is not compatible with the quality of reagents and water used in our solutions.

The titration of the midpoints of the gating curves of Na^+ channels against the extracellular (or intercellular) ionic strength was done by keeping the ionic strength constant at 150 mM in the opposite side of the channel. It should be noticed, however, that the effects of ionic strength in a specific side of the channel do not depend on the ionic strength of the opposite side. Similar behavior has been demonstrated for divalent cations (Cukierman et al., 1988).

Electrophysiological setup

The *cis*-side of the bilayer was kept at ground level while the applied membrane potential and transmembrane currents were measured in the other side (*trans*-side). A patch clamp amplifier (EPC-7; List Instruments, New York) was used for this purpose. Both sides of the bilayer were connected to the amplifier via 3 M KCl-agar bridges. Junction potentials were <1 mV. Transmembrane potentials are expressed using the cell convention ($V_{\text{intracellular}}$-$V_{\text{extracellular}}$). A PC-based hardware and software (Axon Instruments, Foster City, CA) were used to digitize and analyze the data. Single channel recordings were digitized at a 200–300 Hz cutoff frequency using a low-pass Bessel filter (Frequency Devices, Haverhill, MA) and stored in the computer for analysis.

Analysis

In this study, I focus attention on how the gating properties of Na^+ channels are affected by the ionic strength of solutions. Basically, two types of analysis were done. The first one was to measure the voltage shifts on the gating curves (open probability of the channel, P_o, versus membrane potential, V_m) as a function of ionic strength. P_o was measured by the average current method (Cukierman et al., 1988; Cukierman and Krueger, 1990) and the relationship between P_o and membrane potential was fit to the Boltzmann distribution:

$$[(1 - P_o)/P_o] = \exp\,[qF(V - V_{0.5})/RT], \qquad (2)$$

where q, V, and $V_{0.5}$ are the effective gating charge, membrane potential, and the membrane potential, where P_o is 0.5, respectively.

The second approach was to quantify the effects of ionic strength on the opening (k_{co}) and closing (k_{oc}) rate constants of the channel. Dwell time distributions for the open and closed states of the channel were analyzed and fit to single exponentials (Cukierman and Krueger, 1990). The experimental results were found to be consistent with a channel existing in two different states (open and closed). In a simple kinetic scheme like that it can be shown that P_o is given by:

$$P_o = k_{co}/(k_{co} + k_{oc}). \qquad (3)$$

The average current method for calculating P_o (see above), which does not implicitly assume any kinetic scheme, was in excellent agreement with the P_o's calculated by Eq. 3. In view of this, and since the fitting of dwell time distributions was not improved using two exponential components, we assume that the simple open-closed kinetic scheme for Na^+-channel gating is valid under our experimental conditions.

Theory

The midpoints of the gating curves as well as the rate constants X V_m relationships were plotted against salt concentration. The experimental points were fit to the Grahame equation:

$$\sigma^2 = (2\epsilon\epsilon_o RT)\sum_i [C]_i \cdot [\exp\,(-z_i F\psi_o/RT) - 1], \qquad (4)$$

where, σ, ϵ, ϵ_o, and ψ_o are the surface charge density, dielectric constant of the medium (78), the permittivity of the free space $[8.85 \cdot 10^{-12}C^2/(N \cdot m^2)]$ and the surface potential at $x = 0$, respectively. The implicit assumption in this fitting is that the surface charges are located adjacent to the voltage sensing device of the Na^+ channel. For a derivation of Eq. 4 as well as a discussion on its assumptions and limitations, see McLaughlin (1977) and Israelachvilli (1985).

Differences between the midpoints of the gating curves of Na^+ channels in neutral and negatively charged bilayers were plotted against different ionic strengths. Fitting of these experimental points was done by calculating electrostatic potentials at different distances from the membrane-solution interface. The following equations were used for that purpose:

$$\sigma_{\text{effective}} = \sigma/[1 + K_A(\text{Na})\exp\,(\psi_o/RT)]. \qquad (5)$$

Eq. 5 calculates the effective charge density ($\sigma_{\text{effective}}$) of charged bilayers assuming a binding constant (K_A) of 0.6 M^{-1} for Na^+ to PS (Eisenberg et al., 1979). In this equation, σ is $1e^-/107Å^2$. The combination of Eqs. 4 and 5 gives origin to the Stern equation.

Electrostatic potentials at specific distances from the plane of the bilayer were calculated by:

$$\psi_{(x)} = 2RT/F \ln \{[1 + \alpha \exp(-\kappa x)]/[1 - \alpha \exp(-\kappa x)]\}, \quad (6)$$

where

$$\alpha = [\exp(F\psi_o/2RT) - 1]/[\exp(F\psi_o/2RT) + 1]$$

and

$$\kappa = [2F^2(\mathrm{NaCl})/\epsilon\epsilon_o RT]^{1/2}.$$

Fitting of experimental points to different functions was done using nonlinear square methods based on a Marquardt-Levenberg algorithm. The overall quality of fit was determined by three different parameters: standard deviation of the fit, degree of dependency between different parameters present in the equation, and by a small value of the residuals (difference between the fitted curve and dependent variable values).

RESULTS

A. Experiments in neutral bilayers

Fig. 1 shows the effects of increasing the extracellular ionic strength on the gating behavior of a single Na^+ channel reconstituted in a neutral bilayer. The extracellular ionic strength was increased from 150 mM (*upper trace*) to 290 mM (*lower trace*). Two observations are apparent from this figure: (*a*) in the lower recording, the open channel current level is increased in relation to the upper recording. This is due to an increased electrochemical driving force for Na^+; (*b*) most interestingly, when the extracellular ionic strength increased, the open probability of the Na^+ channel decreased.

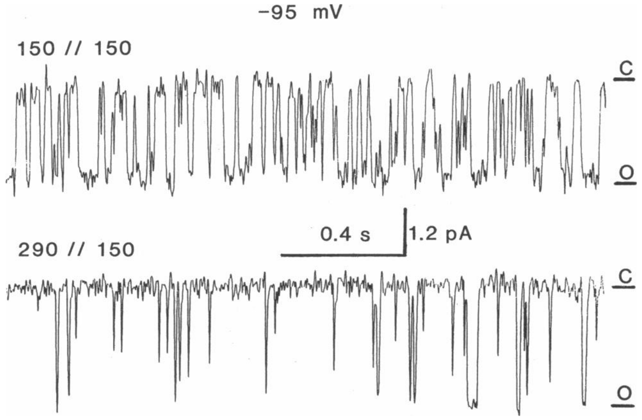

FIGURE 1 Current recordings of a single sodium channel in a neutral bilayer in 150//150 (*upper trace*) and 290//150 (*lower trace*). Unless otherwise mentioned in this and in the following figure legends, those figures refer to NaCl concentrations. Upward deflections in both recordings are closing events which represent the 0-current level.

Fig. 2 shows the effects of changing the extracellular ionic strength on the gating curve of Na^+ channels. Fig. 2 *A* shows that a decrease in ionic strength from 150 (*circles*) to 38 mM (*triangles*) caused a hyperpolarizing voltage shift of 21 mV on the gating curve of a Na^+ channel. Further reduction in the extracellular ionic strength to 18 mM (*squares*) resulted in an extra −7mV shift of the gating curve of this channel. Fig. 2 *B*, obtained from a different Na^+ channel, shows that increasing the extracellular ionic strength from 150 (*circles*) to 600 mM (*squares*) caused an 18 mV depolarizing shift on the channel's gating curve.

Fig. 3 illustrates the result of an experiment where the intracellular ionic strength was increased from 20 to 150 and 600 mM. Panel *A* shows representative segments of single Na^+ channel recordings in a neutral bilayer in three different experimental conditions. Contrary to the effects of extracellular ionic strength, this panel shows that *increasing* the intracellular ionic strength leads to an *increase* in the open probability of the channel. Fig. 3 *B* shows the voltage shifts caused by different intracellular ionic strengths on the gating curve of the same channel illustrated in panel *A*. When the intracellular ionic strength was increased from 20 (*circles*) to 150 mM (*squares*), the gating curve shifted by 19 mV in the hyperpolarizing direction. A further increase in intracellular ionic strength to 600 mM (*triangles*) resulted in a 8-mV shift of the gating curve in the hyperpolarizing direction. It is important to mention that alterations in ionic strength (extracellular or intracellular) did not cause changes in the steepness of the gating curves. Apparently, ionic strength displaces the midpoint of

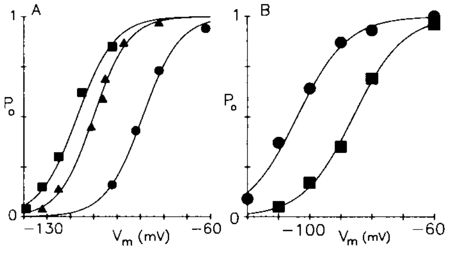

FIGURE 2 Gating curves of single sodium channels in neutral bilayers. (*A*) Circles (150//150), triangles (38//150), and squares (18//150); (*B*) gating curves from a different bilayer in 150//150 (*circles*) and 600//150 (*squares*). Curves fitted to experimental points using Eq. 2 in text with the following values for q and $V_{0.5}$. (*A*) Circles, $q = 3.2$, $V_{0.5} = -89$ mV; triangles, $q = 3.4$, $V_{0.5} = -110$ mV; squares, $q = 3.2$, $V_{0.5} = -117$ mV. (*B*) Circles, $q = 3.3$, $V_{0.5} = -104$ mV; squares, $q = 3.2$, $V_{0.5} = -86$ mV.

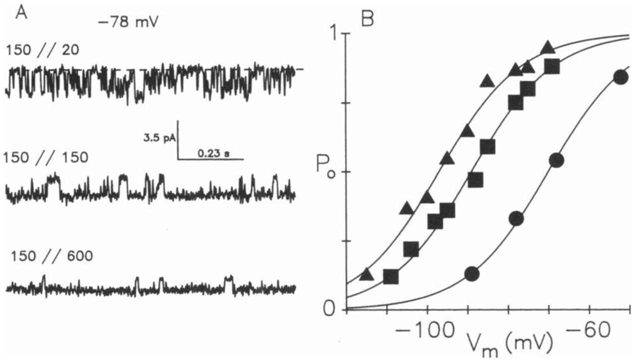

FIGURE 3 Left panel shows single channel recordings from a sodium channel in a neutral bilayer voltage clamped at −78mV. The ionic strengths are indicated on the upper left corner of each recording. The channel closing level is indicated by the dashed trace in the upper recording (150//20), upward deflections in the current level indicating channel closures. The right panel shows the gating curves from the same experiment: circles (150//20), squares (150//150), and triangles (150//600). Curves in right panel were fit to Eq. 2 in text with $q = 2.5$, $V_{0.5} = -70$ mV (*circles*), $q = 2.4$, $V_{0.5} = -89$ mV (*squares*), and $q = 2.3$, $V_{0.5} = -97$ mV (*triangles*).

those gating curves without affecting the number of effective gating charges (see Figs. 4 and 5).

There are different possibilities by which ionic strength could modulate the open probability of Na^+ channels. To discriminate among those possibilities, the effects of ionic strength on the opening (k_{co}) and closing (k_{oc}) rate constants of the channel were investigated. In Fig. 4, the open (panels *A, C, E*) and closed (panels *B, D, F*) dwell time distributions of a single Na^+ channel were plotted under different experimental conditions. Ionic strengths were 150//150 (panels *A, B*), 290//150 (panels *C, D*), and 290//290 (panels *E, F*). Dwell time distributions were analyzed at a membrane potential of −103 mV in those different experimental situations. Increasing the extracellular ionic strength from 150 to 290 caused a decrease in the mean open time from 9.2 to 3.3 ms, while the mean closed time increased from 7.0 to 19.7 ms. When the intracellular ionic strength was increased, the mean open time increased from 3.3 to 4.2 ms while the mean closed time decreased from 19.7 to 15.3 ms. Three significant observations are apparent from this figure: (*a*) alterations in extracellular or intracellular ionic strengths caused changes in both the opening (1/mean closed time) and closing (1/mean open time) rate constants; (*b*) however, both rate constants were affected by the same proportion. In this figure, an increase in extracellular ionic strength caused a decrease in the mean open time by 1/2.79 while the mean closed time increased by 2.81. When the intracellular ionic strength was increased, the mean open and closed times changed by 1.27 and 1/1.29, respectively; (*c*) the effects of changing the extracellular or intracellular ionic strength on the gating of Na^+ channels are not only qualitatively but also quantitatively different. A given alteration in the extracellular ionic strength caused larger effects on the gating curve and on the mean dwell times than similar changes in intracellular ionic strength (see Figs. 2 and 3). In the experiment of Fig. 4 as well as in others, it was not possible to restore the original mean dwell times of the channel by increasing both extracellular and intracellular ionic strengths by the same amount. This result suggests that the density of surface charges affecting Na^+ channel gating are different in the extracellular and intracellular sides of the channel.

In Fig. 5, ln k_{co} (*a*) and ln k_{oc} (*b*) were plotted against membrane potential. An increase in the extracellular ionic strength shifted both curves by the same amount to more depolarized voltages. There was no alteration in the voltage sensitivity of these curves (in this figure ~e-fold/12.5 mV). The effects of ionic strength are limited to shifting both rate constants by the same amount along the voltage axis. Fig. 5 *c* shows the gating curves from the same experiment. The gating curve shifted by approximately the same amount along the voltage axis, as the curves in panels *a* and *b* (~8–9 mV). The experimental P_o's in Fig. 5 *c* were obtained by the average current method and not by calculating them from the kinetic rate constants displayed in panels *a* and *b*. As mentioned before and shown by this figure, there is a good agreement between those two different methods for measuring P_o.

What is important to realize from Fig. 5 is that the effects of ionic strength on the gating curve of single Na^+ channels can be fully accounted by the effects of ionic strength on their kinetic rate constants. Apparently, by changing the ionic strength of the solution, a bias voltage is being added across the voltage sensor of the Na^+-channel molecule: the voltage sensitivity is not changed but the channel responds in such a way as if it were experiencing only a different applied transmembrane voltage.

In Fig. 6, the midpoints of the gating curves as well as the transmembrane voltages where the opening and closing rate constants cross each other, are plotted against the concentration of the extracellular (*a*) or intracellular (*b*) solutions. The experimental points could be adequately fit by assuming negative surface charge densities of $1e^-/533$ Å^2 and $1e^-/1{,}231$ Å^2 for the extracellular and intracellular sides of the Na^+ channel, respectively (Eq. 4). It can be seen that while the midpoints of the gating curves extend from −125 to −80 mV upon changes in the extracellular ionic strength (15–1,000 mM), the same alterations in intracellular

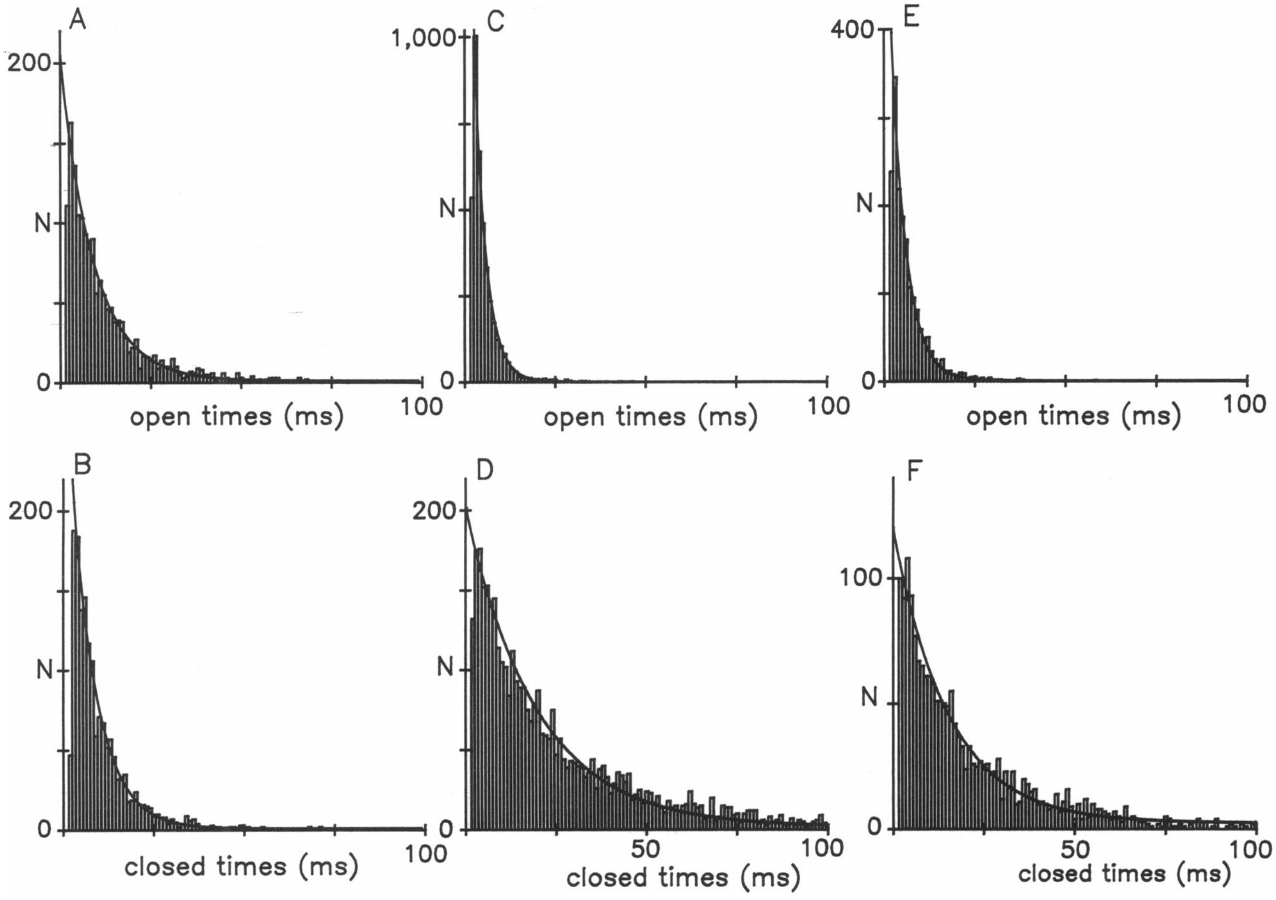

FIGURE 4 Dwell time distributions of a single sodium channel in a neutral bilayer. (*A*, *B*) 150//150; (*C*, *D*) 290//150; (*E*, *F*) 290//290. Membrane potential: −103 mV.

ionic strength lead to a smaller shift in the midpoints of the gating curves (from −75 to −100 mV).

B. Experiments in charged bilayers

Fig. 7 shows the effects of decreasing the extracellular ionic strength on the gating of a single Na^+ channel reconstituted in a charged bilayer. The applied transmembrane voltage is indicated on the left side of each recording while the value of P_0 is displayed on the right. At −124 mV in 150 mM extracellular ionic strength P_0 was 0.27 and when the extracellular ionic strength was decreased to 20 mM, the P_0 became ~1 (at −126 mV). Notice that at −170 mV in 20 mM extracellular ionic strength P_0 was still 0.1.

The qualitative effects of ionic strength on Na^+ channels in charged bilayers were the same as in neutral bilayers. However, important quantitative differences were observed. As an example, I consider what happens when the extracellular ionic strength decreases from 150 to 20 mM. In neutral bilayers, such a decrease in ionic strength shifted the gating curve of Na^+ channels by -34 ± 3.0 mV ($n = 6$) in the hyperpolarizing direction (see Fig. 6*A*). When the same alteration in ionic strength was performed in a Na^+ channel in a negatively charged bilayer, the shift in the gating curve was -42.7 ± 2.8 mV ($n = 3$). These differences (of ~ −9 mV in the case of 20 mM extracellular ionic strength) are related to *extra* voltage shifts induced by ionic strength on the gating curve of Na^+ channels in charged bilayers in relation to neutral bilayers. In Fig. 8, $\Delta V_{0.5}$'s are the additional voltage shifts induced by ionic strength on the gating curve of Na^+ channels in charged bilayers in relation to the gating curve of Na^+ channels in neutral bilayers. They are being plotted against the extracellular (*a*) or intracellular (*b*) salt concentration.

Because the experimental conditions in Fig. 6 were the same as in Fig. 8, the origin of $\Delta V_{0.5}$ at each salt concentration must be associated to the presence of an electrostatic potential generated by membrane surface

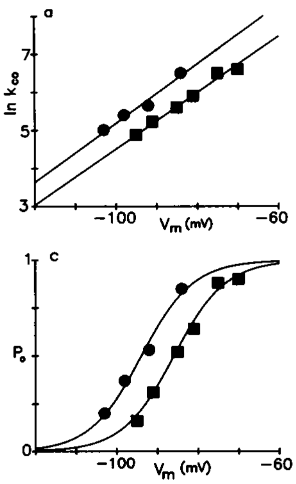

FIGURE 5 Relationships between the opening (*a*) and closing (*b*) rate constants and the membrane potential. Regression lines in *a* and *c* were drawn following the equations: *a*, $\ln k_{co} = 0.0778$ mV + 12.963 (*circles*), and $\ln k_{co} = 0.0740$ mV + 11.91 (*squares*). *b*, $\ln k_{oc} = -0.0826$ mV −2.095 (*circles*) and $\ln k_{oc} = -0.085$ mV −1.613 (*squares*). (*c*) gating curves from the same experiment. (*Circles*) 150//150 and squares: 300//150. Curves in *c* were fit with Eq. 2 with $q = 4.0$, $V_{0.5} = -94$ mV (*circles*), and $q = 4.0$, $V_{0.5} = -86$ mV (*squares*).

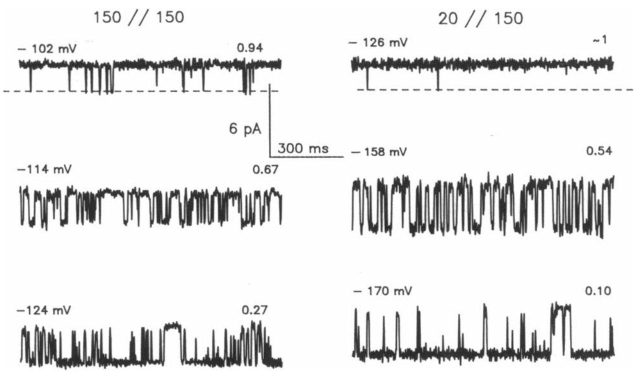

FIGURE 7 Single sodium channel recording in a negatively charged bilayer. Above each recording the membrane potential (*left*) and the open probability of the channel (*right*) are indicated. The dashed traces in the two upper recordings indicate the zero current level. For all recordings, downward deflections are channel closings, which represent the 0-current level.

charges that is sensed by the gating machinery of the channel. In neutral bilayers, when the ionic strength was varied, only surface charges *on the channel* were being screened. In charged bilayers, a change in ionic strength screens *both surface charges on the channel protein and on the membrane.* By subtracting the voltage shifts on the gating curve caused by ionic strength on the Na^+ channel in a neutral bilayer from the voltage shifts caused by ionic strength on a charged bilayer, I am estimating the influence of surface charges present *on the membrane* on the gating of sodium channels. The electrostatic potential generated by a charged membrane is a function of its surface charge density and the ionic strength of the solution. The value of the electrostatic potential decays with distance from the membrane-solution interface (Eq. 6). From the experimental points in Fig. 8, *A* and *B*, it is apparent that $\Delta V_{0.5}$ is not the voltage present at the membrane-solution interfaces ($x = 0$). It seems that the gating machinery of the sodium channel is indeed capable of sensing the potential generated by surface charges on the membrane but at specific distances from the membrane-solution interfaces. Because the electrostatic properties of these charged bilayers are well defined (Eisenberg et al., 1979; Hartsel and Cafiso, 1986;

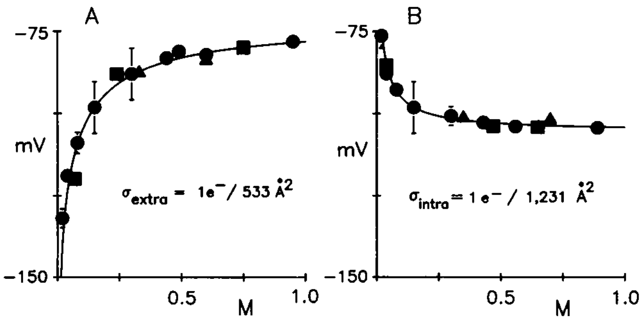

FIGURE 6 Midpoint of the gating curves of sodium channels versus extracellular (*A*) or intracellular (*B*) salt concentration. Each point is the mean of 3–7 different membranes. Standard deviation bars were omitted when smaller than the size of the symbols. Theoretical curves drawn according to Eq. 4 with the surface charge densities indicated in each panel. (*Circles*) NaCl; (*squares*) KCl, and (*triangles*) Na_2SO_4.

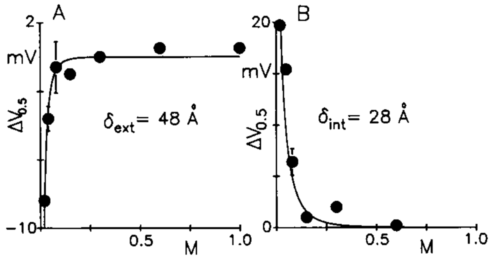

FIGURE 8 Extra voltage shifts ($\Delta V_{0.5}$), in relation to the midpoint of the activation curves of sodium channels in neutral bilayers, Fig. 6, as a function of extracellular (*A*) and intracellular (*B*) salt concentration in negatively charged bilayers. Each symbol represents the mean of 2–6 different observations. The theoretical curves describe the change in potential at distances indicated from the plane of the membrane (Eqs. 5 and 6). In this calculation it is assumed that electrostatic potentials of different origins (membrane and channel molecule) are nearly additive (see Israelachvili, 1985) at the low surface charge densities.

Langner et al., 1990; McLaughlin, 1977, 1989; Winisky et al., 1986, we calculated the electrostatic potentials at given distances from the plane of the bilayer at different ionic strengths. The values of those electrostatic potentials, at distances of 48 and 28 Å from the plane of the membrane, were plotted as theoretical curves in Fig. 8, *A* and *B*, respectively.

Because I am working with symmetrical bilayers, it is interesting to notice that the electrostatic contribution of membrane surface charges on Na^+ channel gating are different between the intracellular and extracellular sides of the channel (notice the different voltage scales in the two panels of Fig. 8). Thus, the effects of charged bilayers on the gating of Na^+ channels can be understood as if the voltage sensor of the Na^+ channel is able to sense the difference between the two following electrostatic potentials generated by surface charges on the bilayer: (*a*) at a distance of 48 Å on the extracellular membrane-solution interface, and (*b*) at a distance of 28 Å from the intracellular membrane-solution interface.

DISCUSSION

This study follows previous investigations on the effects of divalent cations on sodium channel gating (Cukierman et al., 1988; Cukierman and Krueger, 1990). Following those observations, we suspected that the intracellular and extracellular surface charge densities in the Na^+-channel molecule were different. I also noticed that the presence of negatively charged membranes influenced the interaction between external or internal divalent cations with Na^+-channels (Cukierman et al., 1988; Cukierman and Krueger, 1991). However, it was not clear whether the channel's gating machinery was able to sense the electrostatic field created by membrane surface charges or if the presence of a negatively charged membrane affected the interaction of divalent cations with the Na^+ channel by a different mechanism. Moreover, the effects of divalent cations were found to be incompatible with a simple electrostatic effect (Cukierman and Krueger, 1990; Cukierman and Krueger, 1991), thus complicating the analysis of our previous experimental results (see below). In this context, our studies with divalent cations are in agreement with previous observations on the effects of external divalent cations on sodium currents in squid axons (Gilly and Armstrong, 1982*a*, *b*).

In this paper, I have studied the effects of ionic strength on the gating of sodium channels. These experimental findings can be summarized as follows: (*a*) an increase in extracellular ionic strength shifts the activation curve of Na^+ channels to more depolarized membrane voltages; (*b*) an increase in intracellular ionic strength shifts those activation curves to more hyperpolarized voltages; (*c*) the effects of ionic strength (both intra and extracellular) can be accounted by their effects on the voltage shifts of the opening and closing rate constants of the channel; (*d*) the effects of ionic strength on Na^+ channels reconstituted in charged bilayers have qualitatively similar effects as in neutral bilayers. However, the voltage shifts on the gating (or rate constant X V_m) curves are more pronounced in the ionic strength range of 0–150 mM.

A significant feature of one proposed transmembrane segment in the Na^+ channel molecule (S_4, Noda et al., 1986) is the presence of positively charged amino acids every third residue (Noda et al., 1986). There is experimental evidence suggesting that these transmembrane segments are involved in the opening-closing of Na^+ channels (Stuhmer et al., 1989). Computational methods applied to the reconstruction of the Na^+-channel molecule in the membrane suggest that small "helical-screw" movements of this positively charged alpha-helix transmembrane domain could open the pore of the Na^+ channel. When the sodium channel opens, a positively charged residue in an S_4 domain would become exposed to the external side of the channel. The inward movement of a positive charge would result in channel closure (Guy and Seetharamulu, 1986; Guy and Conti, 1990). This representation is compatible with available electrophysiological information (Armstrong, 1981; Armstrong and Bezanilla, 1977).

My experimental results suggest that there are net negative surface charge densities on both the extracellular and intracellular surfaces of the Na^+ channel molecule. The presence of extracellular negative surface charges create an electric field that would help pulling those positively charged residues in the S_4 domain to the extracellular side (channel opening). An analogous reasoning could also be applied to the relationship between intracellular surface charges and the inward movement of positive charges (channel closing). In this sense, when the extracellular ionic strength increases, the negative electrostatic potential generated by those surface charges decreases, leading to a decrease in the open probability of the channel. The effects of increasing the intracellular ionic strength would have an opposite effect on channel gating: by decreasing the electric field caused by those intracellular surface charges, the channel would remain less time in the closed configuration, thus increasing P_o (shift of the gating curve to more hyperpolarized potentials).

It is extremely important to notice that the ionic strength *does not* modify the intrinsic sensitivity of the channel to transmembrane electric fields. Those negatively charged residues do not seem to allosterically influence the basic conformational changes in the channel molecule leading to opening-closing of the pore. However, those negative surface charges on the channel are essential for defining the precise location of the gating curve on the transmembrane potential axis.

Two different surface charge densities were found for the extracellular and intracellular sides of the Na^+ channel. These experimental results are compatible with surface charge densities of $1e^-/533$ Å^2 and $1e^-/1,231$ Å^2 for the extracellular and intracellular sides of the channel, respectively. The underlying assumption in determining those values was that the gating mechanism is located adjacent to those surface charges. The Gouy-Chapman treatment assumes a simple geometry for charge distribution and a uniform charge density. This is a very simple situation that might not occur on the Na^+-channel surfaces. However, in the absence of a detailed picture describing the geometric relationships between surface charges on Na^+ channel and its voltage sensing device, I regard the Gouy-Chapman model as a first *rough* approximation to quantify and understand electrostatic surface effects on Na^+-channel gating. This treatment approximates a complex physical picture into a more manageable model (McLaughlin, 1989). Nevertheless, the use of that model, by proposing new experiments and insights into how Na^+ channels work, can be used as a starting point for future questions.

The extra voltage shifts induced by charged bilayers in relation to neutral bilayers on the gating curve of Na^+ channels were also quantified (Fig. 8). A physical interpretation for this measurement is now being attempted. The extra voltage shifts induced by negatively charged membranes on Na^+-channel gating can be understood by an alteration of the electric field associated with those charges at specific distances from the membrane-solution interfaces. The gating charges which are involved in the opening-closing of the channel's pore, could interact with the electrostatic field created by surface charges on the membrane. The outward movement of positive charges (channel opening) could be modulated by the presence of an electrostatic potential at a distance of 48 Å away from the plane of the bilayer while the inward movement of positive charges (channel closing) might be affected by the potential generated by surface charges on the membrane at a 28-Å distance from the plane of the bilayer. This defines a second asymmetric electrostatic mechanism that occurs during the gating of Na^+ channels: it is conceivable that charge movements occurring during the opening and closing of Na^+ channels are asymmetrically displaced in relation to the two membrane-solution interfaces. However, it is important to notice that because more than one charge moves when the channel opens or closes, the values for the distances between the plane of the bilayer and gating charges are limited by the simplicity of an average or resultant charge model. Another possibility to consider is that gating charges in Na^+ channels sense the membrane electrostatic potentials laterally. In this sense, gating charges could be envisioned as being laterally insulated from the lipids at characteristic distances of 48 and 28 Å in the extracellular and intracellular sides of the channel, respectively (see Bell and Miller, 1984 for a similar reasoning concerning the conduction properties of sarcoplasmic reticulum K^+ channels). These calculated distances cannot be taken too seriously. At this point, all I can say is that the gating of Na^+ channels behaves *as if* it were capable of sensing electrostatic potentials at the given distances from the intracellular and extracellular membrane-solution interfaces.

Following my experimental results I proposed that at least three different electrostatic potentials modulate the opening-closing of Na^+ channels: (*a*) the applied transmembrane potential; (*b*) the potential generated by intracellular and extracellular surface charges on the channel's structure (that was estimated using neutral bilayers, Fig. 6), and (*c*) the potentials generated by membrane surface charges on both the extracellular and intracellular sides of the channel (Fig. 8). The latter seems to be sensed at specific distances from the membrane-solution interfaces on both sides of the channel and not at the membrane-solution interfaces.

It is important to mention that the effects of ionic strength on the gating of Na^+ channels are qualitatively different from the effects of divalent cations. Previous studies (Cukierman and Krueger, 1990) revealed that external divalent cations decreased the opening rate constant by a larger proportion than it increased the closing rate constant. Similar results were obtained with Zn^{2+} in squid axons (Gilly and Armstrong, 1982*a*, *b*). Intracellular divalent cations had an opposite effect: the closing rate constant was considerably more affected than the opening rate constant (Cukierman and Krueger, 1991). These results were intepreted by assuming that divalent cations exert two different effects on channel gating. First, divalent cations are able to change the electric field across the channel's voltage sensor. In addition to this, divalent cations are also able to exert a modulatory effect on Na^+-channel gating. The present results support the idea that surface charges affect the gating behavior of Na^+ channels. However, changes in ionic strength affect both opening and closing rate constants by the same proportion (see Figs. 4 and 5). While I am not in a position to explain the modulatory effect of divalent cations on Na^+ channel gating, our preliminary results show that even in the presence of 1 M NaCl, Ba^{2+} is still capable of inducing shifts in the gating curve of Na^+ channels (Cukierman, unpublished observations). Following the results in this study, the effects of Ba^{2+} in 1 M NaCl cannot be explained by a simple change in surface electrostatic potential as sensed by the gating machinery of the channel, because at this high concentration of NaCl, the surface charges on the channel are almost completely screened. Hopefully, future experimental results will better define the origin of modulatory effects of divalent cations on Na^+ channel gating.

The presented results finally explain why in previous studies (Cukierman et al., 1988) no alteration in the midpoint of sodium channel gating curves was found when the channel was reconstituted in neutral or in negatively charged bilayers: those experiments were conducted at extra- or intracellular ionic strengths higher than 150 mM and, due to the distances between the plane of the bilayer and the gating charges, the electrostatic effects of charged bilayers on the gating of the channel would be negligible. However, the effects of divalent cations on Na^+ channel gating are complex: all divalent cations tested enhanced the shift on the gating curve of Na^+ channels in charged bilayers in relation to neutral bilayers in 150 mM ionic strength solutions (Cukierman et al., 1988; Cukierman and Krueger, 1990; Cukierman and Krueger, 1991). Following the present study, I would not have anticipated such an effect. One possibility to explain why charged bilayers enhance the shift in the gating curve of Na^+ channels in relation to neutral bilayers is that charged membranes increase the binding affinity of sodium channels to divalent cations: charged bilayers could expose more to bathing solutions those negatively charged residues on the surfaces of the channel that bind divalent cations. Experiments to test this hypothesis are now in progress in my laboratory.

It is important to discuss the differences between surface charge effects on the gating and permeation processes in Na^+ channels. The effect of surface charges on ion permeation in Na^+ channels are not clear. My observations together with others (Cukierman and Krueger, 1990; French et al., 1986; Garber and Miller, 1987; Moczydlowski et al., 1984) suggest that the entrance of the Na^+ channel pore does not sense an electric field due to the presence of negative charges either on the channel structure or on the membrane surface. Opposite conclusion was reached by Green et al. (1987) in planar bilayers and by Smith-Maxwell and Begenisich (1987) in squid axons. This issue is of extreme relevance for the structural organization of the Na^+ channel in the membrane. Following our own observations, I proposed that those surface charges responsible for modulating the gating behavior of Na^+ channels are located several Debyes away from the pore entrances on both the extracellular and intracellular sides of the channel.

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