Effects of Membrane Surface Charge and Calcium on the Gating of Rat Brain Sodium Channels in Planar Bilayers

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ABSTRACT The voltage-dependent gating of single, batrachotoxin-activated Na channels from rat brain was studied in planar lipid bilayers composed of negatively charged or neutral phospholipids. The relationship between the probability of finding the Na channel in the open state and the membrane potential (P_o vs. V_m) was determined in symmetrical NaCl, both in the absence of free Ca^{2+} and after the addition of Ca^{2+} to the extracellular side of the channel, the intracellular side, or both. In the absence of Ca^{2+}, neither the midpoint ($V_{0.5}$) of the P_o vs. V_m relation, nor the steepness of the gating curve, was affected by the charge on the bilayer lipid. The addition of 7.5 mM Ca^{2+} to the external side caused a depolarizing shift in $V_{0.5}$. This depolarizing shift was ~17 mV in neutral bilayers and ~25 mV in negatively charged bilayers. The addition of the same concentration of Ca^{2+} to only the intracellular side caused hyperpolarizing shifts in $V_{0.5}$ of ~7 mV (neutral bilayers) and ~14 mV (negatively charged bilayers). The symmetrical addition of Ca^{2+} caused a small depolarizing shift in P_o vs. V_m. We conclude that: (*a*) the Na channel protein possesses negatively charged groups on both its inner and outer surfaces. Charges on both surfaces affect channel gating but those on the outer surface exert a stronger influence. (*b*) Negative surface charges on the membrane phospholipid are close enough to the channel's gating machinery to substantially affect its operation. Charges on the inner and outer surfaces of the membrane lipid affect gating symmetrically. (*c*) Effects on steady-state Na channel activation are consistent with a simple superposition of contributions to the local electrostatic potential from charges on the channel protein and the membrane lipid.

INTRODUCTION

It has long been recognized that Ca^{2+} ions exert a modulatory effect in excitable cells; an increase in Ca^{2+} concentration in the extracellular fluid decreases the level of excitability (c.f., Brink, 1954). This effect of Ca^{2+} was studied quantitatively by Frankenhaeuser and Hodgkin (1957) who showed that external Ca^{2+} caused depo-

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larizing shifts in the conductance-voltage relationships (gating curves) of Na^+ currents in squid giant axons. They suggested that their results could be explained, at least qualitatively, if Ca^{2+} ions could adsorb to negative surface charges present at the outer edge of the membrane.[1] According to this theory, in the absence of Ca^{2+}, the Na-conductance mechanism would sense a transmembrane potential that would be less than the bulk-to-bulk membrane potential because of the fixed negative charges at the external membrane surface. As a consequence of the adsorption of Ca^{2+} to these charges, the gating machinery would sense a steeper electric field, and the gating curve would shift in the depolarizing direction on the voltage axis. Later, Chandler et al. (1965) provided evidence that the internal side of the squid axon membrane also contained fixed negative charges capable of affecting channel gating. The effects of the interaction of cations with negative charges on the inner and outer surfaces of the membrane have been quantitatively explained using the Gouy-Chapman-Stern theory (Gilbert and Ehrenstein, 1969; Mozhayeva and Naumov, 1970; Begenisich, 1975; Hille et al., 1975; McLaughlin, 1977).

The effects of Ca^{2+} on Na channels are, in fact, more complex than an action only on channel gating. For example, it is known that external Ca^{2+} blocks the current that flows through Na channels in a voltage-dependent manner (Woodhull, 1973; Mozhayeva et al., 1982). Single-channel measurements, using patch-clamp and planar-bilayer techniques, have eliminated any ambiguity between the effects of Ca^{2+} on permeation and on gating by measuring discrete, unitary current fluctuations through single channels (Yamamoto et al., 1984; Worley et al., 1986). Thus, any effects of Ca^{2+} on gating (probability of being open) and on permeation (block of single-channel conductance) can be clearly distinguished. Reconstituting Na channels into bilayers of defined lipid composition (Moczydlowski et al., 1984; Green et al., 1987; Worley, J. F., R. J. French, and B. K. Krueger, manuscript submitted for publication) revealed that Na^+ permeation and Ca^{2+} block are not influenced by the charge on the membrane lipids, and offers the further possibility of distinguishing between the effects on Na channel gating of charges on the membrane lipids and charges on the channel protein.

In this study, we have begun to evaluate the influence of membrane phospholipid surface charge on Na channel gating. Specifically, we were interested in (*a*) the extent to which the channel gating machinery senses the charges on the lipids and (*b*) whether charges on the channel protein itself could also affect channel gating. We have approached these problems by measuring Ca^{2+}-induced shifts along the voltage axis of the activation curve for Na channels incorporated into neutral or

[1] An alternative hypothesis that was also suggested by Frankenhaeuser and Hodgkin (1957) was that voltage-dependent gating may be due to a voltage-dependent block by an extracellular cation, such as Ca^{2+}, so that depolarization would relieve block and increase conductance. According to this mechanism, the effects of extracellular Ca^{2+} on channel gating would simply be due to an increased occupancy of the blocking site. This particular hypothesis has been ruled out because the kinetics of Ca^{2+} block are too fast to account for gating and Na channels exhibit voltage-dependent gating in the absence of Ca^{2+} (Hille, 1968; Woodhull, 1973; Hille et al., 1975). However, variations on this mechanism, in which extracellular divalent cations may selectively alter opening or closing rates by binding to exposed gating charges (Gilly and Armstrong, 1982) or latching channels in the closed state (Armstrong and Matteson, 1986), have recently been proposed.

negatively charged lipid bilayer membranes. Our observations indicate that negatively charged groups on both the channel protein and on the membrane lipid affect channel gating by contributing additively to the local electrostatic potential gradient sensed by the channel. Preliminary reports of some of our observations have been given by French et al. (1986) and Cukierman et al. (1988).

MATERIALS AND METHODS

Materials

Phospholipids were purchased from Avanti Polar Lipids, Birmingham, AL. Batrachotoxin (BTX) was provided by Dr. John Daly, National Institutes of Health, Bethesda, MD. All inorganic salts were ultrapure grade (0.9999) and were obtained from Alfa Products, Danvers, MA. Rat brain plasma membranes (P_3) were prepared by the method of Krueger et al. (1979); the specific activity for binding of [^{3}H]saxitoxin was at least 5 pmol/mg protein (Krueger et al., 1979).

Planar Lipid Bilayer Formation

Planar bilayers were formed across a 200–250-μm hole in a Lexan partition separating two identical aqueous solutions. The membrane-forming solution contained either pure lipid or mixtures of 1-palmitoyl 2-oleoyl phosphatidylethanolamine (PE), 1-palmitoyl 2-oleoyl phosphatidylserine (PS), and 1-palmitoyl 2-oleoyl phosphatidylcholine (PC). The following proportions were used: PS, 100% PS; PE/PS, 56% PE and 44% PS; PE, 100% PE; and PE/PC, 80% PE and 20% PC. In general, 100% PS membranes tended to be less stable after channel incorporation. The lipids were dissolved in decane at a final concentration of 60 mg/ml. Once the phospholipid film was formed, its thinning was followed both by visual inspection and by the capacitance increase monitored by an applied triangular voltage signal. Only bilayers with DC-resistances >100 GΩ were used. Command potentials were applied via a Ag/AgCl electrode to the *cis* side. The *trans* side was maintained at virtual ground with a homemade current-to-voltage converter (French et al., 1986). The transmembrane current was recorded on videotape (NeuroData Instruments, Inc., New York, NY) or an FM instrumentation recorder (model B; A. R. Vetter, Co. Rebersburg, PA).

Channel Incorporation and Measurement of Single Na Channel Currents

Channel incorporation was carried out as described by Krueger et al. (1983). The basic solution employed during this study was (in millimolars): 150 NaCl, 10 HEPES, 0.1 $MgCl_2$, and 0.05 EGTA. The pH of the solution was adjusted to 7.0 with NaOH. BTX, a steroidal-alkaloid neurotoxin that removes Na channel inactivation, was added to the *cis* side of the membrane at a final concentration of 120 nM. Similar results were obtained with BTX added only to the *trans* side or when a small aliquot of P_3 was mixed with BTX before its addition to the bath chamber. Symmetrical Mg^{2+} (100 μM) was present in all experiments to facilitate channel incorporation. We have established that the presence of that concentration of Mg^{2+} did not affect the results reported here. Ca^{2+} was added from a 1 M $CaCl_2$ stock solution. All experiments were performed at room temperature (20–22°C).

Once the membrane had thinned, P_3 vesicles were added to the *cis* side at a final concentration of 20 μg of protein/ml. After stirring the solution for a few seconds, the membrane was held at −60 mV until a Na channel incorporated into the bilayer. Incorporation was visualized by a sudden displacement of the current record on the oscilloscope screen. Usually, incorporation could be obtained within 15–30 min after P_3 addition. If no incorporation was

observed after 45 min a new membrane was formed and the entire process was repeated. The intracellular side of the channel was defined as the side to which negative voltages (~−90 mV) induced closing of the Na channel. In most cases, the *trans* side was the intracellular side. Transmembrane voltages (V_m) are defined as the $V_{intracellular} - V_{extracellular}$. Reported values for V_m were corrected for ~±2.5 mV electrode offsets due to asymmetric chloride.

After channel incorporation, a control gating curve was generated by recording the transmembrane current at several potentials from −60 to −120 mV. At each test potential, records were taken for at least 20 s after the capacity transient had settled. Ca^{2+} (7.5 mM) was then added either to the extracellular or intracellular side of the bilayer, and a second gating curve was acquired. In some experiments, a second addition of Ca^{2+} was made to the opposite side and the same channels were then studied with symmetrical Ca^{2+}. In general, the results reported here are based on membranes that contained at most three Na channels, all with the same orientation in the bilayer. For determination of the steepness (q) of the gating curve, only data with a single channel in the bilayer were analyzed to avoid artifactual reduction of the apparent q for an ensemble of more than one channel when the individual $V_{0.5}$'s were not identical.

Data Analysis

Data were digitized in 4,096-point segments at 1 ms per point using a digital oscilloscope (2090-3A; Nicolet Instrument Corp., Madison, WI). Data transcription and analysis were controlled by a microcomputer (Plessey Peripheral Systems, Irvine, CA) based on an LSI-11/23 processor (Digital Equipment Corp., Maynard, MA). Software for data handling and analysis was developed using the interpretative language, DAOS (Laboratory Software Associates, Melbourne, Australia). All records were low-pass filtered at a corner frequency of 100 Hz using an eight-pole Bessel filter (Frequency Devices, Haverhill, MA).

Single Na channel current records were visually monitored on a CRT display during analysis. Limiting open and closed current levels used in determining the fractional open times (P_o) were checked against a whole group of records before carrying out the analysis. If necessary, the selected values were changed to accommodate small shifts in the baseline within a series of records. We excluded abnormally noisy segments of the records from the analysis. Occasionally, we observed spontaneous, abrupt shifts in P_o (Moczydlowski et al., 1984; French et al., 1986; Green et al., 1987). Normally these periods of nonstationarity represented <5% of the total recording and such segments were omitted from the analysis. Experiments exhibiting >~5% nonstationarity were discarded.

P_o values were determined as described by French et al. (1984). A figure illustrating the typical placement of cursors is shown in French et al. (1986). The background leakage current, i_L, across the membrane with all channels closed, was identified by setting a horizontal cursor at the low conductance level of the envelope of the single-channel fluctuations. The maximum current level, i_{max} (all channels open), was determined in an analogous manner. Values of P_o calculated in this manner were highly reproducible among different operators working with the same data set. The average current, $\langle i \rangle$, over at least 30 s, was then computed. P_o is then given by:

$$P_o = (\langle i \rangle - i_L)/(i_{max} - i_L) \quad (1)$$

$V_{0.5}$ was determined by assuming that P_o is a Boltzmann function of the voltage. Data points for $0.05 < P_o < 0.95$ were fit to a linearized form of the Boltzmann relation:

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

where R, T, and F have their usual meanings, and $V_{0.5}$ and q were determined from a linear least squares fit to Eq. 2. Variation among repeated determinations of $V_{0.5}$, for the same

channel under constant ionic conditions, is much smaller (on the order of 1 mV) than the shifts due to Ca^{2+} addition. Changes in the order in which different voltages were applied revealed no significant hysteresis in P_o.

RESULTS

Na Channel Gating in Neutral and Negatively-charged Bilayers

Fig. 1 shows P_o vs. V_m relations for two different Na channels in bilayers with two different net charges (Worley, J.F., R.J. French, and B.K. Krueger, manuscript sub-

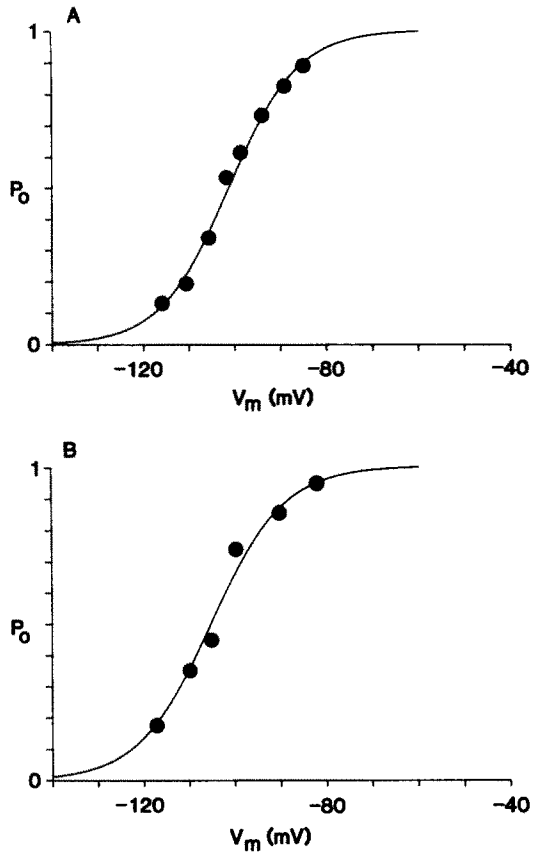

FIGURE 1. P_o vs. V_m curves for single Na channels in (*A*) neutral (PE/PC) and (*B*) negatively charged (PE/PS) bilayers. P_o was computed as described in Materials and Methods. The smooth curves were drawn according to Eq. 2 with values for $V_{0.5}$ and q of (*A*) −102 mV and 3.4, and (*B*) −104 mV and 3.2. (Experiments PEPCZ6 and PS0001.)

mitted for publication), namely near 0 (PE/PC membrane; Fig. 1 *A*) and ~−0.6 charges/lipid headgroup (PE/PS; Fig. 1 *B*). Although there was a small difference in $V_{0.5}$ in the experiments shown in Fig. 1, analysis of a large number of experiments (Table I) revealed that the gating of the Na channels was not significantly affected by the net charge on the bilayer lipids. In 32 different charged bilayers (10 pure PS and 22 PE/PS membranes) $V_{0.5}$ was −98.0 ± 1.4 mV (mean and SEM) whether the bilayer contained only 44% PS (PE/PS) or 100% PS. In 19 different neutral bilayers this value was −100.0 ± 1.7 mV. There was considerable variation in $V_{0.5}$ from channel to channel (standard deviation was ±8–10 mV for all conditions studied), as

TABLE I

$V_{0.5}$ and q Are Independent of Lipid Charge

Lipid	$V_{0.5}$	q
PE/PC and PE	−100 ± 1.7 mV (19)	3.1 ± 0.2 (13)
PE/PS	−97 ± 1.4 mV (22)	3.3 ± 0.3 (11)
PS	−100 ± 3.5 mV (10)	3.2 ± 0.4 (7)

PE and PE/PC bilayers are neutral; PS and PE/PS bilayers are negatively charged. These values were determined from Eq. 2 as described in Materials and Methods. The means ± SEM are given for the number of determinations shown in parentheses. For determination of $V_{0.5}$, bilayers contained one, two, or three channels, all with the same orientation in the bilayer. For determination of q, only data from bilayers with one channel were included in order to eliminate the possibility that two channels with different $V_{0.5}$'s might give an erroneously low apparent q. In the absence of free Ca^{2+}, symmetrically varying the lipid surface charge does not alter $V_{0.5}$ or q.

has been reported for purified sodium channels in planar bilayers (Hartshorne et al., 1985). The effective gating charge, q (from Eq. 2), was also unaffected by the charge on the membrane lipid, being about 3 in both neutral and negatively charged bilayers (Table I).

Effects of Extracellular Ca^{2+} on Na Channel Gating in Neutral and Negatively Charged Bilayers

Fig. 2 shows representative single-channel current records from a Na channel in a neutral bilayer under control conditions at −87 mV (upper record) and after the addition of 7.5 mM Ca^{2+} to the external side (lower record). External Ca^{2+} caused a depolarizing shift in $V_{0.5}$ as indicated by more frequent channel closing events in the presence of external Ca^{2+}. Also, the record shows that Ca^{2+} reduced the single-channel current, presumably reflecting a fast block that was not resolved at this bandwidth. This block was voltage dependent (not shown) as was previously

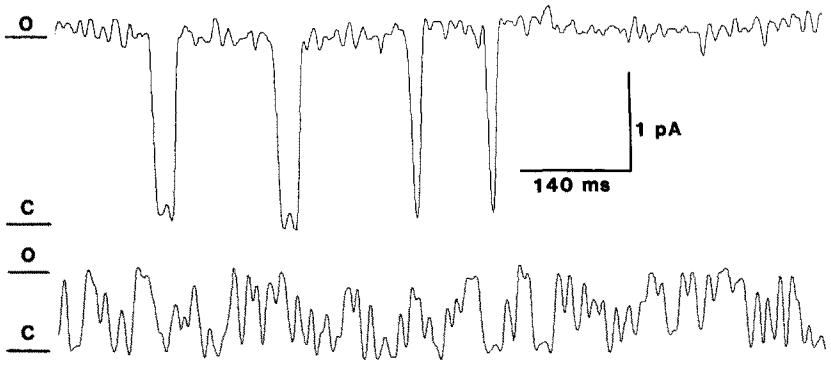

FIGURE 2. Single Na channel currents in a neutral (PE) bilayer (−87 mV) before (upper record) and after (lower record) the addition of 7.5 mM Ca^{2+} to the external side. Computed P_o's in this experiment were 0.90 (upper) and 0.44 (lower). (Experiment PE101.)

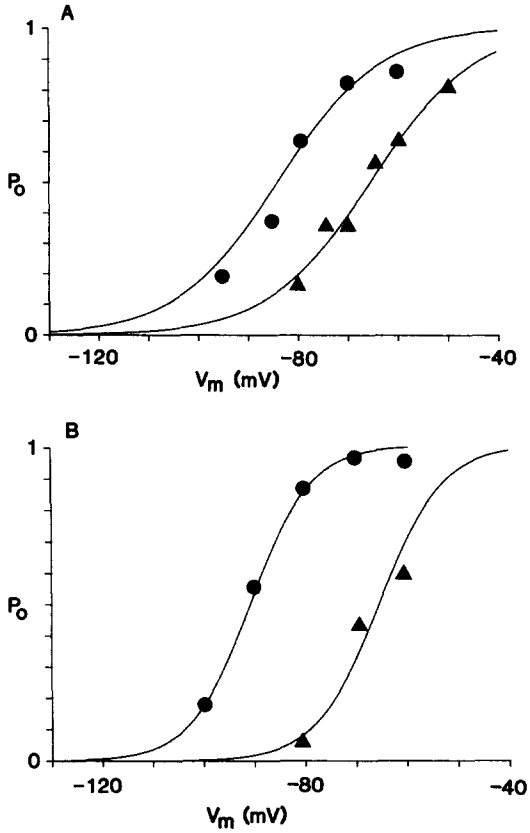

FIGURE 3. P_o vs. V_m curves for single Na channels in (*A*) neutral (PE/PC) and (*B*) negatively charged (PE/PS) bilayers. (●) control; (▲) after the addition of 7.5 mM Ca^{2+} to the external side. P_o was computed as described in Materials and Methods. The smooth curves were drawn according to Eq. 2 with values for $V_{0.5}$ and q of: (*A*) −82 mV and 2.5 (●), and −66 mV and 2.5 (▲); and (*B*) −92 mV and 4.3 (●), and −65 mV and 4.1 (▲). (Experiments PEPCZ3 and PEPSZ3.)

reported (Woodhull, 1973; Worley et al., 1986; Green et al., 1987; Worley, J. F., R. J. French, and B. K. Krueger, manuscript submitted for publication). All of our experiments were carried out with 7.5 mM Ca^{2+}, a concentration that provides a significant shift in $V_{0.5}$ without excessive block. Fig. 3 shows how channel gating (P_o vs. V_m) was affected by 7.5 mM external Ca^{2+} in negatively charged and neutral membranes. The data from all of our experiments are summarized in Table II. The average Ca^{2+}-induced shift in Na channel gating was ~25 mV in negative bilayers

TABLE II

Activation Curve Shifts ($\Delta V_{0.5}$) Produced by External Ca^{2+} with Neutral (PE/PC) or Negatively Charged (PE/PS and PS) Membrane Lipids

Lipid	$\Delta V_{0.5}$ (7.5 mM external Ca^{2+})
	mV
PE/PC	17.2 ± 2.2 (9)
PE/PS	25.8 ± 2.1 (5)
PS	22.3 ± 1.2 (3)

$\Delta V_{0.5}$ is always greater in negatively charged lipids but is about the same in 44% PS (PE/PS) and pure PS. Data shown are mean $\Delta V_{0.5}$ ± SEM. $\Delta V_{0.5}$ for PE/PS and for PS were not significantly different (P = 0.29; pooled Student's t test).

TABLE III

$\Delta V_{0.5}$ for External and Internal Ca^{2+} for Both Neutral and Negatively Charged Bilayers

Ca^{2+} (7.5 mM)	Neutral lipid	Negative lipid	Shift due to negative lipid $\Delta V_{0.5}$ (negative) − $\Delta V_{0.5}$ (neutral)
External	17.2 ± 2.2 (9)	24.5 ± 1.5 (8)	7.3
Internal	−7.3 ± 1.7 (4)	−14.3 ± 2.0 (9)	−7.0
$\Delta V_{0.5}$ (external) + $\Delta V_{0.5}$ (internal)	9.9	10.2	—

Data for PE/PS and PS bilayers were pooled and reported under negative lipid as discussed in the text. Data shown are mean $V_{0.5}$ ± SEM. $\Delta V_{0.5}$'s in neutral bilayers are attributable to charges on the channel protein. $\Delta V_{0.5}$ (negative) − $\Delta V_{0.5}$ (neutral) is the magnitude of the Ca^{2+}-induced shift attributable to the negative charge on the lipid. $\Delta V_{0.5}$ (external) + $\Delta V_{0.5}$ (internal) is the increment by which $\Delta V_{0.5}$ is larger for external Ca^{2+}, which probably reflects a higher negative charge density on the external side of the channel protein. Two important points are clear from this table. First, the shifts in negative bilayers are larger by ~10 mV for both external and internal Ca^{2+} (bottom row). Second, the differences in the magnitudes of the shifts attributable to the negative charge on the lipid do not differ significantly for external and internal Ca^{2+} (fourth column).

and ~17 mV in neutral bilayers. There was a slightly smaller Ca^{2+}_o-induced shift in pure PS than in PE/PS bilayers (Table II), which have a substantially lower net negative surface charge, however, this difference was not significant (see the legend of Table II). When data from 100% PS and PE/PS bilayers are pooled, Ca^{2+}_o induced about a 7-mV larger depolarizing shift in negative bilayers than in neutral bilayers (Table III).

Effects of Intracellular Ca^{2+} on Na Channel Gating

Fig. 4 shows single-channel recordings which illustrate that when Ca^{2+} was added to the internal side of the Na channel, a hyperpolarizing voltage shift in $V_{0.5}$ was observed. Intracellular Ca^{2+} causes a weakly voltage-dependent block (Worley, J. F.,

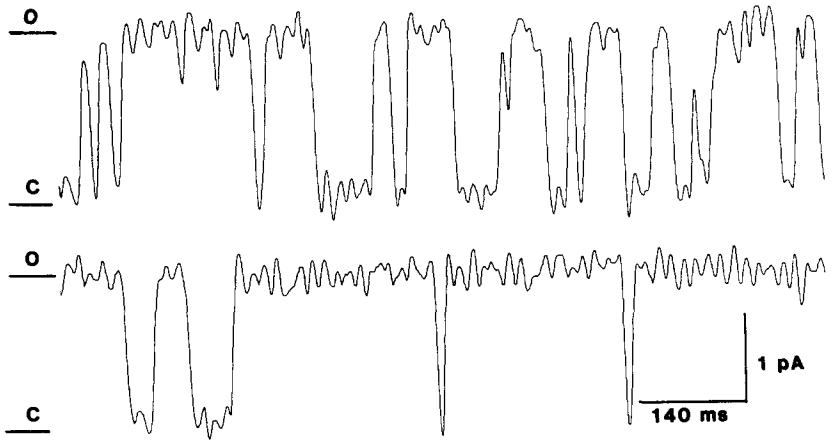

FIGURE 4. Single Na channel currents before (upper record) and after (lower record) the addition of 7.5 mM Ca^{2+} to the internal side. V_m was −90 mV for the upper record and −88 mV for the lower record. Computed P_o's were 0.48 (upper) and 0.90 (lower). (Experiment PEPS02.)

W. F. Wonderlin, B. K. Krueger, and R. J. French, manuscript submitted for publication). Fig. 5 shows a plot of P_o vs. V_m in neutral, PE (Fig. 5 *A*), and negatively charged PE/PS (Fig. 5 *B*) bilayers. The summary of all of our data (Table III) reveals that the average shift in $V_{0.5}$ induced by 7.5 mM internal Ca^{2+} was ~ -14 mV in negatively charged (both PE/PS and pure PS) bilayers and ~ -7 mV in neutral (PE and PE/PC) bilayers. Thus, as was observed for external Ca^{2+}, the shift induced by internal Ca^{2+} was ~7 mV larger in negative than in neutral bilayers. Moreover, the Ca^{2+}_o-induced shift in $V_{0.5}$ was ~10 mV larger than the Ca^{2+}_i-induced shift in both neutral and negatively charged bilayers (Table III, row 3).

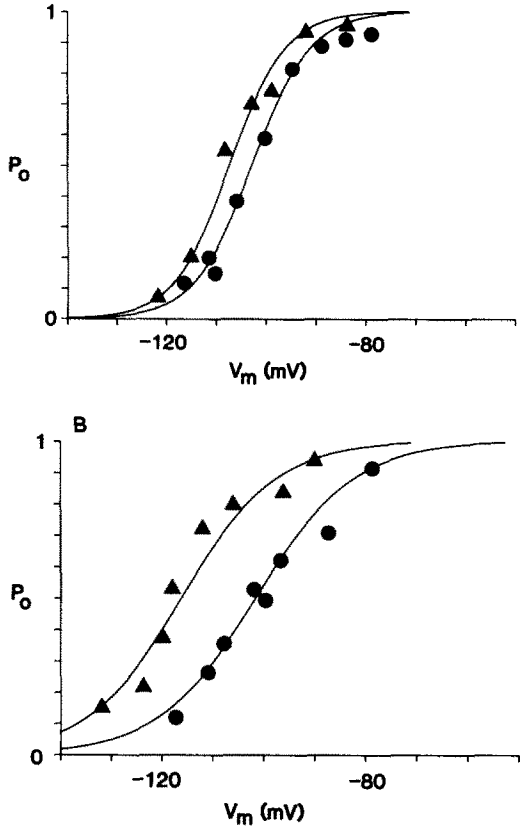

FIGURE 5. P_o vs. V_m curves for single Na channels in (*A*) neutral (PE) and (*B*) negatively charged (PE/PS) bilayers. (●) control; (▲) after the addition of 7.5 mM Ca^{2+} to the internal side of the channel. P_o was computed as described in Materials and Methods. The smooth curves were drawn according to Eq. 2 with values for $V_{0.5}$ and q of: (*A*) −103 mV and 4.5 (●), and −110 mV and 4.4 (▲); and (*B*) −100 mV and 2.6 (●), and −116 mV and 2.7 (▲). (Experiments PEPS05 and PE001.)

Effect of Symmetrical Ca^{2+} on Na Channel Gating

In some experiments with negative bilayers, it was possible to obtain adequate recordings of single Na channel activity with Ca^{2+} added to both sides of the membrane (Table IV; Fig. 6). When Ca^{2+} had been previously added asymmetrically to the external side, $V_{0.5}$ in symmetrical Ca^{2+} moved toward $V_{0.5}$ in control Ca^{2+}-free conditions. When Ca^{2+} had been previously added asymmetrically to the internal side, $V_{0.5}$ in symmetrical Ca^{2+} moved past the control $V_{0.5}$ by ~9 mV. Several difficulties prevented us from obtaining a large number of acceptable experiments. (*a*) Often, the membrane was not stable enough to permit completion of the experi-

TABLE IV

The Shift in the Activation Curve Produced by Symmetrical Ca^{2+} Addition

Lipid	$\Delta V_{0.5}$ (7.5 mM Ca^{2+})		$\Delta V_{0.5}$ (external) + $\Delta V_{0.5}$ (internal)	$\Delta V_{0.5}$ (symmetrical)
	External Ca^{2+}	Internal Ca^{2+}	Predicted	Observed
PE/PS and PS	24.5 ± 1.5 (8)	−14.3 ± 2.0 (9)	10.2 mV	8.8 ± 3.6 (4)

The shift in the activation curve produced by symmetrical Ca^{2+} addition, $\Delta V_{0.5}$ (symmetrical), is equal (within experimental error) to the algebraic sum of the shifts produced by external or internal Ca^{2+} added separately, $\Delta V_{0.5}$ (external) + $\Delta V_{0.5}$ (internal).

ment with symmetrical Ca^{2+} after control data and data in the presence of asymmetric Ca^{2+} had been acquired. (*b*) Because Ca^{2+} also blocks Na channels, reduced single-channel currents in the presence of Ca^{2+} precluded an accurate determination of $V_{0.5}$ in some cases and especially when Ca^{2+} was added to both sides. Fig. 6 shows the effect of intracellular Ca^{2+} followed by symmetrical Ca^{2+} in neutral (Fig. 6 *A*) and negatively charged (Fig. 6 *B*) bilayers. These data are from two experiments where the opening and closing events could be clearly discerned and $V_{0.5}$ could be accurately determined. All five complete experiments (one in neutral, PE/PC, shown in Fig. 6 *B*; four in negative, PE/PS) in which the effects of symmetrical 7.5

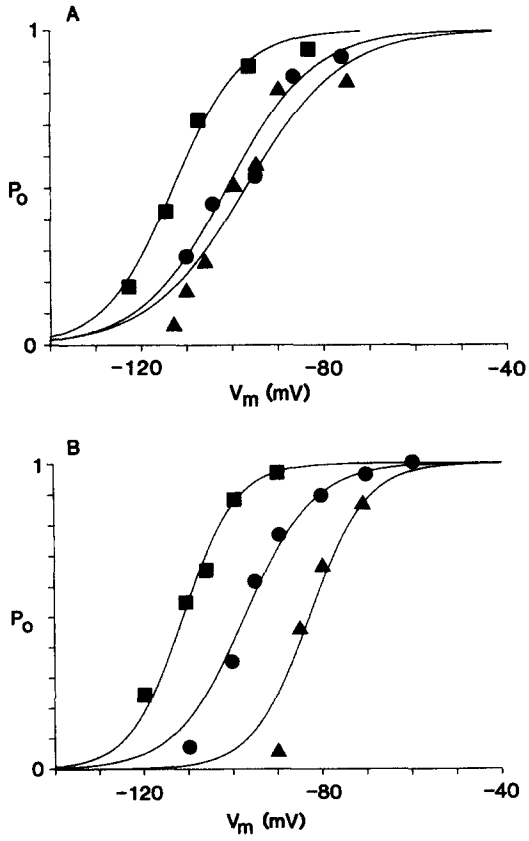

FIGURE 6. P_o vs. V_m curves for single Na channels in (*A*) neutral (PE/PC) and (*B*) negatively charged (PE/PS) bilayers. (●) no divalent cations; (■) 7.5 mM Ca^{2+} added to the internal side; (▲) same channel after the addition of 7.5 mM Ca^{2+} to the external side (symmetrical Ca^{2+}). The smooth curves were drawn according to Eq. 2 with values for $V_{0.5}$ and q of: (*A*) −100 mV and 2.7 (●), −113 mV and 3.4 (■), and −99 mV and 2.4 (▲); and (*B*) −97 mV and 3.3 (●), −112 mV and 4.3 (■), and −82 mV and 4.0 (▲). (Experiments PEPSZ4 and PEPC12.)

mM Ca^{2+} could be determined, showed that symmetrical Ca^{2+} shifted $V_{0.5}$ in the depolarizing direction, as would be expected from the larger effect of external Ca^{2+}. Table IV summarizes the results of the four experiments that were conducted with symmetrical Ca^{2+} addition in negatively charged bilayers. The depolarizing shift induced by symmetrical Ca^{2+} (~9 mV) did not differ significantly from the predicted depolarizing shift $\Delta V_{0.5}$ (external) + $\Delta V_{0.5}$ (internal).

DISCUSSION

In intact cells, extracellular Ca^{2+} depresses excitability, an effect that has been attributed to binding or screening of negatively charged groups on the membrane,

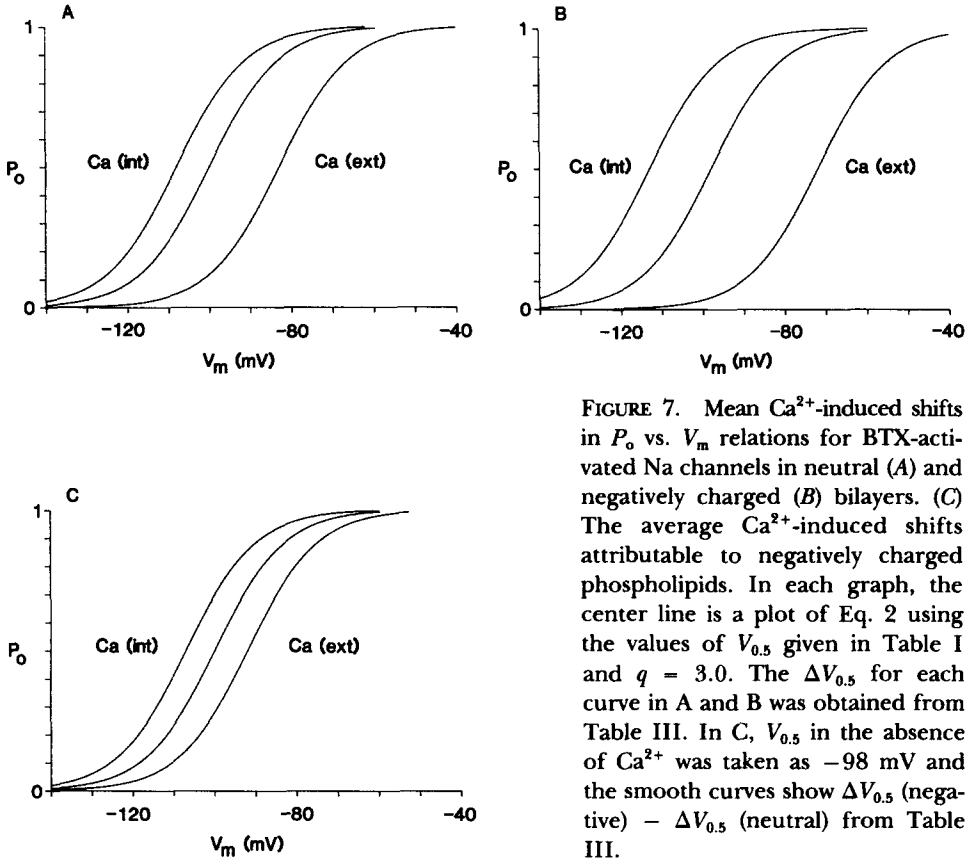

FIGURE 7. Mean Ca^{2+}-induced shifts in P_o vs. V_m relations for BTX-activated Na channels in neutral (*A*) and negatively charged (*B*) bilayers. (*C*) The average Ca^{2+}-induced shifts attributable to negatively charged phospholipids. In each graph, the center line is a plot of Eq. 2 using the values of $V_{0.5}$ given in Table I and q = 3.0. The $\Delta V_{0.5}$ for each curve in A and B was obtained from Table III. In C, $V_{0.5}$ in the absence of Ca^{2+} was taken as −98 mV and the smooth curves show $\Delta V_{0.5}$ (negative) − $\Delta V_{0.5}$ (neutral) from Table III.

and results in an increase in the level of depolarization required for activation of Na channels (Frankenhaeuser and Hodgkin, 1957). Previously, there has been little information about whether these charged groups (or possibly Ca^{2+} binding sites) are associated with the membrane lipids or the channel proteins, or both. The results reported here provide direct evidence for an influence of both lipid and channel-associated charges on gating. Fig. 7 presents a summary of our data (see also Table III) on Ca^{2+}-induced shifts in the midpoint ($V_{0.5}$) of the Na channel, P_o vs. V_m relation. In neutral bilayers, internal and external Ca^{2+} caused hyperpolarizing or depo-

larizing shifts, respectively, in Na channel activation. We believe that these effects of asymmetrically applied Ca^{2+} reflect a direct interaction of Ca^{2+} with negatively charged sites on the two sides of the channel protein. The depolarizing shift caused by external Ca^{2+} was ~10 mV larger than the shift caused by internal Ca^{2+} (Fig. 7 *A*; Table III). Although in negatively charged bilayers asymmetric Ca^{2+} caused larger shifts in channel activation than those observed in neutral bilayers (Fig. 7 *B*), the depolarizing shift induced by external Ca^{2+} was still ~10 mV larger than the hyperpolarizing shift caused by internal Ca^{2+}. We believe that the Ca^{2+}-induced gating shifts observed in negatively charged membranes reflect the sum of the effects due to Ca^{2+} interacting directly with the Na channel protein and to Ca^{2+} interacting with the negatively charged membrane lipids. The effects of asymmetric Ca^{2+} that are due to interactions with the lipids can be estimated by subtracting the shifts in activation in neutral bilayers from those in negative bilayers (Table III). Those shifts were equal for internal and external Ca^{2+} (Fig. 7 *C*).

Ca^{2+} Interacts Directly with the Na Channel to Induce Gating Shifts

A Ca^{2+}-induced shift in the Na channel P_o vs. V_m relation does not require the presence of negatively charged membrane lipids. Separate applications of intracellular and extracellular Ca^{2+} induced oppositely directed voltage shifts in the Na channel gating curves in neutral PE or PE/PC membranes (Table III). It is unlikely that these effects are due to Ca^{2+} interacting with neutral phospholipids.[2] This finding together with the observation that external Ca^{2+} caused a larger shift in $V_{0.5}$ than internal Ca^{2+} (Table III, Fig. 7, and see below), suggests that either external or internal Ca^{2+} can cause a shift in the Na channel gating curve by interacting directly with the Na channel protein itself.

Previous studies have provided evidence suggesting that Ca^{2+}-induced shifts in channel gating may not simply be due to interactions of Ca^{2+} with negatively charged phospholipids. Begenisich (1975) showed different shifts in the activation curves for K^+ and Na^+ currents in *Myxicola* axons resulting from a fourfold increase in the external Ca^{2+} concentration. This suggests that even when the Na channel is in the native membrane, its gating behavior is directly affected by Ca^{2+} ions. However, in those experiments the possibility that Na and K channels are surrounded by lipids of different composition can not be eliminated. In frog node of Ranvier, both the channel-forming antibiotic alamethicin (Cahalan and Hall, 1982) and the lipophilic anion dipicrylamine (Benz and Nonner, 1981) sensed an asymmetric surface charge distribution different from that sensed by Na channels, which suggests that

[2] Ca^{2+} does not bind strongly to the neutral lipids used in this study. McLaughlin et al. (1978) measured a dissociation constant of 1 M for Ca^{2+} binding to PC bilayers, which suggests that at 7.5 mM Ca^{2+}, <1% of the lipid molecules would bind Ca^{2+}. Also, binding of Ca^{2+} to PE does not seem to be significant under our experimental conditions (McLaughlin et al., 1970; Stollery and Vail, 1977). Thus, there is not likely to be any significant interaction of Ca^{2+} with neutral bilayers and the Ca^{2+}-induced shifts in these membranes result from a direct interaction of Ca^{2+} with the channel protein. In contrast to neutral lipids, the dissociation constant for PS-Ca^{2+} binding is 0.03–0.1 M (McLaughlin et al., 1970, 1981; Newton et al., 1978), which suggests that the additional effects of Ca^{2+} reported here for Na channels in negatively charged membranes result from a combination of screening and specific binding to the PS.

charges on the channel protein may affect its voltage-dependent gating. Again, the possibility that each channel type might be surrounded by a different lipid composition in the native nerve membrane cannot be ruled out.

It seems clear that some divalent cations can affect Na channel gating via a direct interaction with the channel protein. Gilly and Armstrong (1982) showed that extracellular Zn^{2+} preferentially slowed the opening of Na channels in squid axons. The authors suggested that Zn^{2+} binds to and stabilizes negative charges exposed on the external side of the channel protein only in the closed configuration. Those results may be contrasted with the observations in frog skeletal muscle by Hahin and Campbell (1983) that Ca^{2+} and Mg^{2+} cause simple shifts in all gating parameters including activation, tail current decay, inactivation, and gating currents, which suggests that the effects are due to changes in surface potential and to the resulting changes in the transmembrane potential gradient sensed by the channel gating machinery. It was pointed out, however, that the differences in potency between Ca^{2+} and Mg^{2+} could result from their binding to charged groups on the channel protein rather than to screening negative charges on the membrane lipids.

There Are More Negative Charges on the Extracellular Side of the Channel than on the Intracellular Side

External Ca^{2+} (7.5 mM) shifted $V_{0.5}$ by ~25 mV in negative and 17 mV in neutral bilayers (Table II; Fig. 7). On the other hand, the same concentration of Ca^{2+} when applied to the internal side of the channel displaces the activation curves by only ~ -14 mV in negatively charged and -7 mV in neutral bilayers (Table III; Fig. 7). In both types of bilayers, the difference in $V_{0.5}$ displacements between external and internal Ca^{2+} additions amounts to ~10 mV (see Table III). This observation provides further evidence that the Na channel, rather than the negatively charged lipid headgroups, is the site of interaction that accounts for the asymmetric effects of Ca^{2+}. The clear asymmetry in the actions of Ca^{2+} on Na channel gating suggests a higher density of Ca^{2+} binding sites on the external side of the channel. In negatively charged bilayers, the symmetric addition of Ca^{2+} induced a small depolarizing shift in $V_{0.5}$ of ~9 mV. The magnitude of this shift was about what was predicted (10 mV) by algebraic addition of the shifts induced separately by internal or external Ca^{2+} (Table IV) as would be appropriate for simple electrostatic effects from Ca^{2+} at each surface. Thus, with equal Ca^{2+} concentrations on both sides of the bilayer, both the ionic composition of the electrolyte solutions and the lipid phase of the membrane are symmetric, leaving only the channel protein to account for the observed depolarizing shift in $V_{0.5}$.

Do Native Lipids Surrounding the Na Channels Mix with Bilayer Lipids?

One possible explanation for the results in neutral bilayers is that the composition of lipids in the immediate vicinity of the channels may not be the same as in the bulk of the bilayer. The possibility exists that the channels enter the bilayer surrounded by an annulus of native lipid that insulates the channel from the bilayer lipids. Except for our finding that some effects of Ca^{2+} on Na channel gating are attributable to the lipid charge (Table III; Fig. 7), there is no direct experimental evidence dealing with Na channels that bears on this question. However, there is some infor-

mation from studies on different channels that demonstrate an influence of bilayer lipid charge on single-channel behavior. Studies of the skeletal muscle sarcoplasmic reticulum K channel (Bell and Miller, 1984), Ca^{2+}-activated K channel (Moczydlowski et al., 1985), and muscle voltage-dependent Ca^{2+} channels (Coronado and Affolter, 1986) showed that the surface charge due to bilayer lipids can be close enough to the mouth of the channel pore to affect permeation as revealed by non-hyperbolic conductance-ion activity relations. No evidence for negative lipid surface charge close to the mouth of the Na channel has been found (Moczydlowski et al., 1984; Green et al., 1987; Worley, J. F., R. J. French, and B. K. Krueger, manuscript submitted for publication), probably because the entrance to the pore is isolated from the membrane lipids by the large channel protein itself. Our results reveal that the amount of shift in the gating curve promoted by Ca^{2+} depends on the bilayer composition: negatively charged membranes showed a greater Ca^{2+}-induced shift than neutral membranes, indicating that the lipid head groups can come close enough to the channel to influence gating (but not permeation).

Symmetric Effects of Lipid Surface Charge and Ca^{2+}-Lipid Interactions on Gating

In the absence of free Ca^{2+}, the gating of BTX-activated Na channels (both $V_{0.5}$ and q) was indistinguishable between negatively charged (PS or PE/PS) and uncharged (PE or PE/PC) membranes (Fig. 1 and Table I). Taken out of the context of our other observations, a possible interpretation of this result would be that the gating mechanism of the channel is electrically isolated from the negative charges on the phospholipids. Our observation of a lipid-dependent component of Ca^{2+}-induced gating shifts (Fig. 7 *C*) indicates that the potential at the sensor is indeed affected by lipid surface charge. While it is convenient to describe channel gating as a function of the directly measurable quantity, transmembrane voltage, we presume that the true variable that controls gating is the local electric field (the potential gradient) in the vicinity of the sensor. Thus, our data suggest that symmetrically altering the membrane surface potential does not affect the field at the sensor, even though the absolute electrostatic potential at the sensor does change.

In contrast to the shifts that are due to interactions of Ca^{2+} with the channel protein, the increments in shift associated with the addition of negative charge to the lipids, were equal for external and internal Ca^{2+}. These extra shifts were ~7 mV (Figs. 3 and 7, and Table III). We believe that this indicates that the gating machinery of the Na channel senses the contribution of the lipid surface charge to the electric field and that when Ca^{2+}, added asymmetrically, screens those charges, the change in surface potential shifts the activation gating curve. The overall effect of Ca^{2+} would be due to a direct interaction with the Na channel protein plus an interaction with the negatively charged phospholipids.

Alternative explanations, in which the influence of the lipid charge is solely indirect, seem unlikely for the following reasons: (*a*) there is no reason that such effects should be symmetric, as our data indicate (Fig. 7 *C*; Table III). In fact, our results with neutral lipids suggest that a lipid charge effect due to an increment in Ca^{2+} concentration at the protein surface should be asymmetric. (*b*) It seems unlikely that the lipid headgroups could have a significant concentrative effect through the bathing solution along the membrane surface and not have an effect on the gating mech-

anism through the channel protein where the range of electrostatic interactions would be expected to be larger than the Debye length in 0.15 M electrolyte. (*c*) Finally, while we cannot rule out that changing the charge on the lipid might alter the structure and Ca^{2+} affinity of the channel protein, there is no reason to suggest that such an effect would be symmetric.

The similarity of the magnitudes of the shifts attributable to PE/PS and to pure PS is consistent with both theoretical calculations and measurements of surface and zeta potentials (Winiski et al., 1986), which suggests a surface potential difference of only ~15% between 100% PS and 44% PS in 0.1 M NaCl. It is unlikely that we would have detected the incremental shifts (~1 mV) that would result from those differences.

Spatial Relationship between the Phospholipid Headgroups and Gating Machinery

Calculations of surface potential (ψ) and published measurements of ψ are consistent with our conclusion that part of the Ca^{2+}-induced shift in $V_{0.5}$ is directly caused by changes in potential at the lipid surface. The magnitude of ψ expected for our negative membranes before Ca^{2+} addition (~−70 mV; cf., Winiski et al., 1986) is large enough to account for the observed shifts. Increasing the divalent cation concentration from 0.1 to 7.5 mM would be expected to decrease the magnitude of ψ by ~30 mV (Ohki and Sauve, 1978; McLaughlin et al., 1981). Such a change in ψ at the lipid would lead to the observed lipid-related shifts in channel activation (~7 mV; Fig. 7 *C* and Table III) if the gating machinery were somewhat further from the lipids than the Debye length of ~1 nm in the electrolyte (150 mN NaCl). Although it is likely that the channel pore may be out of the range of the electrostatic influence of negative charges on the phospholipid headgroups (Moczydlowski et al. 1984; Green et al., 1987; Worley, J. F., R. J. French, and B. K. Krueger, manuscript submitted for publiction), we believe that the electrical sensor controlling gating may be removed from the lipid by only ~1–2 nm, placing it quite close to the outer rim of the large Na channel protein. Thus, our experiments provide a preliminary indication of the geometric location of a specific functional part of the molecule.

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