Lipid surface charge does not influence conductance or calcium block of single sodium channels in planar bilayers

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ABSTRACT We have studied the effects of membrane surface charge on Na^+ ion permeation and Ca^{2+} block in single, batrachotoxin-activated Na channels from rat brain, incorporated into planar lipid bilayers. In phospholipid membranes with no net charge (phosphatidylethanolamine, PE), at low divalent cation concentrations (~100 μM Mg^{2+}), the single channel current–voltage relation was linear and the single channel conductance saturated with increasing [Na^+] and ionic strength, reaching a maximum (γ_{max}) of 31.8 pS, with an apparent dissociation constant ($K_{0.5}$) of 40.5 mM. The data could be approximated by a rectangular hyperbola. In negatively charged bilayers (70% phosphatidylserine, PS; 30% PE) slightly larger conductances were observed at each concentration, but the hyperbolic form of the conductance–concentration relation was retained (γ_{max} = 32.9 pS and $K_{0.5}$ = 31.5 mM) without any preferential increase in conductance at lower ionic strengths. Symmetrical application of Ca^{2+} caused a voltage-dependent block of the single channel current, with the block being greater at negative potentials. For any given voltage and [Na^+] this block was identical in neutral and negatively charged membranes. These observations suggest that both the conduction pathway and the site(s) of Ca^{2+} block of the rat brain Na channel protein are electrostatically isolated from the negatively charged headgroups on the membrane lipids.

INTRODUCTION

The transmembrane movement of cations in excitable cells is controlled by voltage-dependent channels which span the lipid bilayer, providing a pore through which ions are able to traverse the hydrophobic membrane interior (Hodgkin and Huxley, 1952; Hille, 1984). These ion channels are highly selective due to specific interactions between the ions and one or more binding sites in the permeation pathway through the channel protein (Hille, 1975). Another factor that may affect ion permeation is the presence of fixed charges near one or both ends of the channel. When present, fixed negative charges concentrate cations near the channel mouth, with polyvalent cations being preferentially affected over monovalent cations. These charges may be located on the lipid head groups, the channel protein, or both.

Permeation through ion channels is often studied by measuring ionic currents under voltage clamp while varying the concentrations of various permeant and blocking ionic species on one or both sides of the membrane. The results of such experiments have revealed that Na^+ permeation through voltage-dependent Na channels saturates with increasing Na^+ concentration (Hille, 1975; Moczydlowski et al., 1984; French et al., 1986*a*; Green et al., 1987*a*; Sheets et al., 1987), providing direct evidence that Na^+ ions must bind to one or more sites within the channel. Protons and Ca^{2+} ions behave as Na channel blockers when applied from the outside (Woodhull, 1973; Yamamoto et al., 1984, 1985; Mozhayeva et al., 1985; Worley et al., 1986; Green et al., 1987*a*); their voltage-dependent action suggests that these ions block by penetrating the channel pore to a site located a significant distance into the transmembrane electric field.

Most studies of permeation through Na channels have been carried out in intact cells where the macroscopic current through thousands of individual channels is measured. Single channel current measurements, using the patch clamp or planar bilayer techniques (Sakmann and Neher, 1983; Miller, 1986), have the advantage that changes in permeation can more easily be distinguished from alterations in channel opening (gating), which can also be affected by changes in the ionic composition. In

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contrast to experimental protocols using cellular preparations, the planar bilayer technique enables manipulation of the ionic composition on both sides of the membrane simultaneously over ranges greater than would be practical with intact cells. Moreover, the phospholipid composition of the membrane can be systematically varied in order to study channels in membranes with a variety of surface charge densities.

We focus here on the effect of altering the charge of the membrane phospholipid headgroups on Na^+ ion permeation and on block by Ca^{2+} ions. Comparison of channel properties in neutral and negatively charged bilayers demonstrates that negative surface charges on the membrane lipids do not significantly affect the entry of cations into the mouth of the channel.

MATERIALS AND METHODS

Materials

Phospholipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Batrachotoxin (BTX) was a generous gift of Dr. John Daly (National Institutes of Health, Bethesda, MD). Decane was repurified by passage over dry alumina before use. All salts used in this study were reagent grade.

Membrane preparation

Membrane vesicle fraction P3 was prepared from homogenates of rat forebrain by the method of Krueger et al. (1979) as modified by Worley et al. (1986). Fraction P3 contained at least 60% of the [^{3}H]saxitoxin (STX) binding sites found in the crude homogenate with a specific activity of 5–10 pmol/mg protein, representing a 5–10-fold enrichment over the crude homogenate.

Solutions and bilayers

Planar bilayers were formed from bovine brain phosphatidylethanolamine (PE) or mixtures of PE and bovine brain phosphatidylserine (PS) in decane as described by French et al. (1984) and Worley et al. (1986). BTX (60 nM) was present on the *cis* side. The ionic concentrations on both sides of the membrane were identical and normally contained 10 mM Hepes, 150 μM $CaCl_2$, 100 μM $MgCl_2$, and 50 μM EGTA, pH 7.0 (22°C). NaCl was added as needed to bring the final [Na^+] to the value indicated in the figures in the range 10–1,000 mM. After channel incorporation, 200 μM EGTA was added to both sides to lower the Ca^{2+} concentration to <1 μM. After collecting control data under these conditions, $CaCl_2$ was added symmetrically from a concentrated stock solution to the final (free) concentrations indicated in the figures. There were negligible changes in pH as a result of additions of EGTA and divalent cations.

Channel incorporation and electrical recording

Normally, a bilayer was formed and the resistance and capacitance were measured before addition of biological material. Only bilayers with resistances $>10^{11}$ Ω were used for channel incorporation. Capacitances of 0.3–0.5 μF/cm^2 were observed, as is typical of decane-containing "painted" bilayers. Channel incorporation was accomplished by adding P3 (1–10 μg protein) to the *cis* side of the membrane and monitoring transmembrane current at a holding potential of −60 mV. One or more channels generally appeared in the bilayer within 30 min as revealed by stepwise 1.8-pA increases in membrane current with symmetric, saturating Na^+ concentrations.

Holding potentials were applied and trans-bilayer currents were measured via Ag/AgCl electrodes connected to a virtual ground current amplifier circuit (French et al., 1986*b*). Both the circuitry and the bilayer chamber were enclosed in an aluminum box to minimize noise. The bandwidth of the amplifier was ~500 Hz. The current signal was recorded on an FM tape recorder (model B; A. R. Vetter Co., Rebersburg, PA). On playback, data were filtered (low-pass Bessel, model 901F; Frequency Devices Inc., Haverhill, MA) at 20–200 Hz and displayed on a digital oscilloscope (model 2090 or 3091; Nicolet Instrument Corp., Madison, WI). Hard copy was obtained on an oscillographic recorder (model RS2-5P; General Scanning Co., Boston, MA, or model 2200; Gould Inc., Cleveland, OH). Single channel current measurements were made from a digital oscilloscope screen or from a stripchart record.

All applied voltages are expressed in the normal cellular convention, $V_m = V_{in} - V_{out}$. This convention is based on the knowledge that STX and tetrodotoxin (TTX) act only from the extracellular side of Na channels. With this voltage convention, BTX-activated Na channels tend to close at potentials more negative than about −80 mV (Krueger et al., 1983; French et al., 1984), providing a second criterion to confirm their orientation.

Measurement of surface charge density

The net membrane surface charge density was estimated from the measurement of the surface potential by using nonactin as an intrinsic membrane voltmeter (McLaughlin et al., 1970, 1971; McLaughlin, 1977; c.f. Bamberg and Lauger, 1977; Bell and Miller, 1984; and Moczydlowski et al., 1985). Bilayers composed of 100% PE and 70% PS/30% PE were analyzed in the presence of 20–100 mM K^+. K^+ was used instead of Na^+ because of the limited affinity of nonactin for Na^+ (Szabo et al., 1969). Nonactin was added (10^{-6}–10^{-8} M) until a conductance (G) of 10^{-8}–10^{-9} S was obtained. To define the neutral conductance state (G_o) of the membrane, Li^+, which does not interact with nonactin, was added until no further change in the membrane conductance was observed. The increase in the ionic strength neutralizes the membrane surface charge; the bulk salt concentration equals the concentration at the membrane surface at the higher ionic strength. This was repeated at different K^+ concentrations for three to five membranes composed of PE and 70% PS/30% PE. There was no effect of exposing the nonactin-doped bilayer to membrane vesicles (10 μg protein/ml) for 30 min. The membrane surface potential ($\Phi(0)$) was determined for each membrane from $\Phi(0) = (-RT/F)\ln(G/G_o)$. Once the membrane surface potential was determined, the surface charge density was calculated using the Gouy-Chapman-Stern theory as described by McLaughlin (1977) and Bell and Miller (1984).

Table 1 gives the measured surface charge density and net charge per phospholipid head group. Surface charge density for the 70% PS membranes was >10-fold higher than for the "neutral" (actually zwitterionic) PE, in good agreement with measurements of other investigators under similar conditions (McLaughlin et al., 1971; Bell and Miller, 1984).

TABLE 1

Lipid	Surface charge density (elementary charge/nm^2)	Net charge per lipid molecule
70% PS, 30% PE	−0.84 ± 0.05	−0.6
All PE	−0.07 ± 0.04	−0.05

Measurement of surface charge density. Each value, indicated as the mean ± SEM, was obtained from three to five determinations at different K^+ concentrations (20–150 mM) on both sides of the membrane. Net charge per lipid molecule was calculated assuming a molecular area of 0.7 nm^2 per phospholipid (Loosley-Millman et al., 1982).

Calculations of surface charge effects

The fractional occupancy of channel sites by conducting ions (Na^+) or blocking ions (Ca^{2+}) depends on their local concentrations in the vicinity of the channel sites, and hence on the local electrostatic potential. The magnitude of this potential in the vicinity of an electrically charged surface may be reduced by counterions from the solution by two mechanisms: screening by ions in the diffuse double layer, or binding or adsorption of the counterions to the charged surface (for reviews, see McLaughlin, 1977, 1989). Below, we calculate the effects on occupancy of channel sites for the case in which there is a uniform density of negative charge on the membrane surface near cation-binding conduction or blocking sites of the channel. Following Cecchi et al. (1981) and Bell and Miller (1984), we treat the membrane as a planar, uniformly charged surface with the channel sites displaced perpendicularly from the plane of the membrane. In other words, the channel mouth, with sites that control conduction, is considered to project into the solution up to 4.0 nm from the surface of the membrane. This allows a calculation of the potential profiles in one dimension. Although this is obviously an oversimplification, again following the arguments of Cecci et al. (1981) and Bell and Miller (1984), we assume that the qualitative results would not be seriously distorted by this assumption.

Local potential and concentration profiles

To calculate the electrostatic profile near the charged membrane, we used the analytic solution of Abraham-Shrauner (1975, 1977) for the potential near a charged surface bathed in a mixed solution of divalent and monovalent cations with a common monovalent anion. Eq. 1 of Abraham-Shrauner (1975), expressing the potential in the solution in terms of its value at the surface and the bulk solution concentrations, was solved numerically in conjunction with the Grahame equation (see Eq. 11A of McLaughlin, 1977), which expresses the surface potential in terms of the bulk solutions. Concentration profiles near the surface were then calculated by substituting the calculated potential directly in the Boltzmann equation (Eq. 3A of McLaughlin, 1977). Key equations and further details of the calculations are given in the Appendix.

Effects of counterion binding to the lipids

To account for effects of ion binding, a further step was incorporated into the iterative solution described in the previous paragraph. The net surface charge density was expressed in terms of the surface concentrations and the dissociation constants for binding of the counterions, either monovalent or divalent, to the membrane surface (see Appendix). This, in essence, is applying the Gouy-Chapman-Stern theory as described by McLaughlin (1977) without taking into account the finite size of ions involved. All of the lines in Figs. 2 and 4 predicting surface charge effects, including those for which only screening, and not binding, is important, were generated from the Gouy-Chapman-Stern theory. Actual incorporation of the surface charge effects into predictions of conductance–concentration relations, and the fractional block by Ca^{2+}, are outlined in the Discussion.

RESULTS

Dependence of single channel conductance on Na concentration

Fig. 1 *A* shows unitary current fluctuations from single BTX-activated Na channels incorporated into bilayers composed of PE at three different symmetrical Na^+ concentrations in the absence of free Ca^{2+}. The single channel current at −60 mV increased with increasing Na^+. For experiments in low Na^+ ($\leq$0.05 M), 2 nM STX was added to the extracellular side to produce long-lived, nonconducting states to facilitate determination of the single channel current (Fig. 1 *A*, *bottom*). When the single channel current was determined over a range of potentials, linear current–voltage relations were obtained at each Na^+ concentration (Fig. 1 *B*). The single channel conductance (γ) was ~13 pS in symmetrical 0.025 M NaCl and ~29 pS in 0.5 M NaCl.

When γ, determined from the slope of current–voltage relations such as those shown in Fig. 2, was plotted against the Na^+ concentration, a saturating γ vs. [Na] relationship was obtained. Data from PE membranes (*open circles*) are plotted in Fig. 2. A nonlinear, least-squares fit to the data yields a maximal γ of 31.8 pS and a $K_{0.5}$ of 40.5 mM. The observed conductance appears to rise slightly more steeply than a perfectly hyperbolic relationship between 0.5 and 1 M NaCl, perhaps because of some double occupancy of the channels at such high ionic strengths (c.f. Ravindran et al., 1992).

Single-channel conductance in negative and neutral bilayers

We have also studied the properties of BTX-activated Na channels in planar bilayers composed of mixtures of PE and the negatively charged lipid, PS. The single channel conductance in negatively charged (70% PS) bilayers is plotted against Na^+ concentration with the open triangles in Fig. 2. This curve can be fit by a rectangular hyperbola nearly identical to that describing the conductance–concentration relation in neutral membranes, but with a γ_{max} increased to 32.9 pS and a $K_{0.5}$ reduced to 31.5 mM. As was the case for neutral membranes (*open circles*), the observed values of γ

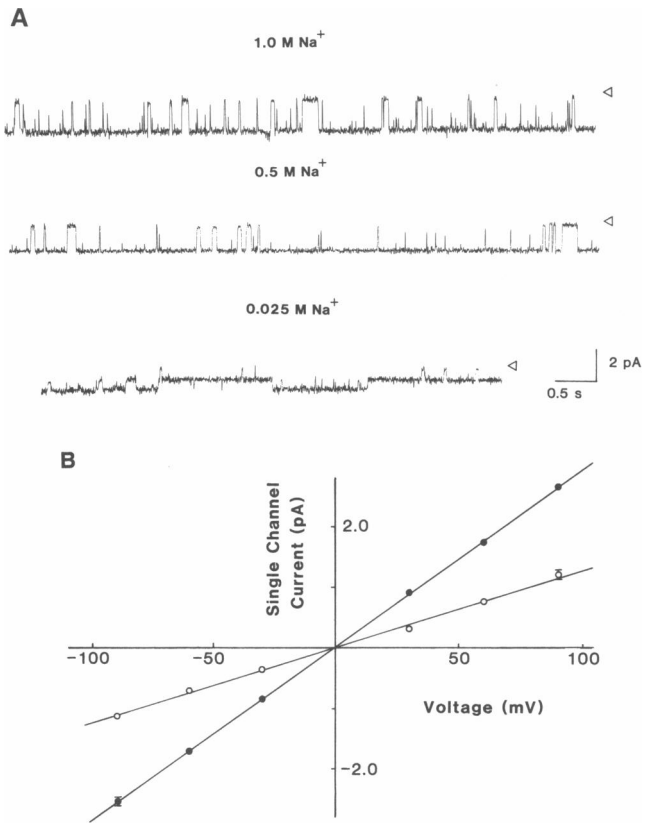

FIGURE 1 Single channel current vs. Na concentration. (*A*) Single channel current records. Na channels were incorporated into PE membranes as described in Materials and Methods. The solutions contained symmetrical 1.0, 0.5, or 0.025 M NaCl and $\leq$ 0.1 mM divalent cations. The membrane potential was −60 mV. Upward current fluctuations represent channel closings and the zero-current levels are indicated by the arrowheads. The membrane at the bottom (0.025 M) contained two Na channels and 2 nM STX was present on the extracellular side. Records were filtered (low-pass, 8-pole Bessel) at 150 Hz. (*B*) Na channels were incorporated into neutral membranes with symmetrical 25 mM NaCl (*open symbols*) or 500 mM NaCl (filled symbols). Single channel currents were measured at the indicated potentials. The data points represent means from 7–16 membranes. Error bars show ± SEM when larger than the dimension of the symbol. The single channel conductances calculated from a linear, least-squares fit to these data were 12.7 ± 0.2 pS for 25 mM Na^+ and 28.9 ± 0.7 pS for 500 mM Na^+.

between 0.5 and 1 M NaCl rose slightly more than predicted by a simple hyperbolic relationship.

Block by Ca^{2+} is independent of the charge on the membrane lipid

In addition, we evaluated the effect of lipid surface charge on block of single Na channel currents by Ca^{2+}. Fig. 3 *A* shows a single channel current–voltage relation recorded in the presence of symmetrical 10 mM Ca^{2+} illustrating the pronounced, voltage-dependent block of inward current at negative potentials. Fig. 3, *B* and *C*, shows the fractional block by symmetrical 10 mM Ca^{2+} at various Na^+ concentrations. The fractional block by Ca^{2+} was essentially the same for negative and neutral bilayers at all Na^+ concentrations tested between 25 and 1,000 mM for both inward currents at −60 mV (Fig. 3 *B*) and outward currents at +60 mV (Fig. 3 *C*). Like the data shown in Fig. 2, these results indicate that the external entrance to the Na channel is well isolated from the lipid head groups in the bulk phase of the bilayer. If a substantial density of fixed negative charges on the lipids had been located close to the site of Ca^{2+} binding, a greater degree of block would have been expected in negative bilayers, because blocking divalent cations (e.g., Ca^{2+}) would be concentrated more than permeant, monovalent Na^+ ions by the negative surface charge. The preferential concentration of Ca^{2+} would be more pronounced at lower Na^+ concentrations (lower ionic strength), but as shown later, the resulting change in the degree of block under our experimental conditions would be greater at intermediate (100–200 mM) concentrations of NaCl (see Fig. 4 and Discussion).

DISCUSSION

Implications of hyperbolic conductance–concentration relations

In neutral bilayers, the single channel conductance varied with Na^+ concentration approximately according to a simple rectangular hyperbola in the range of concentrations from 10 to 1,000 mM (Fig. 2), suggesting that a saturable site limits Na^+ flux at high Na^+ concentrations. Moczydlowski et al. (1984) and Behrens et al. (1989) reported similar observations, but somewhat higher Na^+ affinities ($K_{0.5}$ = 8 and 11 mM for Na^+ channels from rat skeletal muscle and squid nerve, respectively). There may be a slight deviation from a simple, hyperbolic relationship between 0.5 and 1 M Na^+, in that γ appears to continue to rise slowly, rather than reach an asymptotic value. In our data, this tendency was smaller than for canine brain Na channels (Green et al., 1987*a*). We believe that at very high permeant ion concentrations a single binding site may not be sufficient to explain permeation, possibly indicating simultaneous occupancy of the channel by more than one Na^+ ion (see also Ravindran et al., 1992). Alternatively, deviations at high ionic strength ([Na^+] > 0.5 M) may reflect changes in the state of hydration of the pore or slight alterations in the channel's molecular structure induced by the high salt concentrations.

Close apposition of negatively charged lipid near both the internal and external entrances to the pore would be

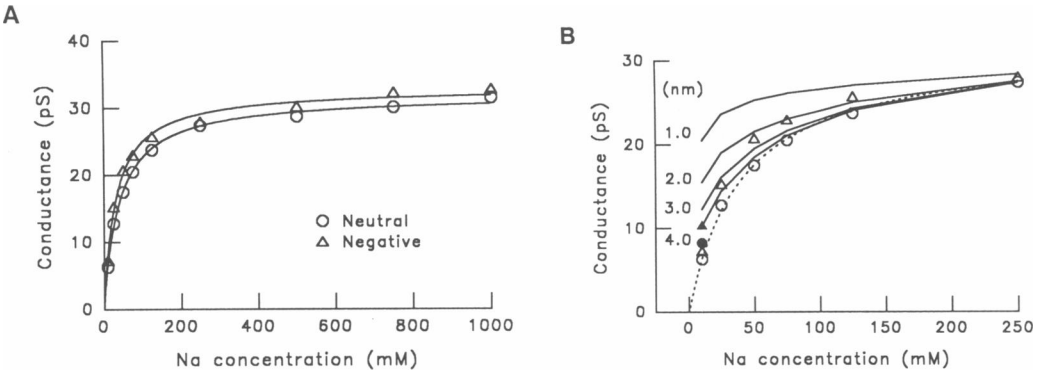

FIGURE 2 Single channel conductance vs. Na^+ concentration. Single channel conductance–concentration relationship for neutral (PE) and negatively charged (70% PS, 30% PE) membranes. Each point represents the slope (γ) of a linear current–voltage curve, as illustrated in Fig. 1 *B*, for Na channels in symmetrical Na^+. Each data point is the mean from 3–30 membranes. Error bars show ± SEM when larger than the dimension of the symbol. (*A*) The lines are rectangular hyperbolae obtained by a least-squares fit to the data using the following parameters:

Lipid	$K_{0.5}$ *mM*	γ_{max} *pS*
PE	40.5	31.8
70% PS	31.5	32.9

Correcting for the small degree of block by the 100 μM Mg^{2+} in the solutions gives estimates of the apparent dissociation constants that are ~10–12 mM lower in each type of lipid than the $K_{0.5}$'s in this table. See part *B* of this figure for conductances determined in the presence or absence of Mg^{2+} at 10 mM Na^+. (*B*) Predicted effects of surface charge (70% PS, 30% PE, σ_o = 1e/1.2 nm^2) near the mouth of the channel on conductance–concentration relations estimated, using the Gouy-Chapman-Stern (GCS) theory, from the best-fit rectangular hyperbola for the PE data (*dotted curve*). Solid line segments connect theoretical points derived from GCS theory at each concentration for which experimental data were collected. Lines are shown for cases in which the mouth of the channel is assumed to be located at distances of 1.0, 2.0, 3.0, or 4.0 nm from the charged membrane surface. See Materials and Methods and Appendix for additional details. In this case, effects of counterion binding to the lipid are small because of the low affinity of Na^+ for the PS ($^{PS}K_{Na}$ = 1 M; McLaughlin et al., 1983) and the low divalent ion concentrations. (Calcium was reduced to negligible levels by addition of EGTA, and an upper limit on the effects of Mg^{2+} binding to the lipid was calculated using a value of $^{PS}K_{Mg}$ = 100 mM; c.f. McLaughlin et al., 1981). Note that even at the lowest ionic strength, where predicted surface charge effects are greatest, there is very little difference between the data points obtained from neutral and from negative membranes. Open symbols represent data points as in *A*. Filled symbols show the means of two experiments, each performed in the absence of any added divalents with [Na^+ = 10 mM; for all other points 100 μM Mg^{2+} was present. Hyperbolic curves drawn through the zero Mg^{2+} points, without changing the γ_{max} values, yield $K_{0.5}$ values that are ~10–12 mM lower for each type of lipid. However, the effects of the Mg^{2+} are too small to alter the conclusions stated regarding the separation between the lipid surface charge and the pore.

expected to result in a conductance–concentration relation that extrapolates to a non-zero ordinate (Bell and Miller, 1984; Bell, 1986). We do not observe such a deviation (but see Neumcke and Stämpfli, 1984; Green et al., 1987*a,b*; and Correa et al., 1991). It should be noted, however, that, as pointed out by MacKinnon et al. (1989), a hyperbolic conductance–activity relation does not necessarily indicate that there is no negative charge at one end of the channel pore. In fact, with ionized groups localized to the channel vestibules, rather than being present as an infinite sheet of charge, the conductance–concentration relation will extrapolate to zero even though both ends of the channel may be charged (Cai and Jordan, 1990). Thus, our observation of a nearly hyperbolic conductance–Na concentration relation is not inconsistent with the presence of a negatively charged carboxyl group associated with the STX/TTX blocking site at the external mouth of the channel (e.g., Worley et al., 1986), even though it precludes close proximity of the membrane lipid charge to the channel mouths.

Expected effects of lipid surface charge on binding of conducting ions

The predicted effects of symmetrical negative lipid surface charge of the γ vs. [Na] relations parallel the calculations of Bell and Miller (1984). The combination of longer Debye length and larger surface potential at low ionic strength would lead to significantly increased conductances and the extrapolation of the conductance toward a non-zero intercept as the Na^+ concentration (and ionic strength) approaches zero. For these experiments, divalent ion concentrations were negligible and

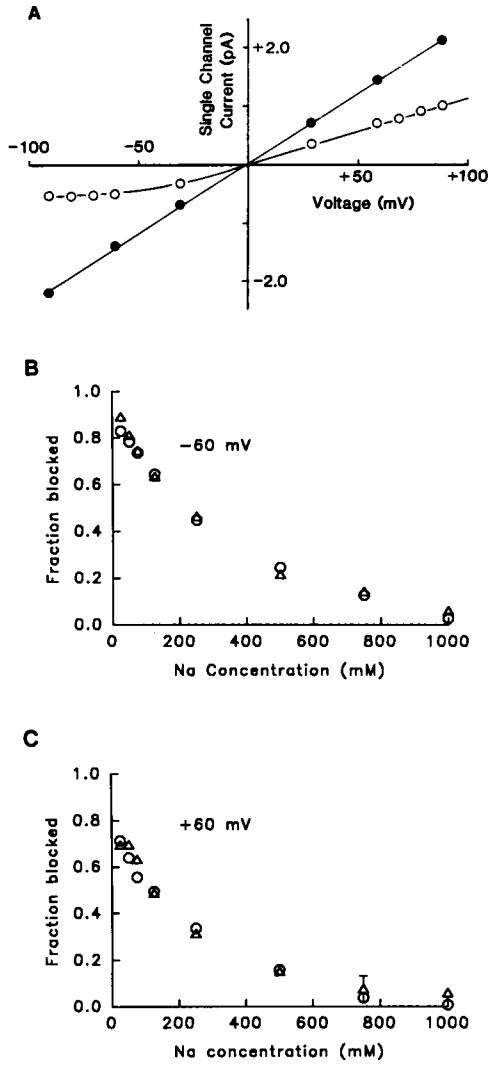

FIGURE 3 Ca^{2+} block is independent of charge on the membrane lipids. (*A*) Single channel current–voltage relation with symmetric 125 mM Na^+ in the absence (*filled symbols*) and presence (*open symbols*) of 10 mM symmetrical Ca^{2+} showing voltage-dependent block at negative membrane potentials. (*B, C*) Ca^{2+} block at various [Na]. The fraction of current blocked by symmetrical 10 mM Ca^{2+} at each Na^+ concentration was computed using the following equation: fraction of current blocked, $F_b = 1 - (i_{Ca}/i_{max})$, where i_{Ca} is the single channel current in the presence of symmetrical 10 mM Ca^{2+} and i_{max} is the single channel current in the absence of Ca^{2+} (0.1 mM Mg^{2+}). The single channel currents were evaluated in neutral (100% PE, *circles*) and negatively charged (70% PS, 30% PE, *triangles*) membranes at −60 mV (*B*) and +60 mV (*C*). Each data point is the mean of four to seven determinations. Error bars show ± SEM when larger than the dimension of the symbol.

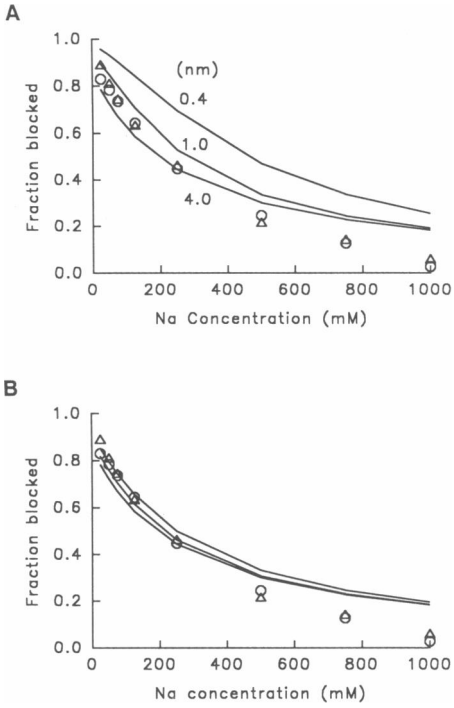

FIGURE 4 Predicted effects of surface charge on Ca^{2+} block with or without counterion binding to the lipid. Same data as in Fig. 3 *B* (−60 mV). Single channel currents were determined in neutral (100% PE, *circles*) or negatively charged (70% PS, 30% PE, *triangles*) membranes. Line segments connect theoretical points at each concentration for which experimental data were collected. The simple competitive model (predictions indistinguishable from the 4.0-nm line) fits the observed fractional block reasonably well up to 250 mM Na^+, but deviates at higher concentrations. The main point of this figure is to illustrate the expected change in the degree of block produced by the surface charge. (*A*) Lines show the predicted effects of surface charge (70% PS/30% PE, σ_o = 1e/1.2 nm^2) on the fraction of channels blocked by Ca^{2+} when the mouth of the channel is located 0.2, 1.0, or 4.0 nm from the charged surface of the lipid bilayer. These predictions are based on the assumption of negligible binding of counterions to the lipid (i.e., essentially pure screening in accordance with the Gouy-Chapman theory). The actual curves were calculated with ${}^{PS}K_{Ca}$ = 10 M and ${}^{PS}K_{Na}$ = 100 M. (*B*) Same as in *A* except that the predicted surface charge effect is smaller because the lines take into account the likely maximal degree of binding of Ca^{2+} to the PS in the bilayer (${}^{PS}K_{Ca}$ = 30 mM, see McLaughlin et al., 1981; ${}^{PS}K_{Na}$ = 1 M, see McLaughlin et al., 1983). See Materials and Methods and Appendix for details.

binding effects were unimportant. While the simple Michaelis-Menten hyperbolic form may not fit the data perfectly (Fig. 2 *A*), it is clear from the minimal effect of changing the membrane lipid from neutral PE to a negative 70% PE/30% PS mixture that the negative surface charge must be distant from the channel mouth.

To estimate the effects of surface potential on the conductance–concentration relations, $[Na^+]$ was calculated for various separations between the charged lipid surface and the channel mouth. The intrinsic dissociation constant for Na binding to the channel was taken to be the $K_{0.5}$ required to fit the conductance–Na concentration relation in PE (virtually uncharged) membranes.

Although PE does have a small net negative charge under the conditions of our experiments, the predicted effects of this surface charge are too small to be detected in our measurements (calculations not shown). The PE/PS data in Fig. 2 *B* generally fall between the Gouy-Chapman-Stern theory predictions based on 2–4-nm separation between the channel mouth and the charged lipid surface. This suggests that the distance from the mouth of the channel to the charged surface must be at least 2 nm, i.e., >2 Debye lengths in physiological solutions.

If the small differences between the PE and the PE/PS curves were due to such a lipid surface charge influence, the largest difference between the two curves should occur at the lowest Na^+ concentration. As this was not observed, the small difference between the $K_{0.5}$'s (neutral membranes, ~40 mM, negative membranes, ~30 mM) probably does not represent a significant surface charge effect at all, but rather a lipid-specific effect that is not primarily electrostatic in mechanism. Thus, we take 2 nm to be a conservative estimate of the minimum separation between the lipid surface charge and the channel entrance. If only the data at the lowest ionic strengths were considered a larger value would be obtained.

Expected effects of lipid surface charge on channel block by calcium ions

Since the divalent Ca^{2+} should be concentrated more strongly than Na^+ near a negatively charged surface, it is possible to test the hypothesis that the site of Ca^{2+} action is also far removed from the lipid surface charge. The qualitative result is immediately clear. There is no significant difference between the degree of Ca^{2+} block in PE and PE/PS membranes (Fig. 3, *B* and *C*).

At −60 mV, block of the channel by Ca^{2+} entering from the external side would be favored; at +60 mV, block by Ca^{2+} entering from the internal side would be favored. Thus, these experiments offer an opportunity to separately estimate the proximity of inner and outer Ca^{2+} binding sites to the lipid. In fact, similar results indicating a lack of influence of lipid surface charge were obtained at both positive and negative voltages, showing that there is no detectable asymmetry in the proximity to the membrane lipid of the sites of Ca^{2+} access (Fig. 3, *B* and *C*).

The addition of Ca^{2+} itself moderates the effect of the surface charge by screening and/or binding. To explore the effects of surface charge on Ca^{2+} block, we fit the fractional block seen in the neutral (PE) membranes as a function of $[Na^+]$ with only one free parameter, the dissociation constant for Ca^{2+} binding to the channel, K_{bCa}, assuming a simple competition between Ca^{2+} and Na^+, using the following equation.

$$F_b = 1/\{1 + (K_{bCa}/[Ca^{2+}])(1 + [Na^+]/K_{0.5})\}$$

The value for $K_{0.5}$ was taken from the fit of the γ vs. $[Na^+]$ relation for PE as described above. This one-site, competitive model is certainly an over-simplification.[1] However, any model in which there is a competitive interaction between Na^+ and Ca^{2+} would be expected to yield qualitatively similar predictions of the surface charge effects. It is in this spirit that we used the simplest model of the Na^+/Ca^{2+} interaction. Predictions of fractional block in the negative (70% PS) membranes were made, using this expression for F_b together with surface potential–modified concentrations of both Na^+ and Ca^{2+}, under the assumptions described in the following paragraph.

We calculated predictions in two limiting cases in order to place limits on the distance between the site of Ca^{2+} action and the lipid charge. A maximal effect of surface charge would be observed if Ca^{2+} only screened the lipid surface charges without binding specifically to them (Fig. 4 *A*). The curves tend to converge at low $[Na^+]$ toward almost complete block, and at high $[Na^+]$ toward no block. At intermediate $[Na^+]$, negative lipids are predicted to induce substantial increases in the degree of block. This results from the stronger local concentrative influence of the negative charge on the divalent Ca^{2+} than on Na^+. When Ca^{2+} can bind to the surface charge, the net charge on the membrane is made more positive. In the limit of saturation, with one Ca^{2+} bound to each PS molecule, the charge on the membrane would reverse. Thus, to determine the maximum effect of Ca^{2+} binding, we repeated the calculations above with a dissociation constant for Ca^{2+}-PS binding of 30 mM (range of experimental values, 30–100 mM; McLaughlin et al., 1981). Under these circumstances, the surface charge is so effectively neutralized by Ca^{2+} that it would be difficult to experimentally detect the effect of the negative lipid unless the site at which Ca^{2+} binds to block the channel were very close (<0.4 nm) to the lipid surface charge (Fig. 4 *B*). A detectable influence would be seen with larger separations if Ca^{2+} binding to the lipid were weaker (e.g., with ${}^{PS}K_{Ca}$ = 100 mM, effects are at least twice as large as for ${}^{PS}K_{Ca}$ = 30).

[1]There must be at least two separate Ca binding sites on the channel, one easily accessible from each side. Furthermore, our recent calculations (French et al., 1992) suggest that the observed degree of inhibition of Ca block at high $[Na^+]$ requires double occupancy of the channel by Na^+ under these conditions, consistent with the conclusions of Ravindran et al. (1992).

The data are thus consistent with the idea that both the site of Na^+ entry to the permeation pathway and the path of Ca^{2+} access to its blocking site are spatially separated from the lipid surface charge. A larger lower limit on the separation is imposed by the minimal effect on the γ vs. $[Na^+]$ relationship, because an influence on the degree of Ca^{2+} block depends on the valence-dependent increase in concentration of Ca^{2+}, relative to Na^+, near the membrane surface. Taking this one step further, to a fair degree of accuracy the combined conductance and blocking data are consistent with a simple competition between Ca^{2+} and Na^{2+} for occupancy of the permeation pathway[1] (see also Woodhull, 1973; Yamamoto et al., 1984).

Conducting pore is distant from the membrane lipid

Addition of a high density of negative surface charge to the bilayer lipids had very little effect on the conductance–Na concentration relationship for BTX-activated Na channels (Fig. 2); moreover, there was no effect on Ca^{2+} block over a wide range of Na^+ concentrations (Fig. 3, *B* and *C*). This lack of effect of negatively charged lipids is in contrast to the results obtained with muscle sarcoplasmic reticulum K channels (pore ~1.2 nm from the lipid head groups; Bell and Miller, 1984), Ca^{2+}-activated K channels (~1 nm; Moczydlowski et al., 1985), and voltage-dependent Ca channels (~2 nm; Coronado and Affolter, 1986). Thus, the mouth of the Na^+ channel appears to be considerably further removed from the lipid surface than those of the other channels that have been studied. It is also of interest that the rank order of block by divalent cations ($Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$; Worley, J.F., unpublished observations) was different from that for binding to PS ($Mn^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$; see Eisenberg et al., 1979; McLaughlin et al., 1981), thus providing further evidence that interactions of divalent cations with the channel pore are not dominated by properties of the charged headgroups on the phospholipids, presumably because the lipids do not approach close enough to the pore.

It is not surprising that the mouth of the Na channel pore should be well isolated from the membrane phospholipid. The mass of the rat brain Na channel is reported to be ~300 kD (Hartshorne and Catterall, 1984), corresponding to a molecular volume of $\sim 4 \times 10^3$ nm^3 (partial specific volume assumed to be 0.72 ml/g). If the channel were represented as a cylinder slightly longer than the thickness of the membrane (8 nm), the diameter of the face of the cylinder would be ~8 nm. It has been estimated that the dimensions of the ion-conducting pore itself are only $\sim 0.3 \times 0.5$ nm (Hille, 1971). Thus, the mouth of a central pore would be located 3.5–4.0 nm from the lipid around the perimeter of the channel. Alternatively, if the channel were long and narrow, extending into the aqueous phase as does the acetylcholine receptor channel (McCarthy et al., 1986; Miller and Garber, 1988), the mouth of the pore would still be substantially removed from the negative charges on the lipid surface. This geometry is implicit in our own calculations and those of Bell and Miller (1984), who computed that lipid negative surface charge densities of ~1 e^-/nm^2 would have little or no effect on the conductance–permeant cation concentration relation if the lipids approached no closer than ~3.5 nm from the mouth of the pore. While the presence of a small amount of tightly bound lipid around the perimeter of the channel cannot be ruled out (see also Levinson et al., 1990), the mass of the Na^+ channel protein itself is sufficient to isolate the entrance to the channel from charges on the membrane lipids.

At least three lines of evidence argue against the retention of a multilayer annulus of native lipid around each channel that would prevent penetration of the bilayer lipids up to the perimeter of the channel protein. First, in the absence of cytoskeletal elements present in the intact cell, there are no known long-range interactions that prevent mixing of native and bilayer lipids. We have not observed any time-dependent changes in the conductance–Na concentration relations that would suggest a slow penetration of bilayer lipids to the channel perimeter. Second, although most channels that have been characterized biochemically bear an overall net negative charge, in all three channel types (SR K channel, Ca^{2+}-activated K channel, and Ca channel; see above) where the effects of lipid charge on permeation have been studied previously, negative charges on the lipid headgroups do affect permeation, behaving as if the lipids can approach as close as 1 nm to the mouth of a channel. Of these three examples, it is the Ca channel for which the pore appeared farthest from the lipid (~2 nm; see Coronado and Affolter, 1986), as might be expected from our own observations and the fact that the DHP-sensitive Ca channel and Na channel proteins are highly homologous. As discussed above, the Na channel protein is large enough to effectively isolate the channel entrance from the bilayer lipids. Third, the activation gating of BTX-modified sodium channels is sensitive to lipid surface charge (Cukierman et al., 1988). Thus, under our experimental conditions, the bilayer lipids are able to approach close enough to have a measurable influence on the gating voltage sensor but not on the conducting pathway of the channel.

APPENDIX

Double-layer calculations for a charged surface bathed by a 2-1-1 electrolyte

For a 2-1-1 electrolyte, such as a mixture of $CaCl_2$ and NaCl, the electrical potential (in units of RT/F) in solution near the charged surface is (Abraham-Shrauner, 1975):

$$\Phi(X) = \ln\{(1 + 3K)\tanh^2[(1 + 3K)^{0.5}(X + G)/2] - K\} - \ln(1 + 2K).$$

Here, X is the dimensionless distance in units of the Debye length, L_D, and K is the concentration ratio of divalent to monovalent cations. The following equation was used to define L_D:

$$L_D^2 = kT\epsilon/(2e^2I),$$

where I is the ionic strength given by

$$I = C(1 + 3K),$$

and C is the concentration of the monovalent salt. Our calculations differ slightly from those of Abraham-Shrauner (1975) in that she defined the Debye length in terms of the concentration of monovalent ions only. The other symbols are defined as follows: k = Boltzmann constant, T = temperature (°K), $\epsilon = \kappa\epsilon_o$, where κ is the dielectric constant for water and ϵ_o is the permittivity of free space, and e = the unsigned magnitude of the electronic charge.

In the expression for Φ, G is a constant of integration determined from

$$G = \{2/(1 + 3K)^{0.5}\}\tanh^{-1}Z,$$

where

$$Z = \{[K + (1 + 2K)\exp(\Phi(0))]/[1 + 3K]\}^{0.5},$$

and $\Phi(0)$ denotes the potential at the surface. The expression is a solution for the corrected equation reported by Abraham-Schrauner (1977).

Using these expressions, the surface charge density is related to the surface potential, σ_o, by Grahame's equation in the form

$$\sigma_o = (kT\epsilon/qL_D)\{1 - \exp[-\Phi(0)]\}\{K + (1 + 2K)\exp[\Phi(0)]\}^{0.5}.$$

The equations were solved numerically to obtain the voltage profile. Then the concentration profiles for Na^+ and Ca^{2+} in the vicinity of the charged surface were calculated applying Boltzmann's equation as follows:

$$Ca(X) = Ca_\infty \exp[-2\Phi(X)] \text{ and } Na(X) = Na_\infty \exp[-\Phi(X)],$$

remembering that Φ and X are normalized potential and distance variables, respectively. Here, $Ca(X)$ and $Na(X)$ are the concentrations at distance X from the charged surface, whereas Ca_∞ and Na_∞ are the concentrations in the bulk solution.

Numerical calculations were made using MathCAD (MathSoft Inc., Cambridge, MA). Routines were written to generate directly the predictions for simple screening of surface charge with no binding of cations to the lipid (Gouy-Chapman) and for the case of counterion binding (Gouy-Chapman-Stern, GCS). It was verified that the latter calculations were equivalent to the former when sufficiently low counterion–lipid binding affinities were used. All figures shown were derived from the full GCS calculation.

An expression for net surface charge density in the presence of multiple charged ligands

S_T = total density of charged sites
S_o = density of unbound sites
S_i = density of sites bound by the ith ligand
$\sigma_o = -eS_T$ = surface charge density in the absence of any ligand
$\sigma_L = e(-S_o + \Sigma_{i\geq 1} S_i z_i)$

where z_i = the net valence of a i-liganded site, and each unbound site has a valence of -1. Thus,

$$\sigma_L = eS_T\left(-f_o + \sum_{i\geq 1} f_i z_i\right), \text{ where } f_o = S_o/S_T \text{ and } f_i = S_i/S_T.$$

The fraction of sites bound by each ligand may be expressed in terms of the dissociation constants, K_i, measured individually in the presence of a single ligand. We consider the case where, for each ligand, there is a 1:1 binding reaction. This leads to the following expression for f_i, the fraction of sites bound by each ligand, in terms of the concentrations and dissociation constants for each of the ligands,

$$f_i = (L_i/K_i)/(1 + \sum_{k\geq 1} L_k/K_k).$$

This expression has a simple physical interpretation. The numerator determines the likelihood that the ith ligand will bind to a free site, and the denominator is a "saturation" term that determines the fraction of sites that will be free with an arbitrary number of ligands present.

Using the earlier expressions for σ_o and σ_L we obtain a general expression for the surface charge density in the presence of bound, charged ligands:

$$\sigma_L = \sigma_o[1 - \sum_{i\geq 1}(z_i L_i/K_i)]/[1 + \sum_{i\geq 1}(L_i/K_i)].$$

This equation is similar to the expression derived by McLaughlin et al. (1981) for a slightly different case. In the case of interest to us, the ligands binding to the surface of the lipid, PS, are Na^+ and Ca^{2+}. The net charges of free, Ca^{2+}-bound, and Na^+-bound sites are then -1, 1, and 0. Then the expression simplifies to

$$\sigma_L = \sigma_o(1 - Ca_s/K_{Ca})/(1 + Ca_s/K_{Ca} + Na_s/K_{Na})$$

where Ca_s and Na_s denote the surface concentrations of Ca^{2+} and Na^+, respectively. When ion binding to the charged sites was considered, the expression for σ_L was used iteratively in conjunction with the expressions in the first section of the Appendix to obtain a numerical value for $\Phi(0)$ and hence calculate the profiles of voltage and concentrations near the membrane surface.

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