

Ion Permeation, Divalent Ion Block, and Chemical Modification of Single Sodium Channels

Description by Single- and Double-occupancy Rate-Theory Models

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ABSTRACT Calcium ions, applied internally, externally, or symmetrically, have been used in conjunction with rate-theory modeling to explore the energy profile of the ion-conducting pore of sodium channels. The block, by extracellular and/or intracellular calcium, of sodium ion conduction through single, batrachotoxin-activated sodium channels from rat brain was studied in planar lipid bilayers. Extracellular calcium caused a reduction of inward current that was enhanced by hyperpolarization and a weaker block of outward current. Intracellular calcium reduced both outward and inward sodium current, with the block being weakly dependent on voltage and enhanced by depolarization. These results, together with the dependence of single-channel conductance on sodium concentration, and the effects of symmetrically applied calcium, were described using single- or double-occupancy, three-barrier, two-site (3B2S), or single-occupancy, 4B3S rate-theory models. There appear to be distinct outer and inner regions of the channel, easily accessed by external or internal calcium respectively, separated by a rate-limiting barrier to calcium permeation. Most of the data could be well fit by each of the models. Reducing the ion interaction energies sufficiently to allow a small but significant probability of two-ion occupancy in the 3B2S model yielded better overall fits than for either 3B2S or 4B3S models constrained to single occupancy. The outer ion-binding site of the model may represent a section of the pore in which sodium, calcium, and guanidinium toxins, such as saxitoxin or tetrodotoxin, compete. Under physiological conditions, with millimolar calcium externally, and high potassium internally, the model channels are occupied by calcium or potassium

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much of the time, causing a significant reduction in single-channel conductance from the value measured with sodium as the only cation species present. Sodium conductance and degree of block by external calcium are reduced by modification of single channels with the carboxyl reagent, trimethyloxonium (TMO) (Worley et al., 1986) *Journal of General Physiology*. 87:327–349). Elevations of only the outermost parts of the energy profiles for sodium and calcium were sufficient to account for the reductions in conductance and in efficacy of calcium block produced by TMO modification.

INTRODUCTION

Deviations from predictions based on the assumption of independent ion movement can provide crucial clues to the physical structure of ion channels. Previously, several aspects of ion conduction across excitable membranes were well described using electrodiffusion theory with the assumption that ions move independently through transmembrane conducting pathways (Goldman, 1943; Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952). However, shortly after these pioneering studies, it was observed for potassium channels that significant interactions between ions did occur such that ions seemed to pass through a channel in single file (Hodgkin and Keynes, 1955). More recently, there have been several observations indicating that ions do not always move independently of one another as they pass through sodium channels. These include voltage-dependent block by permeant and impermeant ions (e.g., Woodhull, 1973; Taylor, Armstrong, and Bezanilla, 1976; Begenisich and Danko, 1983; Yamamoto, Yeh, and Narahashi, 1984; French, Worley, and Krueger, 1986a; French, Worley, Wonderlin, and Krueger, 1986b; Moczydlowski, Uehara, Guo, and Heiny, 1986; Chahine, Chen, Kallen, Barchi, and Horn, 1992; Worley, French, Pailthorpe, and Krueger, 1992), current saturation with increasing concentration of the permeant ion (Hille, 1975a,b; Begenisich and Cahalan, 1980a; Yamamoto et al., 1984; Moczydlowski, Garber, and Miller, 1984a; French et al., 1986a; Worley et al., 1992), and concentration-dependent permeability ratios (Cahalan and Begenisich, 1976; Ebert and Goldman, 1976; Begenisich and Cahalan, 1980b). The application of reaction rate theory by Hille (1975a,b) to describe permeation through sodium channels demonstrated how the energy barriers posed to ion movement by a channel could be interpreted in terms of possible features of the channel's molecular structure.

In this paper, we focus on the interactions of sodium and calcium ions with single sodium channels, reflected by current saturation and voltage-dependent block. Prompted by reaction rate theory calculations, we ask what physical assumptions are necessary to account for the observed behavior. We present new data on the block of the channels by calcium, applied either externally or internally in the presence of symmetric sodium concentrations or sodium/potassium gradients, and attempt to identify a simple, rate-theory model that can adequately describe the functional dependence of single-channel currents on voltage and on the concentrations of sodium, calcium, and potassium. We conclude that there must be two discrete regions in the channel, one easily accessed by external Ca^{2+} , and the other by internal Ca^{2+} , with a rate-limiting barrier to Ca^{2+} permeation in between. Consistent with other

recent analyses (Ravindran, Kwicinski, Alvarez, Eisenman, and Moczydlowski, 1992; Daumas and Andersen, 1993; Naranjo and Latorre, 1993), optimal fits of the competitive interaction between Na^+ and Ca^{2+} were obtained only when a limited degree of double occupancy of the channel was allowed. A novel test of the model demonstrated that small alterations of the energy barrier profiles can account for changes in ion conduction and block observed after chemical modification of single sodium channels by trimethyloxonium (Worley, French, and Krueger, 1986). Preliminary reports of some of our results have appeared (French et al., 1986b; French, Worley, Wonderlin, Kularatna, and Krueger, 1992).

METHODS

Solutions and Single-Channel Methods

The methods of channel incorporation and recording are based on those of Krueger, Worley, and French (1983) and French, Worley, and Krueger (1984). A detailed description has been given by Worley et al., (1986). Examples of raw data records from which the *i-E* data analyzed in this paper were derived can be seen in the preceding references and in Krueger, Worley, and French (1986). For this study, current fluctuations were recorded from single sodium channels incorporated into neutral (phosphatidylethanolamine, [PE]) lipid bilayers bathed by (a) symmetrical sodium chloride solutions or (b) solutions containing 125 mM NaCl and 5 mM KCl (extracellular), and 5 mM NaCl and 125 mM KCl (intracellular) to approximate physiological monovalent ion gradients. All solutions contained 0.1 mM Mg^{2+} . Sodium channel block by Mg^{2+} under these conditions is negligible (see Worley et al., 1992), and is ignored in the rate theory calculations described below. Solutions were buffered to pH 7.0 with 10 mM HEPES. EGTA was used, where required, to reduce the free $[\text{Ca}^{2+}]$ to negligible levels. In all experiments, 60 nM batrachotoxin (BTX) was added to one of the aqueous solutions to remove inactivation, lengthen channel open times, and enable steady-state recording of single-channel current fluctuations. (A more frugal and effective protocol for BTX modification, based on a suggestion of Cukierman and Krueger [1990] has since been described by Becker, Prusak-Sochaczewski, Zamponi, Beck-Sickinger, and French [1992]).

Throughout the text, solute content of solutions is expressed in concentration units (molarity); however, all calculations were performed using solute activities, based on activity coefficients given by Robinson and Stokes (1959) for NaCl, and by Butler (1968) for Ca^{2+} in calcium chloride-sodium chloride mixtures.

Recordings were obtained over a range of -90 to $+90$ mV, in the absence and in the presence of divalent cations. In some cases, to improve the signal-to-noise ratio in single-channel current amplitude measurements, nanomolar saxitoxin (STX) was added to the bath to induce slow unitary fluctuations due to the blocking and unblocking of the channels by STX (Krueger et al., 1983; French et al., 1984). Points plotted on *i-E* curves, in general, are means determined from 2–18 membranes. In most cases, standard errors were <0.1 pA and would fall within the plotted symbols (e.g., Worley et al., 1986, 1992). The worst case estimate of SEM was ± 0.23 pA (250 mM symmetric Na, $+90$ mV, $n = 2$). Voltages are expressed, in the normal physiological convention, as $E = (E_{\text{in}} - E_{\text{out}})$, where the extracellular surface is defined as the side from which STX blocks.

For those experiments where salts of divalent cations were added to only one side, there were slight alterations in the chloride potential. In each figure that illustrates the effects of asymmetric divalent cations on the sodium current, the applied potential was corrected for the

effects of asymmetric Cl^- by the subtraction of the following offset voltage:

$$E_{\text{Cl}} = (RT/zF) \ln ([\text{Cl}_{\text{in}}]/[\text{Cl}_{\text{out}}]) \quad (1)$$

where $z = -1$, and R , T , and F denote the gas constant, the temperature in degrees Kelvin, and the Faraday, respectively. This accounts for the difference between the electrode potentials of the two reversible Ag/AgCl electrodes in contact with solutions having different $[\text{Cl}^-]$. The offset voltage between a pair of Ag/AgCl electrodes in contact with the same solution was < 1 mV.

Rate-Theory Models

A reaction rate theory treatment of transmembrane ionic diffusion considers ion movement as a series of discrete steps between energy minima (wells) separated by energy maxima (Glasstone, Laidler, and Eyring, 1941; Eyring, Lumry, and Woodbury, 1949; Woodbury, 1971). One may simply account for ionic block, including self-blocking effects that lead to saturation at high concentrations, by assuming that only one ion at a time may reside in a particular energy well, which would correspond to a discrete ion-binding site in the channel, and that individual ions cannot pass each other in the channel. Despite theoretical limitations in the assumptions underlying the rate constant expressions as applied to ion transport (e.g., Cooper, Gates, and Eisenberg, 1988), rate theory as formulated by Eyring and his co-workers provides an interpretable and computationally manageable framework for quantitative description of the relatively complex processes of ion permeation and block in channels with restricted occupancy. Other theoretical approaches are certainly possible, and offer different insights into the underlying processes (e.g., Levitt, 1987; Wu, 1992).

Modeling Strategy for Choosing Barrier Profiles to Fit i - E Relations

We adapted the program AJUSTE, developed by Alvarez, Villaroel, and Eisenman (1992), to adjust the energy barrier profiles for Na^+ , K^+ , and Ca^{2+} to obtain a best fit of our observed single channel i - E data. Surface charge effects are included using the Gouy-Chapman formalism as described by Alvarez et al., except that the Grahame equation was used to solve numerically for surface potential in mixed valence solutions. The following FORTRAN subroutines or programs in the AJUSTE suite were modified: AGRANDA, DMATINV, INPUTA, DATFILE, FN3B3I (3B2S model for three-ion species), TOLOTUS, APLOTS. In addition, two new routines were written to calculate the rate constants for a strict single-occupancy 4B3S model with three-ion species, FN4B3I, and a Newton-Raphson solution of the Grahame equation, NEW.

Two different model subroutines were used within AJUSTE to obtain best fits to our data for three different cases:

Three-barrier, two-site (3B2S) double occupancy. For these calculations, interionic repulsion was treated as a single, variable parameter that determined the interaction energy between two adjacent monovalent ions. This repulsion was assumed to be strictly electrostatic, and thus, for other cases, was scaled according to valence: $2\times$ for monovalent-divalent, $4\times$ for divalent-divalent. The 3B2S double-occupancy model provided the best overall fit to our data. Optimal parameter values for this model are given in Table I.

3B2S single occupancy. Calculations for this case were performed using the general 3B2S subroutine, with repulsion between two ions in the channel arbitrarily set ($20 RT$ for two monovalent ions) to make the probability of double occupancy negligible.

Four-barrier, three-site (4B3S), single-occupancy. In order to make the computation more efficient in this case, we replaced the appropriate subroutines in AJUSTE with those based on a strict, single-occupancy, 4B3S model, thus reducing the number of occupancy states in the reaction scheme to 10 (with three ion species present). The tactic employed for the 3B2S

model, of testing both multiple-occupancy and single-occupancy cases by simply adjusting the interaction energies between ions in the channel was not practical with the much larger number of occupancy states (64) in the 4B3S case.

Other practical details in adapting AJUSTE to our problem included the following: as in the recent studies by Correa, Latorre, and Bezanilla (1991) and Ravindran et al. (1992), the

TABLE I
Adjustable Parameters Determined from the Best-Fit 3B2S (Low Repulsion) Model

		Ion energy profiles		
			pre-TMO	post-TMO
Electrical distances				
D1	0.05	Na ⁺	G11	4.83
D2	0.05		G21	5.43
D3	0.34		G31	5.56
D4	0.34		U11	-6.92
D5	0.11		U21	-7.07
Repulsion parameter		K ⁺	G12	7.64
A(11)	1.94		G22	7.86
			G32	9.27
Radius per unit surface charge			U12	-7.01
S1	1000.0		U22	-7.49
S2	1000.0			
		Ca ²⁺	G13	6.83
			G23	11.56
			G33	6.21
			U13	-10.77
			U23	-10.67

Energy profiles for each ion were defined by three peaks, G_{ij} , expressed in RT units relative to a reference state of 55.5M, where i denotes the i th peak from the intracellular side and j refers to the j th ion, and two wells, U_{ij} , where i denotes the i th well and j denotes the j th ion. Distances are numbered from the intracellular side, and are expressed as a fraction of the total transmembrane voltage drop between adjacent energy peaks and wells. A single variable repulsion parameter, $A(11)$, was used to define the repulsion energy, at unit electrical distance, between two monovalent ions. Repulsion was scaled inversely with electrical distance between occupied sites, and in proportion to the product of the valences of the interacting ions, consistent with a purely electrostatic interaction. Thus, in the case shown above, for the monovalent–monovalent repulsion, we used values of $A(11) = A(22) = A(12, 21) = 1.94$, for monovalent–divalent interactions $A(13, 31) = A(23, 32) = 3.88$, and for divalent–divalent interactions $A(33) = 7.77$. Surface charge density is defined by parameters S1 and S2, at the intracellular and extracellular surfaces respectively, which express the mean radius, in Angstroms, of a circle that contain a single elementary charge, in a uniformly smeared sheet of charge. The surface charge densities used here have a negligible effect. Adjustment of only the four indicated parameters (outer peaks and wells for Na⁺ and Ca²⁺) was sufficient to fit the data obtained after TMO modification of the channels (Fig. 8). Other parameters were obtained from a global fit to data of Figs. 1, 3, 4, 5, and 6.

reference energy state corresponds to 55.5 M (the molar concentration of water), and 4.0 RT units should be added to our energy profiles for direct comparison with calculations using a standard state of 1 M. Rate constants were calculated using a preexponential factor of $k_B T/h$ (k_B = Boltzmann's constant, T = temperature in °K, and h = Planck's constant) and assuming a transmission coefficient of 1.

For an n -barrier energy profile, the input parameter files for AJUSTE (Table II of Alvarez et al., 1992) allow specification of $(2n-1)$ independent electrical distances, which are the well-to-peak or peak-to-well distances for the n barriers expressed as fractions of the transmembrane voltage that fall across each segment. The last of the $2n$ distances is chosen so that the sum of all $2n$ is equal to one. We made the simplifying assumption of "centered peaks," setting the adjacent well-to-peak and peak-to-well distances equal for each barrier, so that distances for an n -barrier profile were specified by $(n-1)$ input parameters. Thus, each peak is centered between the two adjacent wells, and spatial asymmetry can be introduced into the overall energy profile by assigning unequal barrier widths (i.e., unequal well-well distances). It can be notoriously difficult to obtain unique convergence when allowing distance parameters to vary (e.g., in the study Naranjo and Latorre [1993] distances were preselected and then energy parameters optimized). In our study, the principal constraints on the distance parameters were provided by the asymmetric, voltage-dependent blocking action of Ca^{2+} .

As noted by Alvarez et al. (1992), even with single-ion calculations, it is not possible to optimize all parameters simultaneously. Thus, our tactics were as follows: where possible, initial guesses were calculated for maximum barrier heights from single-channel conductance, and for maximum well depths from apparent dissociation constants. These values were then optimized as follows.

(a) Barrier profiles for Na^+ were refined by fitting all data collected with Na^+ as the only cation. This required linear i - E relations over the experimental range, with single-channel currents, and a binding affinity for Na^+ , consistent with data for 25–750 mM Na^+ .

(b) Barrier profiles for Ca^{2+} were adjusted to match the Ca^{2+} block represented by data collected in the presence of $\text{Na}^+/\text{Ca}^{2+}$ mixtures. The only case in which relaxation of the single occupancy restriction, by lowering the ionic interaction energies, noticeably improved the fit was in the case of $\text{Ca}^{2+}/\text{Na}^+$ mixtures over a range of $[\text{Na}^+]$. Optimized predictions of both 3B2S and 4B3S, restricted to single occupancy, slightly overestimated the degree of block at the highest $[\text{Na}^+]$.

(c) Barrier profiles were constructed for K^+ using data taken with Na^+/K^+ mixtures with approximately "physiological" and reversed ionic gradients. Optimal fits demanded substantial occupancy by K^+ , as well as an asymmetric profile for K^+ .

(d) All barrier profiles were refined with simultaneous fits to the complete data set (i - E curves determined under 16 different ionic conditions). Optimization was repeated with overlapping subsets of parameters allowed to vary until no further improvement was obtained.

RESULTS

In Symmetric $[\text{Na}^+]$, Current–Voltage Relations are Linear and Conductance Saturates with Increasing $[\text{Na}^+]$

Single-channel i - E relations were linear when the membranes were bathed in symmetric solutions ranging from 10 to 1000 mM. Examples are shown in Fig. 1. The single-channel conductance appears to saturate with increasing $[\text{Na}^+]$ (Fig. 2) and, in fact, may be well approximated by a simple rectangular hyperbola with an apparent dissociation constant ($[\text{Na}^+]$ at half-maximal conductance) of 40.5 mM, and a maximal conductance of 31.8 pS (Worley et al., 1992). The linearity of the i - E relations over this wide range of concentration and voltage requires the energy barrier profile for Na^+ to be approximately symmetric.

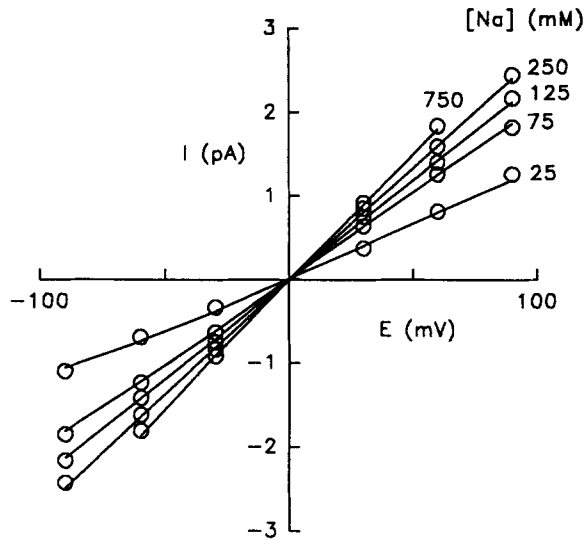


FIGURE 1. Single-channel current-voltage relations for BTX-activated sodium channels bathed by symmetric solutions of NaCl ranging from 25 to 750 mM. The lines are drawn with the 3B2S double-occupancy model described in the text. In the overall fitting strategy, these data provided the initial constraints on the energy profile posed by the channel to permeating Na⁺ ions.

One-sided or Symmetric Addition of Ca²⁺ Causes Voltage-dependent Block of Single-Channel Currents

When Ca²⁺ ions are added, to either side separately, or symmetrically, the single-channel conductance is reduced and the *i-E* relations become distinctly nonlinear. This voltage-dependent block by Ca²⁺ is most dramatic for external Ca²⁺, but is also clearly apparent when Ca²⁺ is applied internally. (Fig. 3; see also Moczydlowski et al., 1986, Table 2, and reports of internal Mg²⁺ block by Albitz, Magyar, and Nilius [1990] and Lin, Conti, and Moran [1991]). Qualitatively, these data are consistent with the idea that Ca²⁺ ions can enter the channels from either side, but are not able to pass easily through the channel. Thus, an increase in the electrochemical gradient favoring Ca²⁺ entry, from either end, leads to an increasing degree of block of the single channel current.

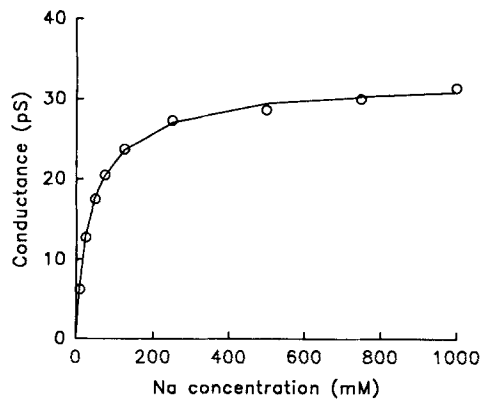


FIGURE 2. Conductance-concentration relationship for single sodium channels. The points are the same data as in Worley et al. (1992). The line connects points calculated from the 3B2S double-occupancy model.

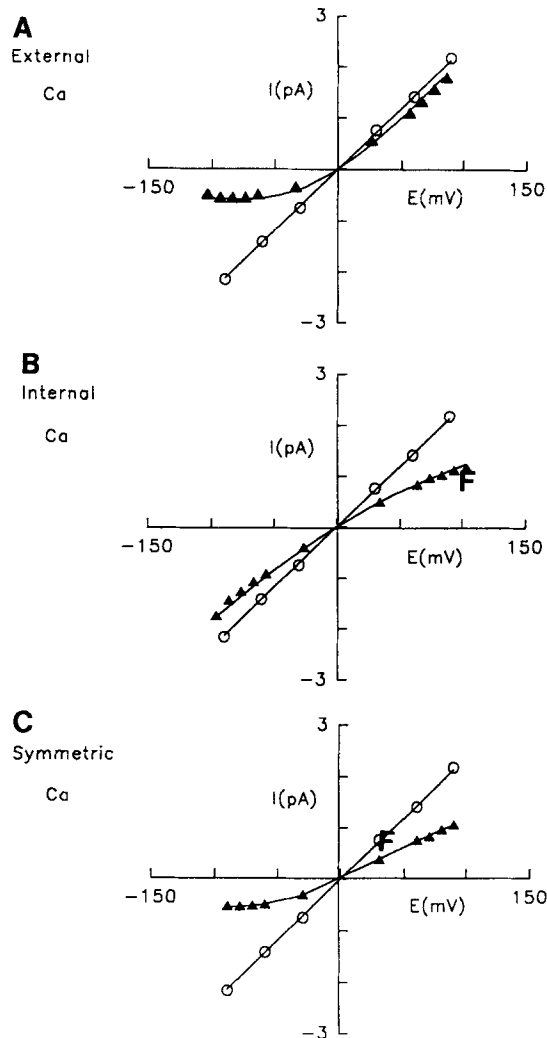


FIGURE 3. Effects of extracellular, intracellular or symmetric Ca^{2+} on current-voltage relations of single, BTX-activated sodium channels in planar lipid bilayers, determined in the presence of 125 mM symmetrical Na^+ . Each point represents the mean of data from two to eight membranes. In each of the frames the smooth curves through the data points were calculated from the 3B2S double-occupancy model. Open circles and filled triangles represent current measured in the absence and presence of Ca^{2+} , respectively. (A) External calcium. Single-channel current-voltage relationships obtained with symmetrical 125 mM Na^+ , in the absence of divalent cations, or with 10 mM Ca^{2+} added to the external side of the membrane. Note that external Ca^{2+} blocks both inward and outward current, with the degree of block increasing as the voltage is made more negative. (B) Intracellular calcium. The same conditions as A, except that 10 mM Ca^{2+} was added to only the intracellular surface of the sodium channel. Internal Ca^{2+} blocks both inward and outward current, with

the degree of block increasing as the voltage is made more positive. (C) Symmetric calcium. The same conditions as A, except that 10 mM Ca^{2+} is added to both sides of the membrane. The i - E relationship is asymmetric, despite the symmetric ionic conditions, consistent with a more potent and more voltage-sensitive block by external Ca^{2+} , as indicated by A and B.

For single-sided application of Ca^{2+} , there is a weaker block of current flowing toward the side on which Ca^{2+} was added, and as the driving force and single channel current increase, the degree of block decreases, suggesting that Ca^{2+} is being forced out of the channel down the electrical potential gradient. Symmetric application of Ca^{2+} leads to an increased degree of block of single channel current in both directions. The i - E relation is asymmetric, qualitatively resembling that for external Ca^{2+} alone. Consistent with the fact that external Ca^{2+} produced more strongly

voltage-dependent block than internal Ca^{2+} , this suggests that external Ca^{2+} penetrates more deeply through the transmembrane voltage to reach its site of action than does Ca^{2+} entering from the cytoplasmic end. Thus, the barrier that limits the rate of Ca^{2+} permeation must be nearer the cytoplasmic end of the channel.

The data presented thus far provide an argument that a minimal model for the Na channel pore must have at least three barriers and two ion-binding sites (a 3B2S model). Extensive calculations with single site (2B1S) models (not shown) revealed no profile that could simultaneously mimic the voltage dependence of Ca^{2+} block when Ca^{2+} was applied from each side of the membrane. This is a consequence of the need for Ca^{2+} to encounter a rate-limiting barrier to permeation immediately beyond the blocking site, in order to produce a substantial voltage dependence of block. For a single-site model, this is possible only for Ca^{2+} entering from one side.

Increasing $[\text{Na}^+]$ Decreases the Degree of Ca^{2+} Block

In the presence of symmetric Na^+ over the range 75–750 mM, the degree of block by 10 mM symmetric Ca^{2+} decreased progressively with increasing $[\text{Na}^+]$ (Fig. 4). Such behavior suggests that Na^+ may be competing with Ca^{2+} for a site or sites within the channel. Worley et al. (1992) indicated that, although competition for a single site provided a reasonable approximation to the data over a restricted range (e.g., $[\text{Na}^+] = 75\text{--}250$ mM), simple Michaelis-Menten competition was inadequate to describe the $[\text{Na}^+]$ dependence of Ca^{2+} block over the full range examined.

Of the models examined here, the 3B2S model with a small but significant probability of double occupancy, was best able to predict the competitive interaction between Ca^{2+} block and Na^+ . Single-occupancy versions of both 3B2S and 4B3S models gave optimal fits that underestimated the ability of Na^+ to compete with Ca^{2+} at high $[\text{Na}^+]$ (Fig. 4). This failure was largely overcome by reducing the ion-ion interaction energies to allow double occupancy in the 3B2S model.

Single-Channel i - E Relations Change Asymmetrically on Reversal of Gradients in Na^+/K^+ Mixtures

A subtle prediction arose from fitting i - E data collected in the presence of Na^+/K^+ mixtures. In the presence of quasi-physiological gradients (in mM, in/out; 125 K^+ , 5 $\text{Na}^+ / 5$ K^+ , 125 Na^+), the i - E relation showed the normal rectification expected when there is a concentration gradient of the more permeant ion from outside to inside. Qualitatively, the relationship reversed when the concentrations were interchanged (in/out: 5 K^+ , 125 $\text{Na}^+ / 125$ K^+ , 5 Na^+ ; see Fig. 5). However, a closer examination of the data at 0 mV shows that the magnitude of the inward current with high $[\text{Na}^+]$ outside is less than the magnitude of the outward current with high $[\text{Na}^+]$ inside. This reflects a general, small asymmetry in the two i - E relations. This asymmetry yields predicted reversal potentials that are of unequal magnitude. In other words, the brain Na channels studied here show a greater selectivity for Na over K when there is high Na and low K on the cytoplasmic side than with physiologically oriented gradients. This is identical with the conclusion reached by Garber (1988) for BTX-activated skeletal muscle channels and suggests that both of these channel isoforms present an asymmetric barrier profile to permeating K^+ ions.

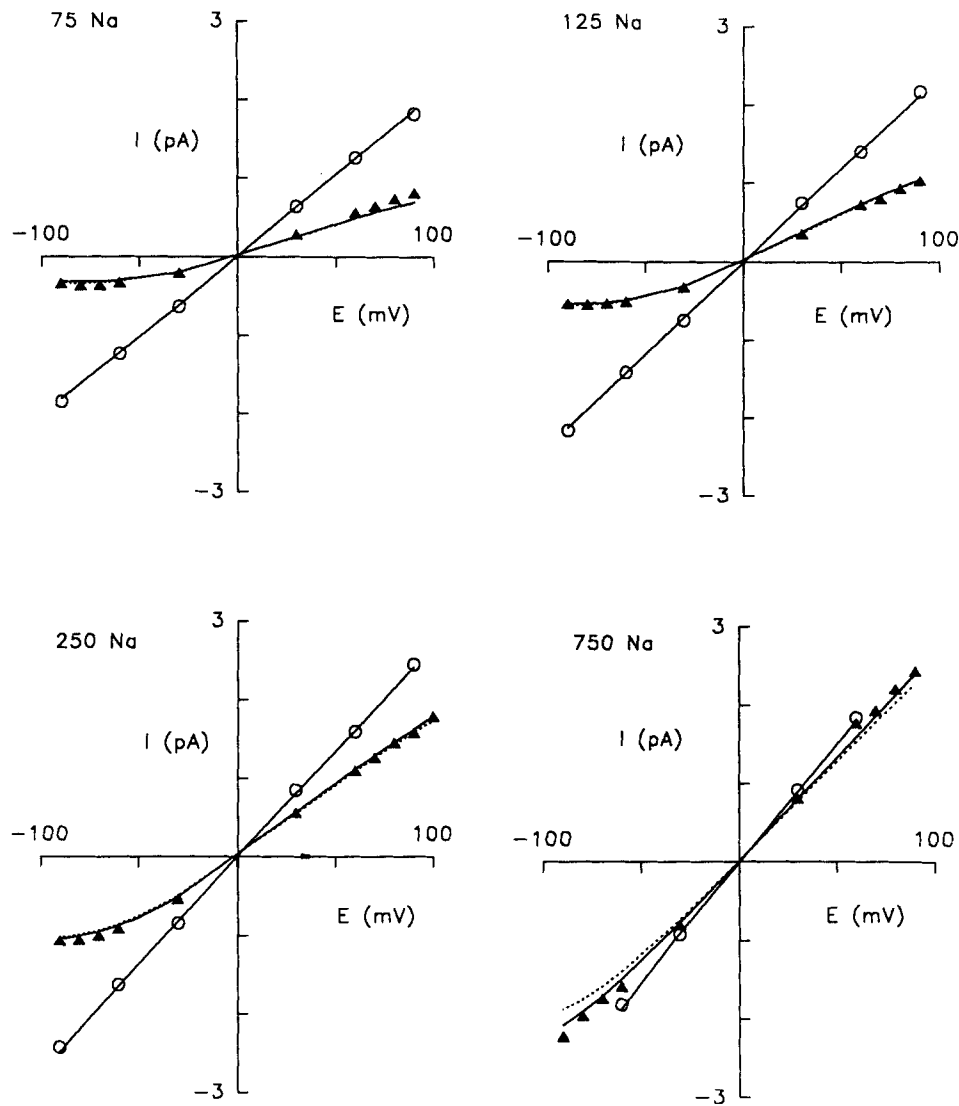


FIGURE 4. Increasing $[\text{Na}^+]$ decreases the degree of block by Ca^{2+} . Single-channel i - E curves were determined under symmetric ionic conditions in the absence (< 100 nM) of free Ca^{2+} (○), or with 10 mM Ca^{2+} present (▲) on both sides of the bilayer. $[\text{NaCl}]$ was varied symmetrically from 75 to 750 mM. The solid lines drawn with the 3B2S model represent the best fit to the data obtained among all the models tested. The dotted lines represent the best fit obtained with the 4B3S single-occupancy model, but deviate from the data points at 750 mM Na^+ . Thus, the 4B3S single-occupancy model gave a less satisfactory fit even though it has more free parameters than the 3B2S, double-occupancy model. The best fit obtained with 3B2S, single-occupancy calculations (not shown) showed a deviation from the data similar to that of the 4B3S calculations shown here.

Ca²⁺ Block in the Presence of Quasi-physiological Na⁺/K⁺ Gradients

We also examined block by Ca²⁺, added externally, internally, or symmetrically, of inward Na⁺ currents in the presence of quasi-physiological Na⁺ and K⁺ gradients (Fig. 6). Block showed similar dependence on voltage and the side(s) of Ca²⁺ application as does block of inward currents in the presence of symmetric Na⁺ (Fig. 3) and was well fit by the same parameters.

Barrier Profiles for Na⁺, K⁺, and Ca²⁺

In Fig. 7, we show the barrier profiles defined by the best-fit parameters from the 3B2S, double-occupancy model, optimized by simultaneously fitting *i*-*E* relations under 16 different sets of ionic conditions (Figs. 1, 3, 4, 5, and 6). We note the following points. The asymmetry of Ca and K profiles is demanded directly by the

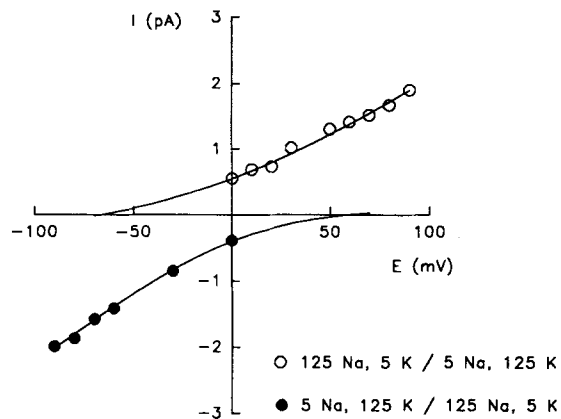


FIGURE 5. Single channel *i*-*E* relations in the presence of Na⁺/K⁺ mixtures. Preliminary constraints on the K⁺ energy profile were provided by fitting these data from Na⁺/K⁺ mixtures, either with quasi-physiological gradients (extracellular: high [Na⁺], low [K⁺]; intracellular: high [K⁺] and low [Na⁺]), or with the reverse. Concentrations (mM) are indicated on the figure as intracellular/extracellular. The lines shown here are drawn using the

3B2S double-occupancy model with parameters refined by fitting the entire data set. Note that the *i*-*E* data do not change symmetrically when the ionic gradients are reversed, and that the reversal potentials predicted by the fit are also asymmetric (-67.5 and 58.5 mV). This appears to represent a slight, but fundamental, asymmetry in the structure of the channel's energy profile for K⁺ (c.f. Garber, 1988).

asymmetry introduced into the *i*-*E* relations when these ions are present. For example, the outer well is placed farther into the channel, in electrical distance, from the outer entrance, than is the inner well from the inner entrance. This is required by the steeper voltage dependence of block by external Ca²⁺ than by internal Ca²⁺. For block to be enhanced by voltages driving Ca²⁺ into the channel from either side, the two wells for Ca²⁺ must be separated by the highest barrier in the profile. Potassium ions enter the channel relatively easily from the inside (i.e., the selectivity for sodium over potassium is slightly weaker at the inner entrance), requiring a lower entry barrier to K⁺ at the inner mouth of the channel.

By contrast, the slight asymmetry of the Na profile arises from the arbitrary simplification that binding sites for all ions be located at the same positions. A perfectly symmetric profile would be slightly better suited (though scarcely noticeable

in our figures) to fitting the data collected in the presence of Na^+ alone. An alternative procedure, in which the precise electrical positions of wells and peaks were allowed to vary independently for each ion (e.g., Waggoner and Oxford, 1987), would multiply the number of free parameters to such an extent as to make the problem computationally unmanageable, even with the many constraints imposed by our diverse data set.

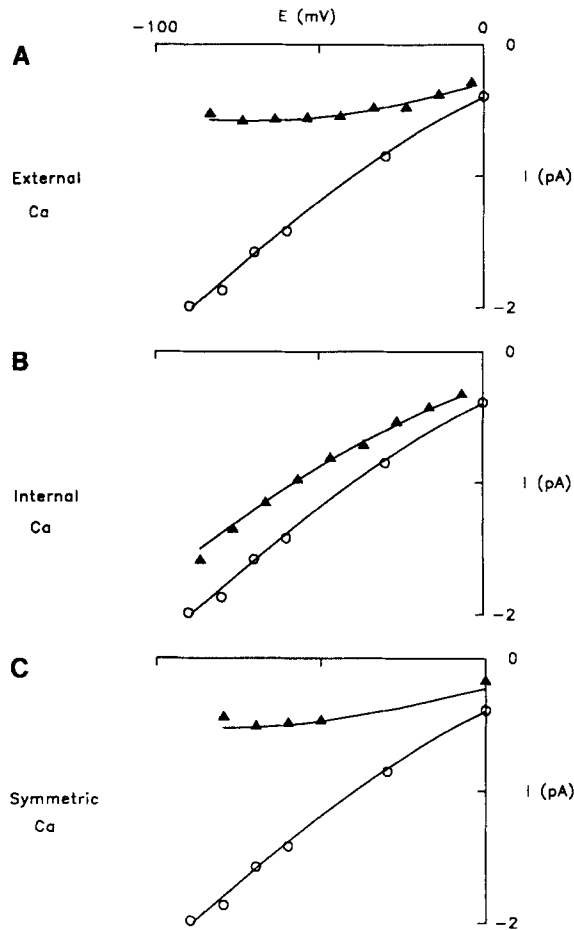


FIGURE 6. Effects of extracellular and intracellular calcium with approximately physiological monovalent cation (Na^+/K^+) gradients. Open circles and filled triangles represent current measured in the absence and presence of Ca^{2+} , respectively. The lines were obtained for the 3B2S double-occupancy model from a global fit to the entire data set (Figs. 1 and 3–6). (A) External calcium. Current–voltage relationships for single sodium channels in the presence of 125 mM NaCl, 5 mM KCl on the extracellular side and 5 mM NaCl, 125 mM KCl on the intracellular side. When 10 mM Ca^{2+} was added to the external side of the membrane the inward current was reduced. The degree of block increased progressively with increasing hyperpolarization (c.f. Fig. 3). (B) Intracellular calcium. The same conditions as in A, except that 10 mM Ca^{2+} was added only to the intracellular side of the sodium channel. The block shows very little voltage dependence, and

the fractional block is less than for external Ca^{2+} . (C) Symmetric calcium. The same conditions as in A, except that 10 mM Ca^{2+} was added to both sides. The solid lines were drawn from the 3B2S, double-occupancy model.

A Minimal Model?

In Table II we compare the total sums of squared deviations of the data from the model predictions for the best fits obtained with three different models. As implemented, the 3B2S model, allowing the possibility of double occupancy, included 18 variable parameters and gave the lowest sum of squares. The best fit by a 4B3S,

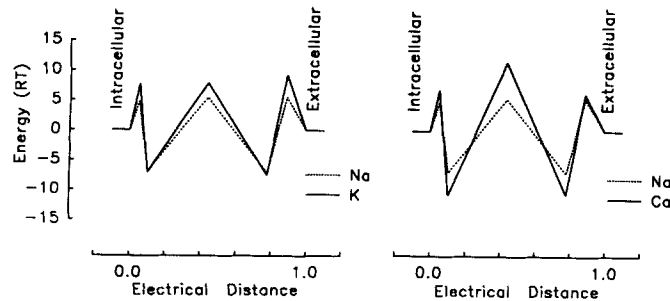


FIGURE 7. Best fit energy barrier profiles posed to Na^+ , K^+ , or Ca^{2+} ion movement through the sodium channel for the 3B2S double-occupancy model, in the unoccupied state. Notice that the profile for Na^+ (dotted line, and repeated in each frame for comparison) is approximately symmetric, whereas those for Ca^{2+} and K^+ are asymmetric, having the highest peak centrally (Ca^{2+}) or at the extracellular end (K^+). It was assumed, for simplicity, that the corresponding peaks and wells for each ion occur at the same electrical distances across the membrane. The peaks were placed at the midpoint of the electrical distance spanned by each barrier.

single-occupancy model, despite having 24 free parameters, showed a sum of squares about $1.6\times$ larger, giving optimal fits that systematically deviated from the data at high $[\text{Na}^+]$ in the presence of Ca^{2+} . When the 3B2S model was restricted to single occupancy (effectively having 17 free parameters), it deviated from the data in a similar manner to the 4B3S single-occupancy model, giving a sum of squares about $1.8\times$ larger than the optimal double-occupancy fit. Thus, the general 3B2S model appears to be the model of minimal complexity that can reasonably quantitatively reproduce the features of our data set.

Reduction in Single-Channel Conductance and Efficacy of Ca^{2+} Block after Trimethyloxonium Treatment

Having identified a relatively simple model capable of describing conduction and block in BTX-activated sodium channels under diverse conditions, we further tested the formalism by asking if the model could be altered in a physically reasonable way to account for the striking alterations in single channel properties resulting from

TABLE II
Sums of Squares for Best Fits Using each of Three Different Models

Model	Datafile	Number of variable parameters	Sum of squares
3B2S (low repulsion)	ACTVJFW	18	pA^2 0.3205
3B2S (high repulsion)	ACTVJFWH	17	0.5749
4B3S (single occupancy)	ACTVJFW	24	0.5325

Contributions to the sum of squares were multiplied by the reciprocal of the magnitude (in picoamperes) of the measured current in order to give reasonable weight to points where currents were reduced by block, or by reduced concentrations of Na^+ . Weighting factors ranged between 0.4 and 6.

modification by the carboxyl reagent, trimethylxonium (TMO), applied externally. TMO abolishes block by STX, reduces single channel conductance, and reduces efficacy of external Ca^{2+} block (Worley et al., 1986). The fact that no single one of these changes was observed separately from the others suggested that all may result from modification, by TMO, of a single group near the mouth of the channel. In the present study, we tested the feasibility (though not the uniqueness) of the hypothesis that changes in both conductance and Ca^{2+} block could arise from a localized modification at or near the outer mouth of the channel. Fig. 8 shows that by raising

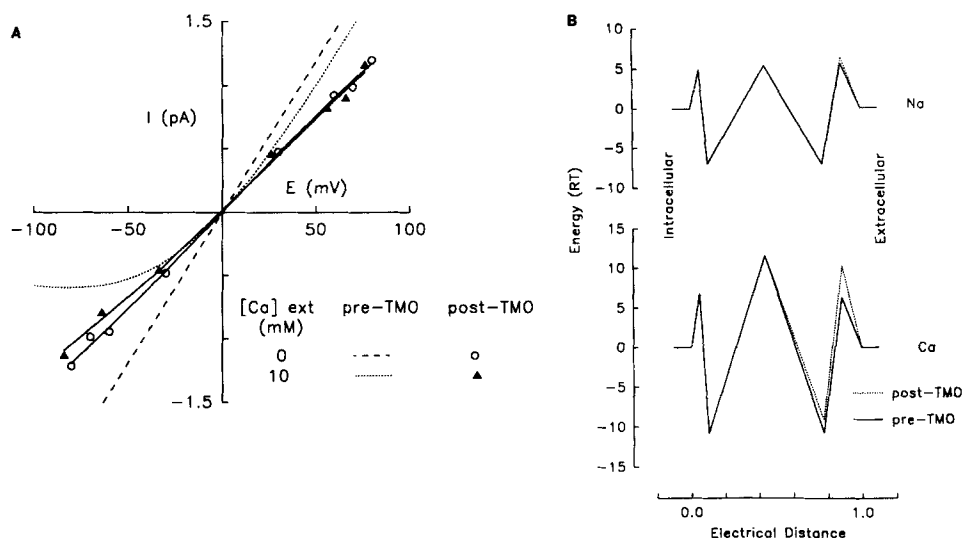


FIGURE 8. (A) Single-channel current–voltage relations after TMO modification. Points are data taken from Worley et al. (1986). Solid lines are the i - E relations calculated using the 3B2S double-occupancy model with the energy profiles of B. Note that the currents in the absence of Ca^{2+} are reduced, and block by Ca^{2+} is much weaker, than observed under similar ionic conditions before TMO modification. Fits to data obtained without TMO modification are indicated by the dashed (0 mM Ca^{2+}) and dotted lines (10 mM Ca^{2+}). Data for the non-TMO-modified channels are shown in Fig. 3. (B) Changes in the barrier profiles for the 3B2S model resulting from TMO modification. Barrier profiles before TMO treatment are represented by the solid lines (see also Fig. 7), and those after TMO treatment are represented by the broken lines. Note that the data for the modified channel were well fit by changing only the parts of the two energy profiles nearest to the extracellular solution.

only the outermost parts of the barrier profiles for Na^+ and Ca^{2+} (the outer barrier for Na^+ , and the outer barrier and well for Ca^{2+}), it was possible to obtain an excellent fit of the i - E relationships determined after TMO modification. While not demonstrating uniqueness of the fit—indeed, uniqueness is unlikely given the simple form and limited scope of the data collected after TMO modification—this exercise does show that an intuitively reasonable modification of the model, at the likely site of TMO attack, can account for our earlier observations.

DISCUSSION

What Does It Mean to Be a Sodium Channel? Predictions of Occupancy and Conduction

A surprising conclusion from our calculations is that, while defined by its ability to selectively conduct Na^+ ions, a sodium channel may, under physiological conditions,

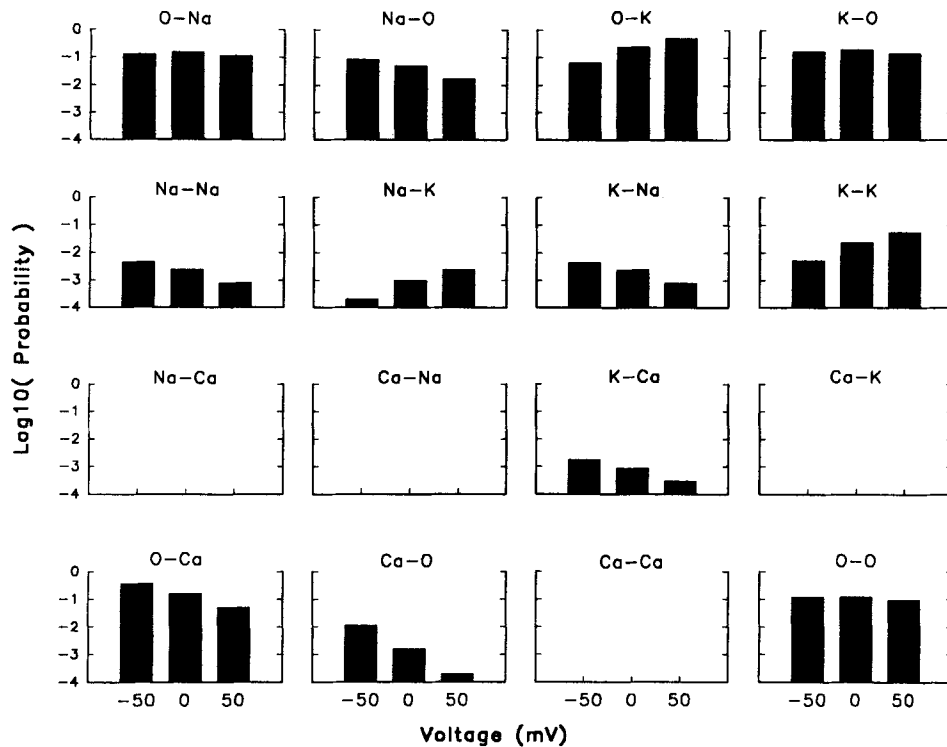


FIGURE 9. Predicted \log_{10} (probability) of all the possible occupancy states of the 3B2S double-occupancy model, plotted against voltage, under quasi-physiological ionic conditions (in mM, inside: 5 Na^+ , 125 K^+ ; outside: 125 Na^+ , 5 K^+ , 3 Ca^{2+}). Occupancy states are identified as follows: O-Na represents the state with the inner well unoccupied and Na^+ in the outer well. The sodium-selective channel is about equally likely to be occupied by K^+ as by Na^+ —in fact, the model channel is more likely to be occupied by K^+ at depolarized potentials. At potentials near normal neuronal or muscle resting levels, each of the three ion species would occupy the channel with a similar probability. Doubly occupied channels are predicted to occur with a low probability (generally, the summed probability for all doubly occupied states is <0.06), but allowing double occupancy leads to a better fit of some subtleties of the i - E relations (e.g., Fig. 4).

be more likely to be occupied by the “blocking” ions, Ca^{2+} and/or K^+ . The inner workings of our best-fit, 3B2S model are illustrated in Fig. 9. For each of the 16 possible occupancy states for the 3B2S model, the probability is shown on a logarithmic ordinate for -50 , 0 , and $+50$ mV. For example, in the upper left panel,

the probability of the channel being singly occupied by a Na^+ ion in the extracellular well (state O-Na) is depicted. From the lower panels, it is clear that occupancy by Ca^{2+} , present only in the extracellular solution, is favored by negative potentials. Overall, in the range of voltage spanned by an action potential, occupancy by K^+ is somewhat more likely than by Na^+ . This is consistent with several observations that K^+ decreases single Na channel currents (Krueger et al., 1983; Green, Weiss, and Andersen, 1987a; Garber 1988). In addition, near the resting potential and firing threshold, occupancy by Ca^{2+} reduces unitary current to <50% of the value in the absence of Ca^{2+} (see also Fig. 6). Considering doubly occupied states, monovalent cation pairs are most probable because of the lower interaction energies. K-K is more likely than Na-Na, both because the energy wells for K^+ are somewhat lower, and because the asymmetry of the K profile favors loading of the channel with K^+ at depolarized potentials (see also Garber, 1988; Ravindran et al., 1992).

While a small degree of Ca^{2+} permeation is important to simultaneously fit the observed block of the channel by internally and externally applied Ca^{2+} , this would not lead to measurable single-channel Ca^{2+} current in pure Ca^{2+} solutions with present techniques (0.023 pS in symmetric 125 mM Ca^{2+} , predicted from our model). This requirement for some degree of Ca^{2+} permeation is consistent with aequorin fluorescence and macroscopic current measurements of Ca^{2+} permeation in squid axon (Baker, Hodgkin, and Ridgway, 1971; Meves and Vogel, 1973) and recent single channel studies (Gangler and Krueger, 1991). Slight Ca^{2+} permeation is also not surprising given the recent demonstration by Heinemann, Terlau, Stühmer, Imoto, and Numa (1992) that most Ca^{2+} channel conduction properties can be conferred on a rat brain Na^+ channel by only one or two amino acid substitutions. In our calculations, the effect of Ca^{2+} permeation is analogous to the more dramatic role of a slight Na^+ permeability shaping macroscopic *i-E* relations for the delayed rectifier of squid (French and Wells, 1977; French and Shoukimas, 1985).

The single channel conductance predicted by the double-occupancy 3B2S model in the presence of 125 mM symmetric K^+ is 0.77 pS. The value is lower than previously observed either in rat skeletal muscle (Garber and Miller, 1987; 5.7 ± 0.5 pS in 500 mM KAc) or in purified rat brain channels (Hartshorne, Tamkun, and Montal, 1986; 3.2 pS in 500 mM KCl), probably in part because in our data set we have no observations in pure potassium salts over a range of concentrations to more precisely constrain the K^+ energy profile. Model predictions of the components of net ionic current under quasi-physiological conditions are given in Table III. These, as expected, indicate that Na^+ is the dominant charge carrier except in the immediate vicinity of the reversal potential, which is slightly negative to the Na^+ equilibrium potential due to the permeation of K^+ .

Symmetric and Asymmetric Properties of the Sodium Channel Pore

Echoing the study of Garber (1988), we conclude that the barrier profile for K^+ is asymmetric, with the highest barrier at the extracellular end of the channel. This leads to asymmetric reversal potentials under biionic conditions (Garber, 1988; and Fig. 5, this paper), as well as nonlinear *i-E* relations in symmetric K^+ , and weakly voltage-dependent block of Na^+ currents by K^+ . While much has been made of the changes wrought in the Na channel by BTX modification, similar properties have

been described for the native Na conductance of invertebrate giant axons (Cahalan and Begenisich, 1976; Ebert and Goldman, 1976). Thus, this subtle asymmetry of the channel with respect to K^+ appears to be a general property of several Na channel isoforms, and is maintained despite modification by batrachotoxin, rather than being caused by it. This is consistent with the conclusion of Garber and Miller (1987) that the changes in pore structure induced by alkaloid modification are discrete, rather than being a general distortion of the whole conducting pathway.

Is the Sodium Channel a Single-Ion Pore?

In common with the studies of Ravindran et al. (1992) and Naranjo and Latorre (1993), we suggest that the Na channel may be occupied by more than one ion, with a significant, but low probability (our predicted lumped probability of all double occupancy states, under quasi-physiological conditions, as in Fig. 9 and Table III, is about 0.04–0.06, depending on voltage). In all three studies, this conclusion rests on small, but distinct deviations from predictions of single occupancy models over a

TABLE III
Contributions to Single-Channel Current by Different Ion Species under Quasi-physiological Ion Conditions (in mM, Inside: 5 Na^+ , 125 K^+ ; Outside: 125 Na^+ , 5 K^+ , 3 Ca^{2+})

V	Single-channel current			Total
	Na^+	K^+	Ca^{2+}	
<i>mV</i>			<i>pA</i>	
-100	-0.828	-0.001	-0.004	-0.833
-50	-0.728	0.002	-0.001	-0.726
50	-0.0656	0.0381	0.0	-0.0275
100	0.0187	0.0631	0.0	0.0818

Values given are predictions of the best-fit 3B2S double-occupancy model.

restricted range of conditions. It is consistent with the weak coupling seen in flux studies (Begenisich and Busath, 1981; Busath and Begenisich, 1982).

Our data provide novel evidence for this conclusion. At high concentrations, Na^+ ions compete more successfully with blocking Ca^{2+} than simple competition predicts (Fig. 4). Neither surface charge effects, which would be most apparent at low, rather than high ionic strengths (c.f. Naranjo and Latorre, 1993), nor addition of an extra binding site (requiring six additional parameters when three ion species are present) in a single-occupancy model, could account for this small, but consistent, deviation. Thus, it was striking that freeing a single parameter, the interaction energy, in the 3B2S model, improved the overall fit (Table II), by largely eliminating this deviation between data and model. This is the only feature of our data that was not well fit within the constraints of single occupancy. The final value that we obtained for the interaction energy, $A(11) = 1.94 RT$, is very close to the values found by Ravindran et al. (1992) to fit their conductance-concentration data from rat skeletal muscle Na channels.

Intrapore Binding Sites and Surface Charges

It is not a trivial task to apportion accurately the effects on channel conduction properties between interactions at intrachannel sites and charges on the surface adjacent to the conducting pore (e.g., Green and Andersen, 1991). No features of our own data, including data at the lowest ionic strength used (10 mM NaCl), demand a significant influence of surface charge (Worley et al., 1992; see also Naranjo and Latorre, 1993, Table 3). This contrasts with observations on other sodium channel isoforms for which conductance vs. $[\text{Na}^+]$ plots appear to extrapolate to a nonzero intercept at zero Na^+ (Green et al., 1987a; Recio-Pinto, Duch, Levinson, and Urban, 1987; Castillo, Villegas, and Recio-Pinto, 1992). Global fits to our data were not improved by allowing the surface charge density to vary. Thus, we conclude, within the Gouy-Chapman formalism, that fixed surface charges do not significantly influence conduction in rat brain sodium channels under our experimental conditions. Treatments which include more realistic geometries for fixed charge placement could be explored further (e.g., Cai and Jordan, 1990; Dani, 1986). Furthermore, our own conclusion does not exclude the possibility that experiments at extremely low ionic strength might provide evidence of some nonspecific surface charge, outside of the immediate conduction pathway, whose influence would be largely screened out at the ionic strengths used in most of our experiments (c.f. Naranjo and Latorre, 1993, Fig. 8). Some preliminary observations of Worley (1985) suggest that block—especially of outward current—may be incomplete at high $[\text{Ca}^{2+}]$, and would be consistent with this possibility.

Changes at the Extracellular End of the Energy Profiles Can Account for TMO Modification of Conductance and Ca^{2+} Block

As noted in Results, simply raising the outer parts of the energy profiles for Na^+ and Ca^{2+} allowed the 3B2S double-occupancy model to accurately mimic the observed TMO-induced reduction in conductance and Ca^{2+} block. This result has no specific requirement for a 3B2S profile or for multiple occupancy. A 4B3S, single-occupancy model, in which the limiting barrier for Ca^{2+} permeation is the third en route from outside to inside, also provides an excellent fit to all data except those in Fig. 4, and allows the TMO modification to be placed even closer to the extracellular solution, as again, only the outermost barriers and wells need be raised to fit the TMO data. We have reported that modification by TMO can simultaneously abolish STX block, and reduce conductance and Ca^{2+} block. Moreover, replacement of one of several negative residues (e.g., E387 in the rat brain II sodium channel) is adequate to eliminate STX/TTX sensitivity (Noda, Suzuki, and Stühmer, 1989; Terlau, Heinemann, Stühmer, Pusch, Conti, Imoto, and Numa, 1991). Thus, the small elevation in the outer end of the barrier profile invoked in modeling the TMO-modified channel may be mimicking the functional effect of neutralization and methylation of a single carboxyl group at the outer mouth of the pore.

TMO modification undeniably produces a change in net charge, as well as increasing the bulk of an amino acid side chain by methylation. Elevation of the energy profile, as used to model the TMO effects, reflects a pore made less favorable to cation entry and/or binding. This provides an operationally accurate description

of the effects, but may not exclude other physical interpretations. Our present suggestion, that the mouth of the actual permeation pathway is modified, provides a parsimonious explanation consistent with both functional observations and molecular biological data, which place critical TMO-modifiable residues at the external mouth of the putative Na channel pore (Noda et al., 1989; Terlau et al., 1991). Further inferential support for this view comes from strong functional and structural homologies between Na channels and K channels, for which either charybdotoxin or tetraethylammonium appear able to bind in the outer pore mouth (MacKinnon and Miller, 1989; Heginbotham and MacKinnon, 1992).

Nonetheless, our interpretation should not be blindly generalized. Recent studies of the action of several carboxyl reagents on different Na channel isoforms show a variety of actions on conductance, toxin sensitivity, and Ca^{2+} block (Chabala and Andersen, 1992; Cherbavaz, 1990; Dudley and Baumgarten, 1993). In addition, placement of the toxin site at the mouth of the pore sidesteps the possible paradox raised by evidence from other preparations that there is a substantial surface charge in the vicinity of the STX/TTX site (canine brain—Green, Weiss, and Andersen, 1987*b*; canine heart—Ravindran and Moczydlowski, 1989; Cai and Jordan, 1990). Our data do not reveal any surface charge influence on conduction. How then, can STX/TTX receptor be in the the pore mouth? Perhaps there are differences among the surface charge densities near the toxin receptors of different channel isoforms, but the complete spectrum of experiments on both ion conduction and toxin block that would be required to resolve this issue, for a single Na channel isoform, still has not been done (see also Miller and Garber, 1988). Placement of the STX/TTX site at the mouth of the channel also prompts a reconsideration of the mechanism by which guanidinium toxins with different net charge show identical voltage dependence. It is possible, as suggested by Moczydlowski, Hall, Garber, Strichartz, and Miller (1984*b*), that no toxin charge enters the transmembrane electric field, and that the voltage dependence arises from a conformational change associated with toxin binding. However, it is now known that voltage dependence of charybdotoxin block of the maxi-K channel—albeit resulting from an interaction with K^+ —depends on one specific lysine out of numerous charged residues present on the toxin molecule (Park and Miller, 1992). We consider these to be unresolved issues, worthy of continuing debate and investigation.

What Are the Essential Properties of a Sodium Channel Pore?

Construction of elaborate kinetic models is of little use if they do not bring us closer to an understanding of the actual mechanism of ion conduction in the channel, either by raising testable questions, or by helping to identify fundamental characteristics. Thus, in concluding our discussion, we attempt to underline the distinction between essential properties of the ion conduction and block, per se, and the characteristics of the models. The former, we wish to understand; the latter are artifices invented in the hope that they will help us to refine our understanding. Sometimes it is difficult to distinguish between them. Nonetheless, we attempt to do so. We consider the following to be fundamental, and at least qualitatively testable, issues. Can more than one ion reside simultaneously in the channel? Are intrachannel binding sites, which are responsible for well-defined actions (e.g., block by externally applied Ca^{2+}), closer

to the the outer or inner entrance? Can a reduction in conductance be attributed solely, or in part, to ion binding within the channel, or to sites nearby, but external to the conduction path?

By contrast, other issues are not amenable to rigorous test. We cannot decide unambiguously, on the basis of available data, whether a model with two energy minima is a more realistic representation of the actual pore than one with three, especially given that the weight of evidence favors only a relatively rare occurrence of occupancy states with more than one ion present. Given that our data do not extend to extremely low ionic strengths (in all cases, $[\text{NaCl}] \geq 0.01 \text{ M}$), we cannot weigh with precision the relative contributions of fixed charges within, and those external to, but near, the pore (see Naranjo and Latorre [1993] and Moczydlowski [1993] for illuminating discussions of this issue). The number of constraints imposed, even by such a complex data set, is not adequate to define the increasing number of parameters in more complex models.

Nonetheless, the 3B2S, double-occupancy model provides the following essential characteristics seen in our own and others' experiments: (a) saturating conductance with increasing concentration (Fig. 2); (b) the possibility of coupling of one-way fluxes, including increasing ion exit rates by interionic repulsion (Fig. 4; also Begenisich and Busath, 1981; Busath and Begenisich, 1982); (c) the possibility of voltage-dependent block by poorly permeant ions entering the channel from either side (Figs. 3, 4, and 6); (d) the possibility to identify potential sites of channel modifications (Fig. 8).

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