

# Divalent Cation Selectivity for External Block of Voltage-dependent Na<sup>+</sup> Channels Prolonged by Batrachotoxin

## *Zn<sup>2+</sup> Induces Discrete Substates in Cardiac Na<sup>+</sup> Channels*

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**ABSTRACT** The mechanism of block of voltage-dependent Na<sup>+</sup> channels by extracellular divalent cations was investigated in a quantitative comparison of two distinct Na<sup>+</sup> channel subtypes incorporated into planar bilayers in the presence of batrachotoxin. External Ca<sup>2+</sup> and other divalent cations induced a fast voltage-dependent block observed as a reduction in unitary current for tetrodotoxin-insensitive Na<sup>+</sup> channels of rat skeletal muscle and tetrodotoxin-insensitive Na<sup>+</sup> channels of canine heart ventricular muscle. Using a simple model of voltage-dependent binding to a single site, these two distinct Na<sup>+</sup> channel subtypes exhibited virtually the same affinity and voltage dependence for fast block by Ca<sup>2+</sup> and a number of other divalent cations. This group of divalent cations exhibited an affinity sequence of Co ≈ Ni > Mn > Ca > Mg > Sr > Ba, following an inverse correlation between binding affinity and ionic radius. The voltage dependence of fast Ca<sup>2+</sup> block was essentially independent of CaCl<sub>2</sub> concentration; however, at constant voltage the Ca<sup>2+</sup> concentration dependence of fast block deviated from a Langmuir isotherm in the manner expected for an effect of negative surface charge. Titration curves for fast Ca<sup>2+</sup> block were fit to a simplified model based on a single Ca<sup>2+</sup> binding site and the Gouy-Chapman theory of surface charge. This model gave similar estimates of negative surface charge density in the vicinity of the Ca<sup>2+</sup> blocking site for muscle and heart Na<sup>+</sup> channels. In contrast to other divalent cations listed above, Cd<sup>2+</sup> and Zn<sup>2+</sup> are more potent blockers of heart Na<sup>+</sup> channels than muscle Na<sup>+</sup> channels. Cd<sup>2+</sup> induced a fast, voltage-dependent block in both Na<sup>+</sup> channel subtypes with a 46-fold higher affinity at 0 mV for heart ( $K_b = 0.37$

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mM) vs. muscle ( $K_b = 17$  mM).  $Zn^{2+}$  induced a fast, voltage-dependent block of muscle  $Na^+$  channels with low affinity ( $K_b = 7.5$  mM at 0 mV). In contrast, micromolar  $Zn^{2+}$  induced brief closures of heart  $Na^+$  channels that were resolved as discrete substate events at the single-channel level with an apparent blocking affinity of  $K_b = 0.067$  mM at 0 mV, or 110-fold higher affinity for  $Zn^{2+}$  compared with the muscle channel. High-affinity block of the heart channel by  $Cd^{2+}$  and  $Zn^{2+}$  exhibited approximately the same voltage dependence ( $e$ -fold per 60 mV) as low affinity block of the muscle subtype ( $e$ -fold per 54 mV), suggesting that the block occurs at structurally analogous sites in the two  $Na^+$  channels. These observations suggest that fast block of  $Na^+$  channels by external divalent cations may involve the production of very brief subconductance states.

#### INTRODUCTION

Previous studies of the interaction of divalent cations with ion channels have yielded important models of function. In the case of voltage-dependent  $Na^+$  channels, the depression of instantaneous macroscopic current and single-channel current by external  $Ca^{2+}$  has been interpreted as a voltage-dependent block due to occupation of a specific divalent cation binding site within the channel (Woodhull, 1973; Yamamoto et al., 1984; Sheets et al., 1987; Nilius, 1988). Such evidence for ion binding sites in the channel has been used to explain nonindependence of ion movement and to support permeation models based on Eyring rate theory.

The recognition of distinct  $Na^+$  channel subtypes with different sensitivities to toxins such as tetrodotoxin (TTX) and  $\mu$ -conotoxin (Trimmer and Agnew, 1989) has motivated functional comparisons to determine whether there might be analogous differences in divalent cation sensitivity. Patch clamp experiments have shown that external  $Ca^{2+}$  block results in an apparent reduction of unitary  $Na^+$  current with a similar voltage dependence and affinity for  $Ca^{2+}$  in TTX-sensitive neuroblastoma cells (Yamamoto et al., 1984) and in TTX-insensitive cardiac cells of mammals (Sheets et al., 1987; Nilius, 1988). On the other hand, the data of Weiss and Horn (1986) suggested that TTX-sensitive  $Na^+$  channels in rat myoblasts are more sensitive to external  $Ca^{2+}$  block than TTX-insensitive  $Na^+$  channels in the same cells. Other workers have also reported that certain TTX-insensitive  $Na^+$  channels are more sensitive to block by external  $Cd^{2+}$  and  $Zn^{2+}$  than TTX-sensitive  $Na^+$  channels (DiFrancesco et al., 1984; Frelin et al., 1986; Baumgarten and Fozzard, 1989).

Our laboratory has previously investigated the kinetic basis for differences in binding affinity of guanidinium toxins to rat muscle and canine heart  $Na^+$  channels that have been incorporated into planar bilayers in the presence of batrachotoxin (BTX), which greatly prolongs  $Na^+$  channel opening (Guo et al., 1987; Ravindran and Moczydlowski, 1989). This system facilitates the functional comparison of channel subtypes in the same lipid environment under a wide range of ionic conditions. Continuing this effort in the present paper, we compared the mechanism and specificity of external divalent cation block for a TTX-sensitive  $Na^+$  channel subtype (rat muscle) and a TTX-insensitive subtype (canine heart). Our first aim was to determine the ionic selectivity for block of the two subtypes by various external divalent cations under identical conditions. If the blocking site for divalent cations shares certain structural determinants with the TTX binding site, one might expect

to observe a different selectivity for divalent cations in the two subtypes. The second question concerned the effect of negative surface charge on Na<sup>+</sup> permeation and divalent cation block. Green et al. (1987a) previously interpreted the conductance–concentration relation for NaCl in canine brain Na<sup>+</sup> channels as evidence for a substantial enhancing effect of negative surface charge on permeation by increasing the local Na<sup>+</sup> concentration near the mouth of the channel. If external Ca<sup>2+</sup> enters the outer mouth of the channel and blocks within the pore, we reasoned that it should be possible to obtain independent evidence for this negative surface charge by analyzing the dependence of Ca<sup>2+</sup> blocking affinity on ionic strength. This dependence on ionic strength should be revealed by the Ca<sup>2+</sup> titration curve itself, since CaCl<sub>2</sub> is known to screen surface charge in a predictable fashion (McLaughlin et al., 1981). Third, we hoped to find an inorganic ion that would induce a discrete block of single Na<sup>+</sup> channels. Such inorganic blockers have served as useful probes of conductance mechanisms in a variety of ion channels: e.g., discrete or flickery block by Ba<sup>2+</sup> and Na<sup>+</sup> in a large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (Vergara and Latorre, 1983; Yellen, 1984); discrete Ca<sup>2+</sup> block of Li<sup>+</sup> current through an L-type Ca<sup>2+</sup> channel (Lansman et al., 1986); and discrete block by Na<sup>+</sup> and Cs<sup>+</sup> in an inwardly rectifying K<sup>+</sup> channel (Fukushima, 1982).

With respect to these objectives, we found that a large group of divalent cations (Mg, Ca, Sr, Ba, Mn, Co, and Ni) displayed virtually identical blocking behavior in the two Na<sup>+</sup> channel subtypes. We observed the expected effect of negative surface charge on the Ca<sup>2+</sup> blocking reaction, with an apparent surface charge density similar to that previously found for the TTX- and saxitoxin (STX)-binding reactions (Green et al., 1987b; Ravindran and Moczydlowski, 1989). We confirmed that the TTX-insensitive subtype is more sensitive to inhibition by Cd<sup>2+</sup> and Zn<sup>2+</sup>, and we also found that Zn<sup>2+</sup> induces a resolvable block in single Na<sup>+</sup> channels from canine heart. However, instead of a discrete block to a fully closed conductance level, Zn<sup>2+</sup> induces the appearance of discrete substate events in heart Na<sup>+</sup> channels. This paper on steady-state behavior of divalent cation block and a companion paper on kinetic aspects of Zn<sup>2+</sup>-induced subconductance events (Schild et al., 1991) focus on the mechanisms underlying this unusual behavior. Parts of this work have been presented in abstract form (Ravindran and Moczydlowski, 1988).

## METHODS

### *Incorporation of BTX-modified Na<sup>+</sup> Channels into Planar Bilayers*

Native plasma membrane vesicles were prepared from rat skeletal muscle and canine heart ventricular muscle as described previously (Guo et al., 1987). Final membrane pellets were resuspended at 1–5 mg protein/ml in 0.3 M sucrose buffer (10 mM MOPS-NaOH, pH 7.4, 0.2 mM EDTA, 3 mM NaN<sub>3</sub>) and stored in small aliquots at –80°C. BTX-modified Na<sup>+</sup> channels could be reliably incorporated from preparations stored in this manner over a period of several months. Canine hearts were obtained through the cooperation of the Department of Internal Medicine (Cardiology) at Yale University School of Medicine. Hearts were salvaged from animals euthanized in the course of experimental surgery according to the guidelines of the Yale Animal Care and Use Committee.

Planar bilayers were formed by spreading a solution of neutral phospholipids in decane (25 mg/ml) over a 200- $\mu$ m hole drilled in a polystyrene partition. The lipid solution in decane was

a mixture of 80% bovine brain phosphatidylethanolamine and 20% 1,2-diphytanoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL). A glass rod fashioned from a fire-polished capillary pipette was used to apply the lipid solution. Bilayer formation was monitored by the capacitance increase during thinning. The final bilayer capacitance ranged from 100 to 200 pF. Bilayers were formed in the presence of a symmetrical solution of 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4, and 0.2 mM EDTA, and experiments were carried out at 21–24°C. Na<sup>+</sup> channels were incorporated by addition of 5–50 µg/ml membrane protein and 0.2 µM BTX to one side of the bilayer.

Channel incorporation was monitored at ±50 mV with constant stirring and usually occurred within 30 min. An abrupt increase of ~1 pA current indicated incorporation of a single Na<sup>+</sup> channel. Channel orientation was initially determined by observing rapid voltage-dependent gating (Krueger et al., 1983) at +90 or –90 mV. Since BTX-modified Na<sup>+</sup> channels from both tissues exhibit a steep voltage dependence of activation in the range of –120 to –80 mV (physiological convention), the internal (intracellular) chamber was assigned to the side that resulted in channel closing with increasing negative voltage. Voltage signs are thus referred to the physiological convention (external [extracellular] = ground).

#### *Blocking Experiments with Inorganic Cations*

Aqueous solutions of the following chloride salts were used in the blocking experiments: MgCl<sub>2</sub>·6H<sub>2</sub>O, CaCl<sub>2</sub>·2H<sub>2</sub>O, SrCl<sub>2</sub>·6H<sub>2</sub>O, BaCl<sub>2</sub>·2H<sub>2</sub>O, MnCl<sub>2</sub>·4H<sub>2</sub>O, CoCl<sub>2</sub>·6H<sub>2</sub>O, NiCl<sub>2</sub>·6H<sub>2</sub>O, ZnCl<sub>2</sub>, CdCl<sub>2</sub>, and LaCl<sub>3</sub>·7H<sub>2</sub>O. The salts were reagent grade obtained from Fisher Scientific Co. (Pittsburgh, PA) or Alfa Products (Danvers, MA) except for the Ni, Zn, Cd, and La salts, which were Puratronic (Ni), Ultrapure (Zn, Cd) or REacton (La) grade from Alfa Products.

To facilitate precise measurements of unitary currents in the absence and presence of fast-blocking inorganic cations, guanidinium toxins were added to the external side of the bilayer to induce discrete blocking events. 20 nM decarbamoylsaxitoxin was used for the rat muscle Na<sup>+</sup> channel and 1 µM TTX was used for the canine heart channel. These toxin concentrations are near the respective K<sub>d</sub>'s at 0 mV and the chosen toxins have mean dwell times of ~2–4 s at 0 mV (Guo et al., 1987). In measurements of the current reduction due to fast block it was possible to use bilayers that contained up to three Na<sup>+</sup> channels oriented in the same direction. The use of the guanidinium toxins also provided unambiguous proof of channel orientation since these toxins only act on the extracellular side of Na<sup>+</sup> channels. A different protocol was required for analysis of Zn<sup>2+</sup> block of heart Na<sup>+</sup> channels since Zn<sup>2+</sup> itself induces discrete closing events. In this case, no guanidinium toxins were present and only single-channel bilayers were used for the analysis of open-state probability.

In all blocking experiments with divalent cations such as Ca<sup>2+</sup> that induce a lower unitary current (fast block), 0.2 mM EDTA was included in the bilayer buffer to chelate any contaminating divalent cations and ensure that control *I-V* curves were taken in the virtual absence of divalent cation block. A typical fast-block experiment on a given bilayer involved recording unitary *I-V* data for the control condition (symmetrical 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4, 0.2 mM EDTA) and for several increasing concentrations of divalent cation added to the external chamber by addition from a stock solution until the bilayer broke or the experiment was otherwise terminated. Free concentrations of divalent cations were calculated by subtraction of 0.2 mM from the total concentration to correct for chelation by 0.2 mM EDTA. This approximation is valid because the stability constants for EDTA-Me<sup>2+</sup> formation are > 10<sup>7</sup> M<sup>-1</sup> for the divalent cations studied (Skoog and West, 1969). Since most of the fast blocking divalent ions act with low affinity (K<sub>b</sub> > 10 mM), this 0.2 mM correction is only significant for the lowest concentrations of added divalent cations (1–5 mM). The release of protons that occurs upon divalent cation complexation with EDTA lowered the pH of the bilayer buffer (10 mM MOPS-NaOH, pH 7.4) by <0.1 pH units. This pH change has an

insignificant effect on the unitary conductance of the Na<sup>+</sup> channels under investigation. Since micromolar concentrations of Zn<sup>2+</sup> block the heart Na<sup>+</sup> channel, EDTA was omitted completely in these experiments and reported Zn<sup>2+</sup> concentrations are based on the total amount of Zn<sup>2+</sup> added.

#### *Data Acquisition and Analysis*

Three different amplifiers were used to measure single-channel currents, depending on availability and application. For fast-block experiments, a simple home-built amplifier with a frequency compensation circuit similar to patch clamp design was used as described previously (Guo et al., 1987). For blocking experiments with cardiac Na<sup>+</sup> channels and Zn<sup>2+</sup>, a Yale patch clamp (Yale Physiology Department Electronics Shop) or a List EPC-7 patch clamp (Medical Systems, Inc., Greenvale, NY) was used for improved resolution of Zn<sup>2+</sup>-induced fluctuations. Single-channel data were recorded on FM or VCR tape for subsequent analysis.

The amplifier headstage was connected with two Ag/AgCl<sub>2</sub> wire electrodes to two separate wells containing 1 M KCl. These wells were connected to the two bilayer chambers with small U-shaped bridges formed from capillary pipettes that were filled with 2% agar in 0.2 M KCl, 1 mM EDTA.

Electrode asymmetry and junction potentials were monitored by direct measurement before bilayer formation and by single-channel *I-V* curves taken in the presence of symmetrical control buffer (0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4, 0.2 mM EDTA) for each experiment. Since the control *I-V* curve of BTX-modified Na<sup>+</sup> channels is approximately linear in the voltage range of ±80 mV in the absence of divalent cations (Figs. 1 *B* and 2 *B*), the reversal potential of the control *I-V* curve can be conveniently used to correct for net junction potential by subtraction from the applied voltage. Such corrections were always <5 mV in these experiments. With this method of correcting the junction potential, none of the tested divalent cations appeared to measurably permeate BTX-modified Na<sup>+</sup> channels under the conditions of our experiments, since there were no obvious shifts (>2 mV) from the control reversal potential when divalent cations salts were added to the external chamber.

Analysis of fast-block experiments involved construction of *I-V* curves from unitary currents in the range of ±80 mV at 10-mV intervals. Single-channel currents were measured from data filtered at 100 Hz (-3 db corner frequency; low-pass, eight-pole Bessel filter) and recorded on a strip chart recorder (4500 Microscribe; Houston Instrument, Austin, TX) at 10 or 20 cm/min. Measurement of unitary currents from records such as those shown in Figs. 1 *A* and 2 *A* was facilitated with the use of a TG1017 digitizing tablet (Houston Instrument). At least five unitary current transitions between closed (toxin blocked) and open levels at each voltage and divalent cation concentration were measured to obtain a mean current value. The standard deviation of such measurements was typically <2%. In the analyses of voltage dependence (e.g., Figs. 1 *C* and 2 *C*) or concentration dependence (e.g., Figs. 7, *A* and *B*, and 8 *A*) of fast block, the relative current reduction,  $I/I_0$ , was measured with respect to a control current,  $I_0$ , in the absence of divalent cations for every bilayer.

Equilibrium analysis of discrete block of heart Na<sup>+</sup> channels by Zn<sup>2+</sup> involved measurement of the time-averaged probability of being unblocked,  $P_{un}$ .  $P_{un}$  was calculated as the ratio of the open-state probability in the presence of Zn<sup>2+</sup> to that in the absence of Zn<sup>2+</sup>. Open-state probability was calculated from a 1–2-min segment of digitized single-channel data using a 50% threshold criterion to distinguish open from substate and closed events. Single-channel records were digitized at a sampling rate of 1 or 2 kHz for data filtered at 100 Hz. Digitization and computation of open-state probability was performed with an Indec Systems LSI 1173 computer (Sunnyvale, CA) or an Atari computer system (Instrutech, Elmont, NY).

The fitting of data to theoretical functions was performed by linear regression analysis in cases where functions could be linearized with respect to one independent variable, such as Eq.

5 and the Hill plot analysis of Fig. 8 *B*. Such linear regression fits generally resulted in correlation coefficients  $>0.99$ . Fitting of fast-block titration curves for  $\text{CaCl}_2$  (Fig. 7, *A* and *B*) and  $\text{ZnCl}_2$  (Fig. 8 *A*) to Eqs. 6–8 is a problem that involves finding values of two independent parameters,  $\sigma$  and  $K_d$ , that provide the best fit of titration data (observed  $I/I_0$  values vs. bulk  $[\text{CaCl}_2]$ ). This nonlinear fit was performed by an iterative, least-squares method similar to that described by Ravindran and Moczydlowski (1989). Since the fast-block titration curves at  $-50$  mV cover the most complete range of current inhibition (10–80% inhibition), data points at this voltage were first fit to obtain values of  $\sigma$  and  $K_d$  ( $-50$  mV) that resulted in a minimum value of a chi-squared parameter indicating goodness-of-fit (Ravindran and Moczydlowski, 1989). The best-fit value of  $\sigma$  obtained at  $-50$  mV was then used as a constant in fitting the respective data points at  $+50$  mV to obtain a best-fit value of  $K_d$  ( $+50$ ).

## RESULTS

### *Voltage-dependent Block of Muscle and Heart $\text{Na}^+$ Channels by External Divalent Cations*

In squid axons perfused internally with CsF and bathed in  $\text{CaCl}_2$ , Meves and Vogel (1973) detected small, TTX-sensitive, inward currents attributed to permeation of  $\text{Ca}^{2+}$  through voltage-dependent  $\text{Na}^+$  channels. In contrast, cation selectivity studies of  $\text{Na}^+$  channels in frog muscle fibers failed to detect any inward  $\text{Ca}^{2+}$  current under similar experimental conditions (Campbell, 1976). From such studies, Campbell (1976) estimated an upper limit of the  $\text{Ca}^{2+}/\text{Na}^+$  permeability ratio of  $P_{\text{Ca}}/P_{\text{Na}} < 0.093$  in frog muscle; however, the absence of inward current suggested that the rate of  $\text{Ca}^{2+}$  permeation is so low that  $\text{Ca}^{2+}$  is often described as a blocker of  $\text{Na}^+$  current rather than as a permeant ion. To account for such blocking behavior, Woodhull (1973) proposed a model involving binding of a blocking ion to a site located in the transmembrane electric field. This model was shown to simulate the voltage-dependent inhibition of  $\text{Na}^+$  currents in frog nerve by external  $\text{H}^+$  and  $\text{Ca}^{2+}$  (Woodhull, 1973).

More recent patch clamp studies on single  $\text{Na}^+$  channels in cell membranes have shown that external  $\text{Ca}^{2+}$  causes a reduction in unitary  $\text{Na}^+$  current that is enhanced by hyperpolarization (Yamamoto et al., 1984; Weiss and Horn, 1986; Sheets et al., 1987; Nilius, 1988). A similar effect of external  $\text{Ca}^{2+}$  and other divalent cations such as  $\text{Zn}^{2+}$  has also been observed for BTX-modified  $\text{Na}^+$  channels in planar bilayers (Krueger et al., 1986; Moczydlowski et al., 1986; Worley et al., 1986; Green et al., 1987a; Behrens et al., 1989). Such a reduction in unitary current by an impermeant or slightly permeant ion is often described as a “fast block” (Hille, 1984).

To provide an interpretation of external  $\text{Ca}^{2+}$  block based on the energetics of ion permeation, several laboratories have used an Eyring barrier model that includes four barriers and three binding sites with single-ion occupancy (Yamamoto et al., 1984; Sheets et al., 1987; Nilius, 1988). Following Hille's analysis (Hille, 1975), these workers showed that  $\text{Na}^+$  permeation could be described by a nearly symmetric energy profile for  $\text{Na}^+$ , and the blocking effect of  $\text{Ca}^{2+}$  could be described by an asymmetrical barrier profile with a high barrier for inward  $\text{Ca}^{2+}$  movement from a site located at an electrical distance of 0.31–0.39 from the outside of the membrane. This barrier model predicts an undetectable current of  $\sim 10^{-3}$  pA for  $\text{Ca}^{2+}$  through the  $\text{Na}^+$  channel (Nilius, 1988), which is consistent with the observation that no  $\text{Na}^+$

channel current is observed by patch clamp recording when Na<sup>+</sup> is completely replaced by Ca<sup>2+</sup> (Yamamoto et al., 1984). The success of both the Woodhull blocking model and Eyring permeation models in describing Ca<sup>2+</sup> block is expected, since these two mechanisms become essentially indistinguishable in the low permeation limit of the occluding ion (i.e., an infinitely high internal barrier).

To further probe the mechanism and possible subtype differences of Na<sup>+</sup> channel block by external divalent cations, we studied the effect of a series of external divalent

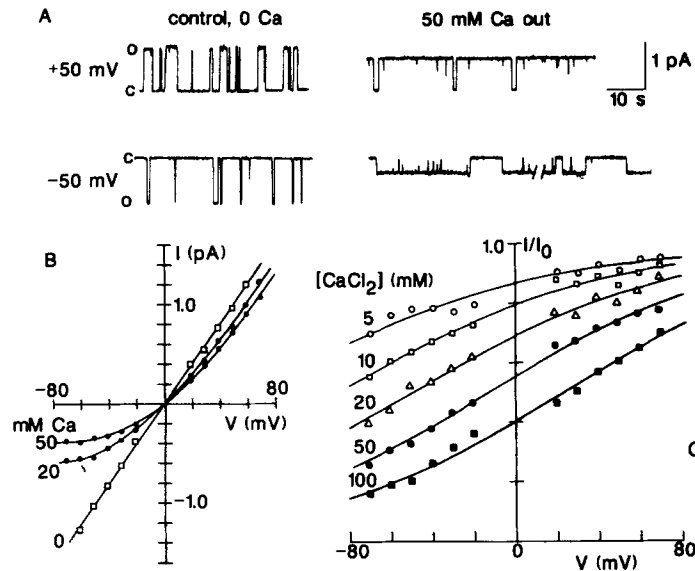


FIGURE 1. Effect of external Ca<sup>2+</sup> on single BTX-modified Na<sup>+</sup> channels from rat skeletal muscle. (A) Current fluctuations at  $\pm 50$  mV of a rat muscle Na<sup>+</sup> channel recorded in the presence of 0.2 M symmetrical NaCl, 10 mM MOPS-NaOH, pH 7.4, 0.2 mM EDTA, 20 nM external decarbamoylsaxitoxin before (left) and after addition of 50 mM external CaCl<sub>2</sub> (right). In the record at the lower right, 43 s of a long opening event were omitted at the slash marks. c, closed current level; o, open current level. (B) *I-V* relations of a single rat muscle Na<sup>+</sup> channel measured as in A with 0, 20, or 50 mM external CaCl<sub>2</sub> as indicated. (C) Ratio of unitary current in the presence of Ca<sup>2+</sup>, *I*, to that measured for the same channel in the absence of Ca<sup>2+</sup>, *I*<sub>0</sub>, plotted as a function of voltage and [CaCl<sub>2</sub>]. Solid lines in C were drawn according to Eq. 4 using best-fit values of *K<sub>b</sub>(0)* and *z'* determined by linear regression (Eq. 5). Mean *K<sub>b</sub>(0)* and *z'* parameters for such fits are listed in Table I.

cations on BTX-modified Na<sup>+</sup> channels from muscle and heart in planar lipid bilayers. We will first illustrate our methods of analysis and general findings with a comparison of the effect of Ca<sup>2+</sup> on both Na<sup>+</sup> channel subtypes.

Fig. 1A shows control records of a single BTX-modified Na<sup>+</sup> channel from rat muscle at  $\pm 50$  mV in the presence of 0.2 M symmetrical NaCl and 0.2 mM EDTA. After addition of 50 mM CaCl<sub>2</sub> to the external chamber, unitary currents are reduced by 27% at +50 mV and by 66% at -50 mV. Fast block by external Ca<sup>2+</sup> thus has the

effect of converting a nearly ohmic  $I$ - $V$  behavior to an outward rectifying  $I$ - $V$  behavior as summarized by the single-channel  $I$ - $V$  curves of Fig. 1 *B* in the presence of 0, 20, or 50 mM  $\text{CaCl}_2$ . In addition, these experiments did not provide evidence of  $\text{Ca}^{2+}$  permeation under these conditions, since the 0 mV reversal potential did not significantly shift toward positive voltages in the presence of symmetrical 0.2 M NaCl with 20 or 50 mM external  $\text{CaCl}_2$  (Figs. 1 *B* and 2 *B*) or 100 mM external  $\text{CaCl}_2$  (not shown).

To provide a quantitative but phenomenological description of the fast blocking effect of  $\text{Ca}^{2+}$  and other divalent cations, we adopted the simple approach of Woodhull (1973). This model was used to obtain empirical parameters for the voltage dependence and affinity of the blocking ion that can be compared for various divalent cations and channel subtypes. We favor this approach for the purpose of the present study because Eyring permeation models involve many more adjustable parameters and have the uncertainty of whether single-ion or multi-ion occupancy (Begenisich and Cahalan, 1980) is applicable.

If the blocking action of  $\text{Ca}^{2+}$  is due to occupation of a single binding site, then the ratio,  $I/I_0$ , of unitary current measured in the presence of blocker,  $I$ , to that measured in the absence of blocker,  $I_0$ , is equal to the probability that the site is unblocked,  $P_{\text{un}}$ . This latter function should ideally be described by Eq. 1 (Eq. 3 of Woodhull [1973]), where  $K_{\text{B}}(V)$  is the voltage-dependent equilibrium dissociation constant for the blocker and  $[B]$  is the blocker concentration:

$$P_{\text{un}} = I/I_0 = K_{\text{B}}(V)/([B] + K_{\text{B}}(V)) \quad (1)$$

Eq. 1 is simply an inverted form of the well-known Langmuir saturation isotherm for ligand binding to a single site. Woodhull (1973) expressed  $K_{\text{B}}(V)$  as a Boltzmann relationship with respect to voltage as summarized by Eq. 2, where  $K_{\text{B}}(0)$  is the equilibrium dissociation constant at 0 mV,  $z'$  is a slope parameter,  $F$  is Faraday's constant,  $V$  is the applied voltage,  $R$  is the gas constant, and  $T$  is absolute temperature:

$$K_{\text{B}}(V) = K_{\text{B}}(0) \exp(z'FV/RT) \quad (2)$$

In Woodhull's formulation, the slope parameter,  $z'$ , is equal to the product of the actual valence of the blocking ion,  $z$ , and the fraction of membrane potential (or electrical distance),  $\delta$ , acting on an ion in the site:

$$z' = z\delta \quad (3)$$

Following the treatment of Woodhull (1973) and that of others adapted for single channels (e.g., Coronado and Miller, 1982; Green et al., 1987a), Eq. 4 below is a combined form of Eqs. 1 and 2 and Eq. 5 is a log-linearized form of Eq. 4 which is often used to fit the voltage-dependent reduction of unitary current by linear regression analysis:

$$P_{\text{un}} = I/I_0 = K_{\text{B}}(0) \exp(z'FV/RT)/([B] + K_{\text{B}}(0) \exp(z'FV/RT)) \quad (4)$$

$$\ln \{(I_0 - I)/I\} = \ln \{[B]/K_{\text{B}}(0)\} - z'FV/RT \quad (5)$$

Fig. 1 *C* compares theoretical curves based on Eq. 4 using best-fit values of  $K_{\text{B}}(0)$  and



$z'$  with the measured  $I/I_0$  ratios at various voltages and  $\text{CaCl}_2$  concentrations. The fitted curves of Fig. 1 C show that Eq. 4 provides an adequate description of the voltage dependence of external  $\text{Ca}^{2+}$  block at fixed  $\text{Ca}^{2+}$  concentrations ranging from 5 to 100 mM in the voltage range of  $\pm 70$  mV.

To compile a quantitative comparison of blocking-ion selectivity for a TTX-sensitive  $\text{Na}^+$  channel (rat muscle) and a TTX-insensitive  $\text{Na}^+$  channel (dog heart), experiments similar to those of Fig. 1 were performed with various divalent cations for the two channel subtypes. Fig. 2 summarizes an analysis of  $\text{Ca}^{2+}$  block of the heart  $\text{Na}^+$  channel, which may be directly compared with the data of Fig. 1 for the muscle  $\text{Na}^+$  channel. In the absence of  $\text{Ca}^{2+}$ , we found that the heart channel has a slightly larger single-channel conductance,  $\gamma$ , than the muscle channel in the presence of

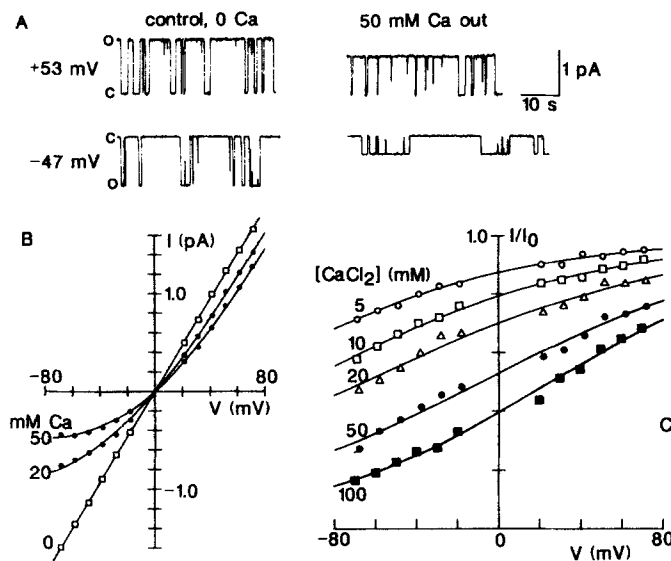


FIGURE 2. Effect of external  $\text{Ca}^{2+}$  on single BTX-modified  $\text{Na}^+$  channels from canine heart. Conditions and descriptions are similar to those presented in Fig. 1, except that canine heart  $\text{Na}^+$  channels were recorded in the presence of  $1 \mu\text{M}$  TTX.

symmetrical 0.2 M NaCl and 0.2 mM EDTA: for muscle  $\gamma = 19.7 \pm 1.2$  pS ( $\pm\text{SD}$ ,  $n = 14$ ) and for heart,  $\gamma = 23.2 \pm 1.1$  pS ( $\pm\text{SD}$ ,  $n = 11$ ). Aside from this difference and the previously documented differences in affinity for guanidinium toxins (Guo et al., 1987), the two subtypes displayed striking similarity in the quantitative aspects of fast  $\text{Ca}^{2+}$  block.

Table I lists results of fitting the observed current reduction to Eq. 5 in the presence of various  $\text{Ca}^{2+}$  concentrations ranging from 5 to 100 mM. At any given  $\text{Ca}^{2+}$  concentration the apparent  $K_B(0)$  and  $z'$  for  $\text{Ca}^{2+}$  binding to the blocking site are quite similar for the two channel subtypes. However, for both subtypes we find that the apparent  $K_B(0)$  obtained by this method increases as a function of the  $\text{Ca}^{2+}$  concentration used in the blocking experiment; e.g.,  $K_B(0) = 33 \pm 3$  mM at 5 mM

$\text{CaCl}_2$  and  $K_b(0) = 70 \pm 5$  mM at 100 mM  $\text{CaCl}_2$  for the muscle channel. This latter behavior indicates that external  $\text{Ca}^{2+}$  block does not obey the simple Langmuir concentration dependence of Eq. 1 based on  $\text{CaCl}_2$  concentration in the bulk solution. In contrast, the slope parameter,  $z'$ , is clearly independent of  $\text{CaCl}_2$  concentration for both channel subtypes (Table I).

TABLE I  
*Kinetic Parameters for Fast Block by Inorganic Cations*

Cation	Concentration	Rat skeletal muscle		Canine heart	
		$K_b(0)$	$z'$	$K_b(0)$	$z'$
	<i>mM</i>	<i>mM</i>		<i>mM</i>	
$\text{Mg}^{2+}$	10	47 ± 4	0.37 ± 0.01	50 ± 6	0.34 ± 0.02
	20	59 ± 5	0.41 ± 0.04	59 ± 5	0.33 ± 0.02
	40	63	0.38	71 ± 3	0.33 ± 0.02
$\text{Ca}^{2+}$	5	33 ± 3	0.42 ± 0.06	34 ± 5	0.37 ± 0.08
	10	39 ± 3	0.42 ± 0.03	40 ± 5	0.34 ± 0.03
	20	46 ± 5	0.42 ± 0.03	47 ± 4	0.34 ± 0.03
	50	59 ± 4	0.44 ± 0.01	59 ± 5	0.38 ± 0.05
	100	70 ± 5	0.43 ± 0.01	67 ± 2	0.40 ± 0.04
$\text{Sr}^{2+}$	10	70 ± 12	0.35 ± 0.07	73 ± 12	0.31 ± 0.05
	20	90 ± 14	0.38 ± 0.05	94 ± 14	0.31 ± 0.02
$\text{Ba}^{2+}$	10	87 ± 7	0.26 ± 0.02	75 ± 9	0.30 ± 0.01
	20	110 ± 6	0.31 ± 0.02	92 ± 9	0.31 ± 0.02
	40	132 ± 1	0.31 ± 0.02	104 ± 6	0.30 ± 0.02
$\text{Mn}^{2+}$	10	18 ± 1	0.50 ± 0.02	19 ± 0.4	0.42 ± 0.04
$\text{Co}^{2+}$	10	13 ± 1	0.51 ± 0.03	14 ± 1	0.45 ± 0.03
$\text{Ni}^{2+}$	10	15 ± 0.03	0.50 ± 0.01	13 ± 1	0.46 ± 0.01
$\text{Zn}^{2+}$	1	7.5 ± 0.3	0.47 ± 0.04	*	*
	2	8.2 ± 1.3	0.50 ± 0.06	*	*
	5	9.0 ± 0.6	0.52 ± 0.06	*	*
	10	11 ± 1	0.56 ± 0.01	*	*
$\text{Cd}^{2+}$	5	17 ± 2	0.48 ± 0.03	*	*
$\text{La}^{3+}$	2	6.8 ± 0.5	0.45 ± 0.04	4.1 ± 0.4	0.22 ± 0.01
	5	9.2 ± 0.3	0.49 ± 0.04	6.8 ± 0.9	0.29 ± 0.03
	10	12 ± 0.02	0.53 ± 0.02	11 ± 0.4	0.27 ± 0.01

Values are mean ± SD as determined from three to eight bilayers. Divalent cation concentrations listed are total concentrations added to bilayer buffer containing 0.2 mM EDTA. Unchelated concentrations are calculated by subtraction of 0.2 mM from total.

\*Parameters for high affinity block of the heart channel by  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  are given in the text.

If the departure from Langmuir behavior were due to occupation of more than one  $\text{Ca}^{2+}$  blocking site, one might expect that  $z'$  would also increase with  $\text{Ca}^{2+}$  concentration, as observed for certain cases of  $\text{Cs}^+$  block in  $\text{K}^+$  channels that behave as multi-ion pores (Adelman and French, 1978; Cecchi et al., 1987). Since this is not the case, one might alternatively propose that Langmuir behavior is not observed in the

range of 5–100 mM CaCl<sub>2</sub> simply due to the change in ionic strength under these conditions. This idea is pursued in a later section of this paper, where the Ca<sup>2+</sup> concentration dependence of block is fit to a simple model of surface charge in the vicinity of the Ca<sup>2+</sup> blocking site.

Table I also lists the results of similar fast-block experiments for several other divalent cations besides Ca<sup>2+</sup> and for a trivalent cation, La<sup>3+</sup>. All of the tested divalent cations induced a fast block similar to that of Ca<sup>2+</sup> shown in Figs. 1 and 2, except in the case of Zn<sup>2+</sup> block of the heart Na<sup>+</sup> channel, which is described later in more detail. In every case where divalent ion concentration was varied, we observed that  $K_B(0)$  increased with blocker concentration and  $z'$  remained essentially constant

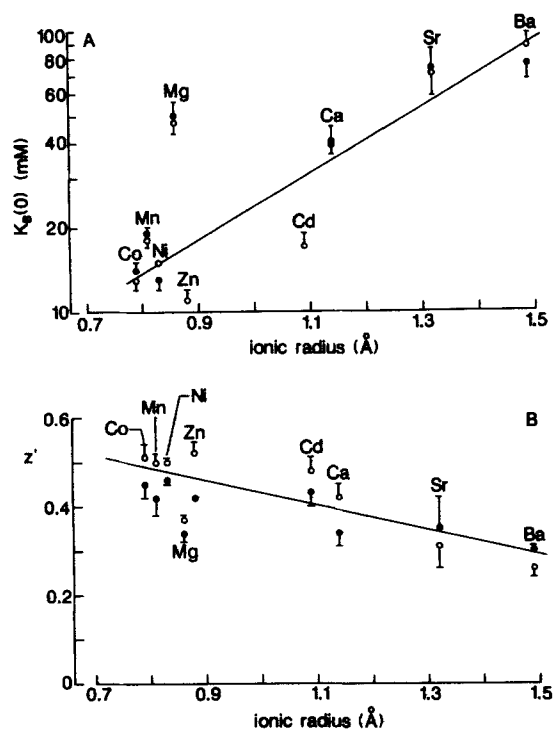


FIGURE 3. Apparent blocking affinity and voltage slope parameter of various divalent cations for muscle and heart Na<sup>+</sup> channels. The zero-voltage dissociation constant ( $K_B(0)$ ; A) and the slope parameter ( $z'$ ; B) were determined at 10-mM concentrations of chloride salts of Mg, Ca, Sr, Ba, Mn, Co, and Ni for rat muscle (○) and canine heart (●).  $K_B(0)$  and  $z'$  values for Zn<sup>2+</sup> and Cd<sup>2+</sup> were determined at lower concentrations of these divalent cations as discussed in the text. Points and error bars refer to the mean  $\pm$  SD of data from three to eight bilayers. The data are plotted vs. ionic radii for the various divalent cations, based on crystallographic data assuming a coordination number of 6 for each of the metals (Shannon, 1976).

(Table I). Thus, this behavior is a general characteristic of external divalent cation block for heart and muscle Na<sup>+</sup> channels. By comparing  $K_B(0)$  values at fixed conditions of 0.2 M NaCl and 10 mM XCl<sub>2</sub>, one can obtain an indication of the relative blocking selectivity for different divalent cations at the same ionic strength. These data are summarized in Fig. 3, where values of  $K_B(0)$  and  $z'$  for muscle and heart subtypes are plotted as a function of ionic radius of the blocking ion.

The data of Fig. 3 indicate that the  $K_B(0)$  and  $z'$  parameters for external block by Mg<sup>2+</sup>, Ca<sup>2+</sup>, Sr<sup>2+</sup>, Ba<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, and Ni<sup>2+</sup> are practically indistinguishable for muscle vs. heart Na<sup>+</sup> channels. Fig. 3 also shows a positive correlation between  $K_B(0)$  and ionic radius of these ions. Neglecting Mg<sup>2+</sup>, which behaves anomalously, we

obtain a correlation coefficient of  $r = 0.94$  for the logarithm of  $K_b(0)$  vs. ionic radius. If we assume that these particular cations induce block by binding to the same site, then ion selectivity theory (Eisenman and Horn, 1983) suggests that the blocking site is a high field-strength binding site, where a strong electrostatic interaction between a small ion and a small anionic site predominates over ion dehydration energy in determining selectivity.

However, the  $z'$  data in Table I and Fig. 3 B indicate an anomaly with respect to the Woodhull (1973) interpretation of the slope parameter,  $z'$ , for voltage-dependent binding to a site in an electric field (Eq. 3). Since the blockers in Fig. 3 B are all divalent cations ( $z = +2$ ), they should have the same  $\delta$  and  $z'$  if they bind at the same site. Instead, we observe that the divalent cation with the smallest ionic radius and highest affinity,  $\text{Co}^{2+}$  ( $\delta = 0.23$  heart; 0.26, muscle), exhibits a significantly larger  $\delta$  than the largest tested ion,  $\text{Ba}^{2+}$  ( $\delta = 0.15$ , heart; 0.13, muscle). The data of Fig. 3 B indicate that there is actually a negative correlation between the absolute value of  $z'$  and ionic radius (correlation coefficient,  $r = -0.87$ , neglecting  $\text{Mg}^{2+}$ ). Also, the  $z'$  values for  $\text{La}^{3+}$  block in Table I imply that  $\delta$  for  $\text{La}^{3+}$  lies in the range of 0.15–0.18 for muscle and 0.07–0.10 for heart. These  $\delta$  values for  $\text{La}^{3+}$  are less than or equal to those of the various divalent cations. In contrast, the Woodhull mechanism predicts that  $z'$  for  $\text{La}^{3+}$  should be 1.5-fold greater than that of  $\text{Ca}^{2+}$  or other divalent ions binding in the same site. In terms of a physical interpretation, these observations imply that the smallest ions, such as  $\text{Co}^{2+}$ , enter the channel more deeply, about twice the electrical distance of the largest ion,  $\text{Ba}^{2+}$ . In other words, the location of the binding site varies for the different metal ions. This result may seem at odds with the usual picture of fixed wells or locations of ion binding sites within channels; however, on a molecular scale, the distances underlying the  $z'$  measurements could be quite small. For example, if 100% of the field were to fall across a distance of 10 Å, as proposed for a sarcoplasmic reticulum  $\text{K}^+$  channel (Miller, 1982), then the differences in  $\delta$  observed for  $\text{Co}^{2+}$  and  $\text{Ba}^{2+}$  would be on the order of only 1 Å, assuming a linear potential drop.

#### *Differential Sensitivity to $\text{Ca}^{2+}$ and $\text{Zn}^{2+}$ in Muscle vs. Heart $\text{Na}^+$ Channels*

Frelin et al. (1986) previously reported that TTX-insensitive  $\text{Na}^+$  channels were ~25-fold more sensitive to  $\text{Cd}^{2+}$  and 40-fold more sensitive to  $\text{Zn}^{2+}$  than TTX-sensitive  $\text{Na}^+$  channels as measured by  $^{22}\text{Na}^+$  flux assays in cultured cells. The single-channel data of Figs. 4–6 confirm a similar differential sensitivity of  $\text{Na}^+$  channel subtypes to these particular metal ions and reveal a novel type of blocking mechanism in the case of  $\text{Zn}^{2+}$  inhibition of the heart channel.

The current records of Fig. 4 illustrate the effect of external  $\text{Cd}^{2+}$  on single muscle and heart  $\text{Na}^+$  channels at  $\pm 50$  mV.  $\text{Cd}^{2+}$  reduced the unitary current of both subtypes in the fashion of a fast block, but 40-fold lower concentrations were effective for the heart channel, relative to the muscle channel. We also observed that the open state of the heart channel exhibited more current noise in the presence of  $\text{Cd}^{2+}$  than in control records; however, discrete blocking events were not resolvable at 200-Hz filtering. Eq. 5 was used to fit the voltage-dependent current reduction induced by 4.8 mM  $\text{Cd}^{2+}$  for the muscle channel and that induced by 0.12 mM  $\text{Cd}^{2+}$  for the heart channel. This analysis showed that  $\text{Cd}^{2+}$  block has nearly the same voltage depen-

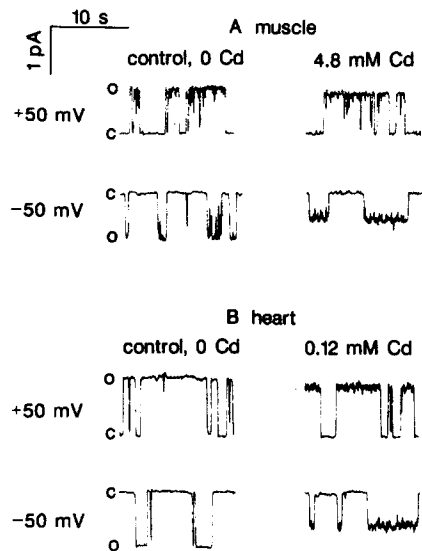


FIGURE 4. Differential sensitivity of muscle and heart Na<sup>+</sup> channels to Cd<sup>2+</sup>. (A) Rat muscle Na<sup>+</sup> channel. (B) Canine heart Na<sup>+</sup> channel. In records labeled "control," current fluctuations of a single BTX-modified Na<sup>+</sup> channel were recorded at  $\pm 50$  mV in the presence of symmetrical 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4, 0.2 mM EDTA, and external guanidinium toxins as described in Methods. 5 mM (A) or 0.32 mM (B) total CdCl<sub>2</sub> was added to the external side to give final free concentrations of 4.8 mM Cd<sup>2+</sup> (A) or 0.12 mM Cd<sup>2+</sup> (B) after taking chelation by EDTA into account.

dence for the two subtypes ( $z' = 0.48 \pm 0.03$  muscle;  $z' = 0.43 \pm 0.03$ , heart,  $n = 3$ ), but the heart channel has 46-fold greater affinity as measured by  $K_B(0)$  (muscle:  $K_B(0) = 17 \pm 2$  mM; heart:  $K_B(0) = 0.37 \pm 0.02$  mM,  $n = 3$ ).

A similar comparison of the effect of external Zn<sup>2+</sup> on the two channel subtypes is shown in Figs. 5 and 6. Millimolar concentrations of Zn<sup>2+</sup> produce a fast block of the

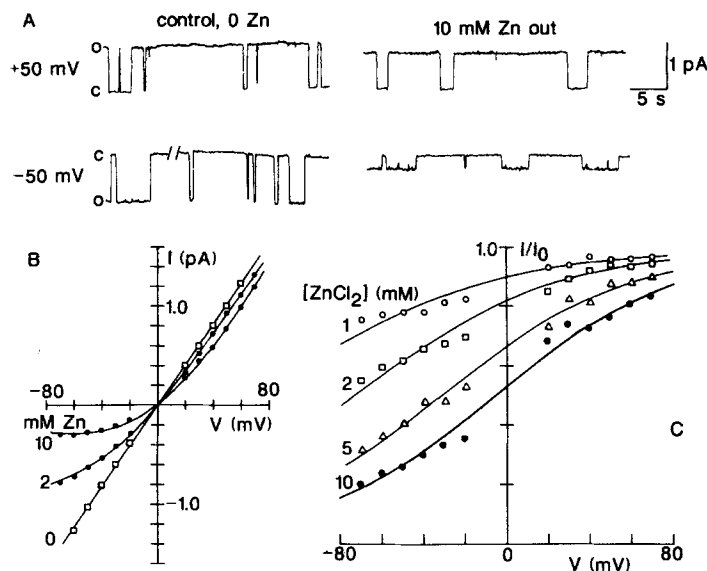


FIGURE 5. Effect of external Zn<sup>2+</sup> on BTX-modified Na<sup>+</sup> channels from rat skeletal muscle. Conditions and descriptions are similar to those of Fig. 1, except that external Zn<sup>2+</sup> was the divalent cation tested at the indicated concentrations.

muscle channel with approximately the same  $z'$  and  $K_B(0)$  as observed for ions of similar size such as  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ni}^{2+}$  (Figs. 3 and 5, Table I). In contrast,  $\text{Zn}^{2+}$  concentrations as low as  $5 \mu\text{M}$  produce brief, resolvable interruptions in the unitary current of heart channels at  $-70 \text{ mV}$ . Fig. 6 shows current records from an experiment where a single heart channel was recorded at  $\pm 70 \text{ mV}$  in the absence and presence of  $\text{Zn}^{2+}$  concentrations ranging from 10 to  $640 \mu\text{M}$ . This experiment was performed in the absence of any guanidinium toxins, and the closing events observed in the control records are normal closings associated with channel gating under these conditions. It can be seen that the frequency of the  $\text{Zn}^{2+}$ -induced flickering events increases as a function of  $\text{Zn}^{2+}$  concentration. In addition, higher concentrations of  $\text{Zn}^{2+}$  are required to produce an equivalent effect at  $+70 \text{ mV}$  compared with  $-70$

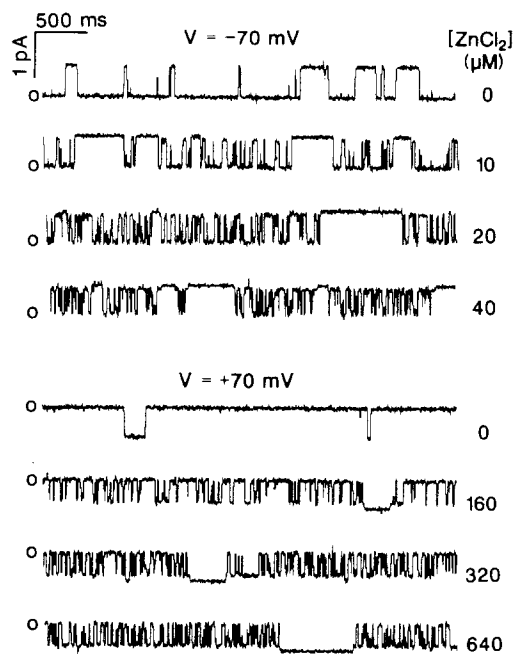


FIGURE 6. Discrete blocking events induced by external  $\text{Zn}^{2+}$  in a BTX-modified  $\text{Na}^+$  channel from canine heart. Current fluctuations of a single heart  $\text{Na}^+$  channel were recorded at  $-70 \text{ mV}$  and  $+70 \text{ mV}$  in the presence of symmetrical  $0.2 \text{ M NaCl}$ ,  $10 \text{ mM MOPS-NaOH}$ ,  $\text{pH } 7.4$ , and the indicated final concentrations of external  $\text{ZnCl}_2$ .  $o$ , open current level.

$\text{mV}$ . Careful inspection of these records also indicates that the  $\text{Zn}^{2+}$ -induced flickering events do not close to the fully closed current level defined by normal closings in the absence of  $\text{Zn}^{2+}$ . This is particularly evident for the record at  $+70 \text{ mV}$  and  $640 \mu\text{M Zn}^{2+}$ , where a single well-resolved closing event separates two bursting periods of  $\text{Zn}^{2+}$  substate activity. Current amplitude histograms of such data indicate that resolved  $\text{Zn}^{2+}$  flickers close to  $\sim 23\%$  of the full open-state current at  $+70 \text{ mV}$  (Schild et al., 1991). Thus, the mechanism of  $\text{Zn}^{2+}$  block in the heart  $\text{Na}^+$  channel involves conversion of the channel to a low conductance conformation or a substate, as opposed to complete occlusion of the channel by  $\text{Zn}^{2+}$ .

*Ca<sup>2+</sup> Titration Curves Suggest the Presence of Negative Surface Charge Near the Divalent Cation Blocking Site*

Studies of Green et al. (1987a) previously suggested that external divalent cations such as Ca<sup>2+</sup> and Zn<sup>2+</sup> decrease unitary currents of Na<sup>+</sup> channels by two mechanisms: (a) screening of fixed negative charges at the channel entrance, and (b) voltage-dependent block by binding to a distinct site in the pore (i.e., the Woodhull blocking mechanism). If, in their blocking action, divalent cations bind to a site that is influenced by local negative surface charges, one would predict that the titration curve for current inhibition by divalent cations Ca<sup>2+</sup> should reflect this surface charge effect. Such an effect may account for the deviation of fast block behavior from a Langmuir isotherm that was previously suggested by the apparent increase of  $K_B(0)$

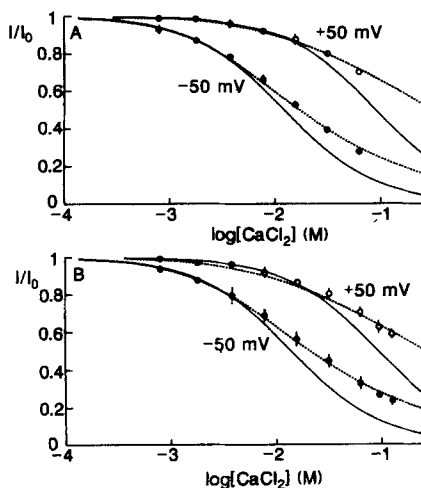


FIGURE 7. Concentration dependence of external Ca<sup>2+</sup> block. (A) Rat muscle Na<sup>+</sup> channel. (B) Canine heart Na<sup>+</sup> channel. Unitary currents of BTX-modified Na<sup>+</sup> channels were measured at -50 mV (●) or +50 mV (○) in the presence of symmetrical 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4, and the indicated concentrations of external CaCl<sub>2</sub>. The ordinate axis,  $I/I_0$ , is the ratio of unitary current in the presence of CaCl<sub>2</sub> to that measured in the absence of divalent cations. Data points and error bars represent the mean  $\pm$  SD of data from three or four bilayers. Solid lines are drawn according to an inverted Langmuir isotherm (Eq. 1) fit to the data

points at low CaCl<sub>2</sub> concentration.  $K_d$  values for the solid line fits are: (A)  $K_d = 12$  mM, -50 mV;  $K_d = 90$  mM, +50 mV; (B)  $K_d = 14$  mM, -50 mV;  $K_d = 93$  mM, +50 mV. Dashed lines are theoretical fits to the surface charge model of Eqs. 6–8 with best-fit parameters for  $\sigma$  and  $K_d(V)$  given in the text.

with divalent cation concentration documented in Table I. In the following sections we analyze titration curves for Ca<sup>2+</sup> and Zn<sup>2+</sup> to assess the possible influence of surface charge on the interaction of these blocking ions with the channel.

The data of Fig. 7 describe the relative decrease of unitary current ( $I/I_0$ ) at  $\pm 50$  mV as a function of increasing external [CaCl<sub>2</sub>] in the bulk solution. The current reduction due to fast Ca<sup>2+</sup> block in Fig. 7 was measured under the same conditions as the experiments of Figs. 1 and 2 in the presence of symmetrical 0.2 M NaCl. Direct comparison of this data for muscle (Fig. 7A) and heart (Fig. 7B) reinforces the previously noted similarity in the affinity and voltage dependence for Ca<sup>2+</sup> block of the two Na<sup>+</sup> channel subtypes. Inasmuch as the analyses of Figs. 1C and 2C test the compatibility of the Ca<sup>2+</sup> blocking data with the Boltzmann dependence on voltage specified by Eq. 2, the titration curves of Fig. 7 may be used to directly examine

compatibility with the Langmuir-type relationship of Eq. 1. In these experiments it was possible to gather data only up to  $\sim 100$  mM  $\text{CaCl}_2$  because the planar bilayers became mechanically unstable under these conditions. To illustrate the departure of these  $\text{Ca}^{2+}$  titration curves from one-site or Langmuir-type binding, theoretical Langmuir saturation curves (Eq. 1) are superimposed (solid lines) on the  $\text{Ca}^{2+}$  titration data in Fig. 7. When plotted in the log-concentration fashion shown in Fig. 7, Langmuir saturation curves have the same shape regardless of the  $K_b$  dissociation constant. By superimposing Langmuir saturation curves to fit the data at low  $\text{Ca}^{2+}$  concentrations, it is clear that the data at high  $\text{Ca}^{2+}$  concentrations depart significantly by exhibiting less steepness than one-site behavior.

One possible interpretation of this deviation from Langmuir behavior is that there are two or more independent classes of  $\text{Ca}^{2+}$  blocking sites with different affinities for  $\text{Ca}^{2+}$ . However, if this were the case and both sites sensed a certain fraction of the electric field, the slope factor,  $z'$ , which reflects the total overall voltage dependence, might be expected to increase as the multiple  $\text{Ca}^{2+}$  sites become occupied at high  $\text{Ca}^{2+}$ . As noted previously, the constant  $z'$  value at different  $\text{Ca}^{2+}$  concentrations suggests that there is only one  $\text{Ca}^{2+}$  blocking site.

Another interpretation of the deviation from one-site behavior involves an effect of surface charge on  $\text{Ca}^{2+}$  binding. As  $\text{CaCl}_2$  concentration is increased in the titrations of Fig. 7, there is an increase in ionic strength that can result in increased screening of surface potential at the  $\text{Ca}^{2+}$  binding site. One might attempt to deal with this problem by keeping the total ionic strength constant by replacement with an "inert" salt. Alternatively, the theory of membrane surface charge as reviewed by McLaughlin (1989) has been previously applied to many phenomena involving binding of ions to sites on charged phospholipid bilayers and ion channel proteins. This theory does predict the departure of  $\text{Ca}^{2+}$  titration curves from Langmuir behavior for  $\text{Ca}^{2+}$  binding to a site located at a protein/ $\text{H}_2\text{O}$  interface in the presence of a negative surface potential. To test the applicability of a surface charge model, we used a simplified version of this theory to fit the  $\text{Ca}^{2+}$  titration curves.

This model is based on one previously used by McLaughlin et al. (1981) for  $\text{Ca}^{2+}$  binding to a negatively charged phospholipid bilayer. We assume that  $\text{Ca}^{2+}$  binds to a single blocking site on the protein that is surrounded by a smeared plane of negative charge. In this situation the local concentration of  $\text{Ca}^{2+}$  at the site,  $[\text{Ca}]_i$ , is related to its bulk solution concentration,  $[\text{Ca}]_b$ , and the surface potential at the site,  $\Psi_s$ , by the Boltzmann equation:

$$[\text{Ca}]_i = [\text{Ca}]_b \exp(-2F\Psi_s/RT) \quad (6)$$

The binding of  $\text{Ca}^{2+}$  as measured by fast block is given by the following modification of Eq. 1, where the bulk blocker concentration,  $[B]$ , has been replaced by  $[\text{Ca}]_i$  and the observed binding constant,  $K_b(V)$ , is replaced by an intrinsic binding constant,  $K_d(V)$ , which applies in the absence of surface potential:

$$I/I_0 = K_d(V)/\{[\text{Ca}]_i + K_d(V)\} \quad (7)$$

The surface potential,  $\Psi_s$ , in Eq. 6 is related to surface charge density,  $\sigma$ , and the concentration of ions in the bulk solution by the Grahame (1947) equation from the Gouy-Chapman theory as cited in McLaughlin et al. (1981):



$$\sigma = \pm \{2\epsilon_r\epsilon_0 RT \sum_i c_i [\exp(-z_i F \Psi_s / RT) - 1]\}^{0.5} \quad (8)$$

where  $\epsilon_r$  is the dielectric constant for H<sub>2</sub>O,  $\epsilon_0$  is the permittivity constant, and the summation is taken over the concentration,  $c_i$ , of all ions of valence  $z_i$  in the bulk aqueous solution.

To analyze the results of Fig. 7 according to the model described by Eqs. 6–8, we first obtained best-fit parameters for  $\sigma$  and  $K_d(-50 \text{ mV})$  for the more complete titrations at  $-50 \text{ mV}$  as described in Methods. We then used this value of  $\sigma$  as a constant in fitting the data at  $+50 \text{ mV}$  to obtain a best-fit value for  $K_d(+50 \text{ mV})$ . Although the titrations at  $+50 \text{ mV}$  do not extend to sufficiently high CaCl<sub>2</sub> concentrations to adequately test the model's behavior in this limit, we include these data for comparative purposes to demonstrate the similar behavior of the muscle and heart Na<sup>+</sup> channels.

Theoretical curves (dashed lines) shown in Fig. 7 are based on Eqs. 6–8, using best-fit parameters for  $\sigma$  and  $K_d$ . For the muscle channel (Fig. 7 A) we obtained values of  $\sigma = 1e^{-}/440 \text{ A}^2$ ,  $K_d(-50 \text{ mV}) = 160 \text{ mM}$ , and  $K_d(+50 \text{ mV}) = 950 \text{ mM}$ ; for heart (Fig. 7 B),  $\sigma = 1e^{-}/420 \text{ A}^2$ ,  $K_d(-50 \text{ mV}) = 200 \text{ mM}$ , and  $K_d(+50 \text{ mV}) = 930 \text{ mM}$ . The close approximation of the fitted curves to the actual data suggests that negative surface charge in the vicinity of the fast-blocking site can account for the departure from Langmuir behavior. Independent analysis of the muscle and heart data by Eqs. 6–8 yielded very similar values of the surface charge density for both channel subtypes,  $\sim 1e^{-}/400 \text{ A}^2$ . This value of  $\sigma$  is similar to that previously found for the effect of surface charge in enhancing the association rate of guanidinium toxins such as TTX<sup>+1</sup> and saxitoxin<sup>+2</sup> binding to an external site on canine brain (Green et al., 1987b) and canine heart Na<sup>+</sup> channels (Ravindran and Moczydlowski, 1989). This value of  $\sigma$  is also similar to that estimated by Green et al. (1987a) for the influence of negative surface charge on the Na<sup>+</sup> permeation process in canine brain Na<sup>+</sup> channels ( $1e^{-}/263 \text{ A}^2$ ).

*Comparison of Fast Block of Muscle Na<sup>+</sup> Channels by Zn<sup>2+</sup> with Substate Block of Heart Na<sup>+</sup> Channels by Zn<sup>2+</sup>*

Fig. 8 compares the titration curve for fast block of the muscle Na<sup>+</sup> channel by external Zn<sup>2+</sup> at  $\pm 50 \text{ mV}$  (Fig. 8 A) with a similar titration of Zn<sup>2+</sup> substate block of the heart channel at  $\pm 70 \text{ mV}$  (Fig. 8 B). Analysis of fast block for the muscle subtype by Zn<sup>2+</sup> was performed in the same manner as that described for fast block by Ca<sup>2+</sup> (Fig. 7). The results for the heart channel are expressed as the probability of being unblocked (analogous to  $I/I_0$ ), which is the open-state probability in the presence of Zn<sup>2+</sup> normalized to that in the absence of Zn<sup>2+</sup>.

We found that the Zn<sup>2+</sup> titration curves for fast block of the muscle channel only exhibit a slight departure from Langmuir-type behavior. This is illustrated by theoretical Langmuir curves (solid lines) at  $\pm 50 \text{ mV}$ , which are drawn to fit Eq. 1 in the region of low [ZnCl<sub>2</sub>]. Application of the surface charge model (Eqs. 6–8) to these same data results in values of  $\sigma = 1e^{-}/400 \text{ A}^2$ ,  $K_d(-50 \text{ mV}) = 52 \text{ mM}$ , and  $K_d(+50 \text{ mV}) = 360 \text{ mM}$ . Thus, the shape of the titration curve for Zn<sup>2+</sup> block of the muscle Na<sup>+</sup> channel is consistent with a value for surface charge density similar to that found for the Ca<sup>2+</sup> titrations.

The Grahame equation (Eq. 8) of surface charge theory describes the decrease in surface potential that is due to charge screening as ionic strength is increased. This dependence on ionic strength accounts for the larger deviation from Langmuir behavior of the  $\text{CaCl}_2$  titration curves at  $-50$  mV (Fig. 7) than the  $\text{ZnCl}_2$  titration of the muscle subtype at  $-50$  mV (Fig. 8A) since this latter  $\text{Zn}^{2+}$  block occurs over a lower concentration range. Inspection of Fig. 8B indicates that the  $\text{ZnCl}_2$  titration curves for the heart channel span  $\sim 100$ -fold lower  $\text{ZnCl}_2$  concentrations than those required to inhibit the muscle channel (Fig. 8A). Thus, at  $0.2$  M NaCl we expect that the  $\text{Zn}^{2+}$  titration of the heart channel should exhibit little or no distortion due to the surface charge effect caused by addition of  $< 1$  mM  $\text{ZnCl}_2$  to  $0.2$  M NaCl.

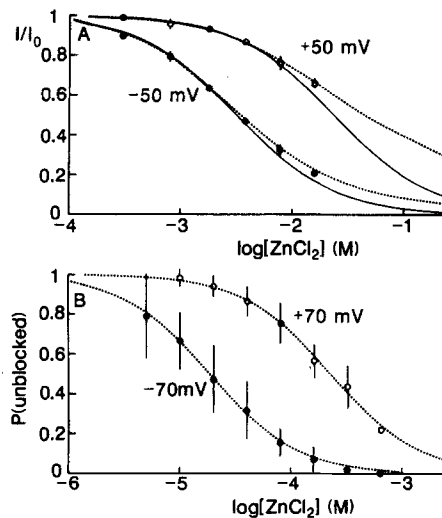


FIGURE 8. Concentration dependence of external  $\text{Zn}^{2+}$  block. (A) Rat muscle  $\text{Na}^+$  channel. (B) Canine heart  $\text{Na}^+$  channel. In A, the ratio  $I/I_0$  of unitary current in the presence of  $\text{ZnCl}_2$  to that in the absence of divalent cations at  $-50$  mV ( $\bullet$ ) and  $+50$  mV ( $\circ$ ) was measured and analyzed as described in Fig. 7. In B,  $\text{Zn}^{2+}$  inhibition of the canine heart  $\text{Na}^+$  channel at  $-70$  mV ( $\bullet$ ) and  $+70$  mV ( $\circ$ ) is plotted as  $P(\text{unblocked})$ , the ratio of the open-state probability measured at various  $\text{Zn}^{2+}$  concentrations to the open state probability measured in the absence of  $\text{Zn}^{2+}$ . The symbols and error bars are mean  $\pm$  SD of data from three to eight bilayers. Solid lines in A are drawn according to an

inverted Langmuir isotherm (Eq. 1) fit to the data points at low  $\text{ZnCl}_2$  concentration.  $K_B$  values for the solid line fits are:  $K_B = 3.2$  mM,  $-50$  mV;  $K_B = 24$  mM,  $+50$  mV. Dashed lines in A are theoretical fits to the surface charge model of Eqs. 6–8 with values for  $\sigma$  and  $K_d(V)$  given in the text. Dashed lines in B are drawn according to fits to the Hill equation:  $P_{\text{un}} = K_B^n / ([B]^n + K_B^n)$ , using  $K_B = 17.9$   $\mu\text{M}$ ,  $n = 1.11$  at  $-70$  mV ( $\bullet$ ) and  $K_B = 218$   $\mu\text{M}$ ,  $n = 1.12$  at  $+70$  mV ( $\circ$ ).

This expectation was borne out in the observation that  $\text{ZnCl}_2$  titrations of the heart channel (Fig. 8B) more closely approximate Langmuir-type behavior than the other divalent cation titrations involving fast block (Figs. 7, A and B, and 8A). For the  $\text{ZnCl}_2$  titrations of the heart channel we performed a Hill plot analysis to examine the molecularity of the  $\text{Zn}^{2+}$  blocking reaction. The Hill equation used is given in the legend of Fig. 8. This analysis yielded values for the Hill coefficient of  $n = 1.11$  at  $-70$  mV and  $n = 1.12$  at  $+70$  mV. The negative surface charge model of Eqs. 6–8 can only give rise to titration curves more shallow than Langmuir behavior or an apparent Hill coefficient  $< 1.0$  and cannot result in a steeper concentration dependence than that for  $n = 1$ . Although values of Hill  $n$  slightly greater than 1.0 were obtained in the majority of such titrations for the heart channel, relatively large standard deviations in these determinations lead to uncertainty about whether the

small deviation from  $n = 1.0$  is significant. Since the Hill coefficients for  $Zn^{2+}$  inhibition of the heart  $Na^+$  channel are very close to 1.0, we tentatively conclude that  $Zn^{2+}$ -induced substate block approximates a one-site system under the present conditions.

The experiments of Fig. 6 and Fig. 8 B also show that the discrete blocking effect of  $Zn^{2+}$  is voltage dependent in the heart channel. To analyze this voltage dependence we measured apparent dissociation constants for  $Zn^{2+}$  either by fits to the empirical Hill equation or by fits of  $P_{un}^{-1}$  vs.  $[Zn^{2+}]$  in the region of low  $Zn^{2+}$  concentrations (not shown). The results shown in Fig. 9 are plotted on a logarithmic  $K_d$  scale vs. voltage and are fitted to Eq. 2. This analysis gives an apparent dissociation constant for  $Zn^{2+}$  in the range of 58–67  $\mu M$  at 0 mV and a slope factor of  $z' = 0.42$ – $0.47$  for the voltage dependence of the blocking reaction (legend to Fig. 9). Comparison with the analogous parameters observed for fast block of the muscle  $Na^+$  channel by  $Zn^{2+}$  (Table I) indicates that the  $Zn^{2+}$  blocking process has practically the

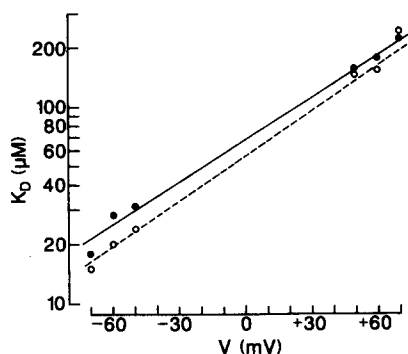


FIGURE 9. Voltage dependence of the apparent equilibrium dissociation constant for  $Zn^{2+}$  block of canine heart  $Na^+$  channels. (●)  $K_d$  determined by linear regression fits of  $Zn^{2+}$  titrations to a Hill equation as in Fig. 8 B. (○)  $K_d$  determined from the slope of linear fits of  $P_{un}^{-1}$  vs.  $[Zn^{2+}]$  (reciprocal of Eq. 1) at low  $Zn^{2+}$  concentrations. Fits to an exponential function of  $K_d$  vs. voltage (Eq. 2) are: (●, solid line)  $K_d(0) = 67 \mu M$ ,  $z' = 0.42$ ; (○, dashed line)  $K_d(0) = 58 \mu M$ ,  $z' = 0.47$ .

same voltage dependence for the two subtypes ( $e$ -fold per 55–60 mV), but the heart channel exhibits at least 100-fold higher affinity than the muscle  $Na^+$  channel.

## DISCUSSION

### *Comparison with Previous Studies of Divalent Cation Block of Na<sup>+</sup> Channels*

Woodhull (1973) originally explained the depression of macroscopic  $Na^+$  currents by extracellular  $H^+$  and  $Ca^{2+}$  on the basis of a voltage-dependent block due to binding of these ions at a site located at an electrical distance of  $\delta = 0.26$  from the outside. While some studies of macroscopic  $Na^+$  currents have provided evidence of actual  $Ca^{2+}$  permeation through normal (Meves and Vogel, 1973) or BTX-modified (Khodorov and Revenko, 1979)  $Na^+$  channels, to our knowledge  $Ca^{2+}$  currents mediated by  $Na^+$  channels have not yet been observed at the single-channel level. Considering the possibility that  $Ca^{2+}$  and other divalent cations may actually permeate at a very low rate, we must emphasize that our use of the modified Woodhull (1973) model that omits permeation must be considered as an approxi-

mate description of the effect of  $\text{Ca}^{2+}$  and other fast blocking divalent cations. A more complete discussion of the paradox of a strongly binding permeant ion acting as a voltage-dependent blocker in the presence of other permeant ions may be found in the work of Cukierman et al. (1985) on  $\text{Cs}^+$  block of a sarcoplasmic reticulum  $\text{K}^+$  channel.

In more recent patch clamp studies on TTX-sensitive and -insensitive subtypes, similar values of electrical distance in the range of  $\delta = 0.31\text{--}0.37$  were reported for neuronal and cardiac cell types by analysis of the  $\text{Ca}^{2+}$ -dependent reduction of unitary conductance using a four-barrier, three-site Eyring permeation model (Yamamoto et al., 1984; Sheets et al., 1987; Nilius, 1988). Investigations of BTX-activated  $\text{Na}^+$  channels in planar bilayers based on the modified Woodhull (1973) model have reported somewhat lower values of  $\delta$  for  $\text{Ca}^{2+}$  block ( $\delta = 0.18\text{--}0.23$ ; Worley et al., 1986; Green et al., 1987a; this work) or  $\text{Zn}^{2+}$  block ( $\delta = 0.21\text{--}0.26$ ; Green et al., 1987a; this work) than those cited for patch-clamp studies of cultured cells. Despite the small difference in apparent voltage dependence, it is clear that the cellular and bilayer experiments encompass the same phenomenon.

The studies of external  $\text{Ca}^{2+}$  block cited above indicate that the affinity and voltage dependence of  $\text{Ca}^{2+}$  block are essentially the same for TTX-sensitive  $\text{Na}^+$  channels of neurons and muscle compared with TTX-insensitive  $\text{Na}^+$  channels of heart. One disagreement is the study of Weiss and Horn (1986), which reported that TTX-insensitive  $\text{Na}^+$  channels in cultured muscle cells were also insensitive to  $\text{Ca}^{2+}$  block. Our own comparison of  $\text{Ca}^{2+}$  block of TTX-sensitive and -insensitive subtypes from denervated rat muscle in planar bilayers revealed no significant difference (Guo, X., and E. Moczydlowski, unpublished observations).

In contrast to the lack of subtype discrimination by  $\text{Ca}^{2+}$ , our results do confirm previous reports of greater sensitivity of TTX-insensitive  $\text{Na}^+$  channels to the group IIb metals,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  (DiFrancesco et al., 1984; Frelin et al., 1986). Results of Frelin et al. (1986) suggested that the selective action of  $\text{Cd}^{2+}$  was only observed for TTX-insensitive  $\text{Na}^+$  channels modified by veratridine or batrachotoxin; however, Baumgarten and Fozzard (1989) reported that  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  are potent inhibitors of unmodified whole-cell cardiac  $\text{Na}^+$  currents at submillimolar concentrations.

#### *Influence of Negative Surface Charge on Divalent Cation Block*

Green et al. (1987a) previously suggested that there is a substantial negative surface charge density in the vicinity of the external mouth of canine brain  $\text{Na}^+$  channels. They also proposed that inhibition of unitary current by extracellular divalent cations was due to a combination of voltage-dependent block and screening of negative surface potential at the mouth of the channel. In this context the term "screening" refers to the decrease in negative surface potential that occurs by addition of ions to the bulk solution according to the Grahame equation (Eq. 8) as opposed to direct complexation of divalent cations with negative surface charges.

Our results on the ionic selectivity of divalent cations in the inhibition of unitary  $\text{Na}^+$  currents confirm that this effect cannot be due solely to simple charge screening. In addition to the difficulty of accounting for the voltage dependence of block, a surface charge mechanism without binding requires that screening is independent of the ionic species (Eq. 8). In contrast, we find a selectivity sequence that roughly

follows an inverse relationship between the blocking affinity and ionic radius (Fig. 3 A). In addition, extracellular voltage-dependent block of the heart Na<sup>+</sup> channel (Fig. 6) occurs at micromolar concentrations of Zn<sup>2+</sup> that are expected to produce insignificant surface potential changes by simple charge screening in the presence of 0.2 M NaCl.

Thus, our results confirm that the phenomenon of extracellular block by divalent cations must involve specific binding site(s). It is noteworthy that our selectivity sequence of fast block of the muscle Na<sup>+</sup> channel is similar to that reported for the effectiveness of shifting gating activation of Na<sup>+</sup> channels in myelinated nerve: Zn > Ni > Co = Mn > Ca > Mg > Ba = Sr (Hille et al., 1975). Hille et al. (1975) previously used this finding to conclude that specific binding sites must be involved in the mechanism by which these divalent ions lower surface potential and cause voltage shifts of gating parameters. McLaughlin et al. (1981) observed similar selectivity sequences for low affinity binding of these metals by phosphatidylcholine or phosphatidylserine. Such comparisons suggest that the particular type of weak selectivity and low affinity binding involved in fast voltage-dependent block by divalent cations may be obtained by rather simple complex formation with a single charged group such as a carboxyl group. Additional evidence for involvement of a carboxyl group was obtained by Worley et al. (1986), who showed that chemical modification by the carboxyl methylating reagent, trimethyloxonium, virtually abolished the voltage-dependent block by Ca<sup>2+</sup> in rat brain Na<sup>+</sup> channels.

Although the role of a specific divalent cation-blocking site now seems to be firmly established, it is difficult to evaluate the exact contribution of surface charge screening by divalent cations on the Na<sup>+</sup> permeation process. This is because screening of negative surface charge not only decreases the local Na<sup>+</sup> concentration available for permeation, but screening also decreases the local divalent cation concentration available for binding to the blocking site. Thus, a comprehensive model of unitary current inhibition by Ca<sup>2+</sup> (i.e., Fig. 7) must incorporate a permeation model for Na<sup>+</sup>, a blocking model for Ca<sup>2+</sup>, and a surface charge model for both of these processes.

Although it is possible to design such a model within the framework of current theory, there are certain issues that must first be addressed. One issue is whether a permeation model based on single or multiple ion occupancy is more appropriate for BTX-activated Na<sup>+</sup> channels. While previous studies of BTX-modified Na<sup>+</sup> channels have used a single-ion occupancy model with some success (Green et al., 1987a; Garber, 1988), our own data on the conductance-concentration behavior of muscle and heart Na<sup>+</sup> channels with respect to Na<sup>+</sup> suggests that a multi-ion model for Na<sup>+</sup> permeation may be more appropriate (Ravindran, A., H. Kwiecinski, and E. Moczydlowski, unpublished observations). Also, if fast block by divalent cations involves rapid substate production instead of complete block, it would seem worthwhile to pursue a more thorough understanding of the substate process before undertaking such a modeling effort. For these reasons, we have not opted to develop a comprehensive model of fast Ca<sup>2+</sup> block in this paper. Instead, we used a greatly simplified treatment of the effect of negative surface charge on a Ca<sup>2+</sup> binding reaction (Eqs. 6–8) to fit the Ca<sup>2+</sup> titration curves of fast block in Fig. 7.

The purpose of this exercise was to show that the Gouy-Chapman theory of surface charge can qualitatively predict the type of distortion of divalent cation titration curves from Langmuir behavior that is observed for  $\text{Na}^+$  channel block. The values of surface charge density and the intrinsic  $\text{Ca}^{2+}$  binding constants that we obtain by such fitting should not be considered as quantitatively correct parameters of the actual blocking reaction, but as empirical parameters that can be compared for different channels. With this qualification, our results show that the  $\text{Ca}^{2+}$  titration curve deviates in the manner expected for low affinity binding ( $K_d \sim 200$  mM at  $-50$  mV,  $K_d \sim 900$  mM at  $+50$  mV) of  $\text{Ca}^{2+}$  to a site located on a surface with a negative charge density of  $\sim 1e^-/400 \text{ \AA}^2$  for both muscle and heart subtypes. While we also recognize that the Gouy-Chapman assumption of an infinite plane of negative charge is an improper geometry for an extracellular  $\text{Ca}^{2+}$  binding site on a membrane protein, more realistic models of surface charge predict qualitatively similar behavior under the ionic strength conditions (0.2 M NaCl) of our experiments (Cai and Jordan, 1990).

Our analysis of the  $\text{Zn}^{2+}$  titration curves for the muscle  $\text{Na}^+$  channel is consistent with a negative surface charge density similar to that required to fit the shape of the  $\text{Ca}^{2+}$  titration curve for muscle and heart. In contrast, discrete  $\text{Zn}^{2+}$  block of the heart channel follows a titration curve slightly steeper than, but basically approximating one-site binding. This result does not necessarily imply that  $\text{Zn}^{2+}$  block of the heart channel does not sense the surface potential. It only means that the titration curve is not significantly broadened by the change in ionic strength from 10 to 640  $\mu\text{M}$   $\text{ZnCl}_2$ . For a negative surface charge density of  $1e^-/400 \text{ \AA}^2$  and a NaCl concentration of 0.2 M, the Grahame equation (Eq. 10) gives a value of  $-36$  mV for the surface potential in the absence of divalent cations. This equation predicts that the addition of 1 mM  $\text{ZnCl}_2$  or a similar salt will only decrease the surface potential by about  $+1$  to  $-35$  mV. Since the concentration of  $\text{Zn}^{2+}$  varied in our titrations of the heart channel covers the range of  $10^{-5}$ – $10^{-3}$  M  $\text{ZnCl}_2$ , it is clear that these concentrations are too low to have any significant screening effect under the present conditions. To test for an effect of negative surface charge on discrete  $\text{Zn}^{2+}$  block of the heart channel, the surface potential would have to be varied by an alternative method, for example by increasing or decreasing NaCl concentration.

The observation of discrete subconductance block by  $\text{Zn}^{2+}$  leads to the question of whether  $\text{Ca}^{2+}$  block may involve the production of very fast substates rather than complete occlusion of the channel. If this were the case, one might have expected the  $\text{Ca}^{2+}$  titration curve of the fast block to saturate at non-zero current levels at high  $\text{Ca}^{2+}$ . However, if the reduction of single channel current involves both substate production and screening of surface charge, then very high  $\text{Ca}^{2+}$  concentrations would inevitably reduce the substate current by the screening mechanism and diminish the possibility of detecting such a non-zero saturation. Therefore, the fact that we did not clearly observe such a saturation does not eliminate the possible involvement of very brief substates in the mechanism of fast block by  $\text{Ca}^{2+}$  or other divalent cations.

Since the binding of TTX<sup>+</sup> and saxitoxin<sup>2+</sup> has been shown to respond to a similar negative surface charge density as that estimated here for the fast  $\text{Ca}^{2+}$  block, it is possible that  $\text{Ca}^{2+}$  block and toxin block occur in sites of close proximity. Worley et al.

(1986) and Green et al. (1987b) previously showed that Ca<sup>2+</sup> and Zn<sup>2+</sup> competitively inhibit STX binding to TTX-sensitive Na<sup>+</sup> channels by lowering the toxin association rate with little or no effect on the dissociation rate. Since these experiments suggest that Ca<sup>2+</sup> and Zn<sup>2+</sup> may bind to a subsite within or near the TTX/STX binding site, we recently examined the interaction between STX binding and Zn<sup>2+</sup> binding as monitored by the Zn<sup>2+</sup>-induced substate effect in heart Na<sup>+</sup> channels. Our results indicate that there is indeed a strong competitive interaction between STX and Zn<sup>2+</sup> binding in heart Na<sup>+</sup> channels at micromolar concentrations of Zn<sup>2+</sup> (Schild, L., and E. Moczydlowski, manuscript submitted for publication).

*A Structural Hypothesis for High Affinity Binding of Cd<sup>2+</sup> and Zn<sup>2+</sup> by the Heart Na<sup>+</sup> Channel*

Given the remarkable similarity of the blocking parameters  $K_b(0)$  and  $z'$  for fast block of the muscle and heart Na<sup>+</sup> channels by Mg, Ca, Sr, Ba, Mn, Co, and Ni, one might legitimately conclude that the high affinity block of the heart channel by Zn<sup>2+</sup> and Cd<sup>2+</sup> occurs at a completely different site with a unique specificity. However, the similar voltage dependence of high affinity Zn<sup>2+</sup> block and low affinity Ca<sup>2+</sup> block suggests that these two divalent cations may be acting at the same site. To explain this paradox, a structural model can be proposed which requires substitution of only one or two amino acids in the fast block site for the muscle channel to explain the high affinity observed for Zn<sup>2+</sup> and Cd<sup>2+</sup> in the heart channel. In Zn<sup>2+</sup>-binding proteins whose structures have been obtained at high resolution, Zn<sup>2+</sup> has been found to be coordinated by a combination of carboxyl groups of glutamate or aspartate residues, sulfhydryl groups of cysteine, imidazole groups of histidine, and H<sub>2</sub>O ligands in a distorted four- or five-coordinate geometry (Brown et al., 1983). Since brain and muscle Na<sup>+</sup> channel subtypes are homologous proteins that differ by amino acid substitutions interspersed throughout the primary sequence (Trimmer et al., 1989), it is possible that substitution of a single residue, such as a cysteine or histidine group, into the divalent cation binding site responsible for fast block of the muscle channel could have occurred in the heart subtype. The introduction of one or two such residues might have minimal or no effect on the binding of group IIa metals (Mg, Ca, Sr, and Ba) or on binding of first-row transition metals (Mn, Co, and Ni), but could account for the increase in affinity for Zn<sup>2+</sup> and Cd<sup>2+</sup> by 100- and 40-fold, respectively. Partial support for this hypothesis has recently been obtained with the observation that Zn<sup>2+</sup> substate block of heart Na<sup>+</sup> channels can be abolished by treatment with iodoacetamide, a specific alkylating reagent for cysteine sulfhydryl groups (Schild, L., and E. Moczydlowski, manuscript submitted for publication).

*Possible Physiological Significance of Zn<sup>2+</sup> Block*

Assuming similar behavior of normal and BTX-modified Na<sup>+</sup> channels, the results of Fig. 9 indicate that the cardiac Na<sup>+</sup> channel has a dissociation constant near 16 μM for Zn<sup>2+</sup> block at -70 mV. If Zn<sup>2+</sup> can bind with similar affinity to the resting conformation of normal Na<sup>+</sup> channels, Zn<sup>2+</sup> would be a relatively potent inhibitor of heart Na<sup>+</sup> channels under physiological conditions. Total Zn<sup>2+</sup> concentrations in human serum and heart tissue are estimated at 12 and 500 μM, respectively, as determined by atomic absorption measurements (Bjorksten et al., 1978; Huxtable et

al., 1984). However, most of this  $Zn^{2+}$  is undoubtedly bound to various metalloenzymes and proteins. Thus, it seems unlikely that  $Zn^{2+}$  could have a significant effect on heart  $Na^+$  channels under physiological conditions, except possibly in the case of  $Zn^{2+}$  release from local regions of damaged heart tissue.

In the central nervous system  $Zn^{2+}$  has been implicated as a possible modulator of synaptic transmission. Histochemical studies have shown that chelatable  $Zn^{2+}$  is selectively localized in certain neuronal pathways in the brain. At the ultrastructural level  $Zn^{2+}$  precipitates formed with sulfide stain have been localized to vesicular structures that resemble synaptic vesicles (Perez-Clausell and Danscher, 1985). Additional studies have demonstrated  $Ca^{2+}$ -dependent release of  $Zn^{2+}$  from synaptic terminals by depolarization. These studies estimated that the concentration of  $Zn^{2+}$  in such synapses could reach levels as high as 300  $\mu M$  (Assaf and Chung, 1984). Possible mechanisms for removing free  $Zn^{2+}$  from the synapse may involve high and low affinity transport systems for  $Zn^{2+}$  that have been identified in studies of hippocampal slices (Howell et al., 1984). A possible functional role for neuronal  $Zn^{2+}$  release was recently suggested by electrophysiological studies that showed that 50–100  $\mu M$   $Zn^{2+}$  selectively blocks the NMDA-subtype of excitatory glutamate-activated channels in brain neurons (Peters et al., 1987; Westbrook and Mayer, 1987). This accumulated body of evidence suggests that  $Zn^{2+}$  release may be a potential mechanism for modulating excitatory neurotransmission. Although our results on a high affinity  $Zn^{2+}$  block are confined to cardiac  $Na^+$  channels, TTX-insensitive  $Na^+$  currents have been reported in cerebellar neurons and in a variety of peripheral ganglia (Rogart, 1986; Ikeda and Schofield, 1987). If such TTX-insensitive  $Na^+$  channels of neurons are similarly susceptible to  $Zn^{2+}$ , high affinity  $Zn^{2+}$ -block may be important in the regulation of these particular  $Na^+$  channels.

We thank Dr. John Daly for the gift of batrachotoxin and Dr. Sherwood Hall for decarbamoylsaxitoxin.

This work was supported by grants from the National Institutes of Health (AR-38796 and HL-38156), the Searle Scholars Program/Chicago Community Trust, an Established Investigator award to E. Moczydlowski from the American Heart Association, and a fellowship to L. Schild from the Swiss National Science Foundation.

*Original version received 31 August 1989 and accepted version received 30 July 1990.*

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