Competitive binding interaction between Zn^{2+} and saxitoxin in cardiac Na^+ channels

Evidence for a sulfhydryl group in the Zn^{2+}/saxitoxin binding site

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ABSTRACT Mammalian heart Na^+ channels exhibit ~100-fold higher affinity for block by external Zn^{2+} than other Na^+ channel subtypes. With batrachotoxin-modified Na^+ channels from dog or calf heart, micromolar concentrations of external Zn^{2+} result in a flickering block to a substate level with a conductance of ~12% of the open channel at −50 mV. We examined the hypothesis that, in this blocking mode, Zn^{2+} binds to a subsite of the saxitoxin (STX) binding site of heart Na^+ channels by single-channel analysis of the interaction between Zn^{2+} and STX and also by chemical modification experiments on single heart Na^+ channels incorporated into planar lipid bilayers in the presence of batrachotoxin. We found that external Zn^{2+} relieved block by STX in a strictly competitive fashion. Kinetic analysis of this phenomenon was consistent with a scheme involving direct binding competition between Zn^{2+} and STX at a single site with intrinsic equilibrium dissociation constants of 30 nM for STX and 30 μM for Zn^{2+}. Because high-affinity Zn^{2+}-binding sites often include sulfhydryl groups as coordinating ligands of this metal ion, we tested the effect of a sulfhydryl-specific alkylating reagent, iodoacetamide (IAA), on Zn^{2+} and STX block. For six calf heart Na^+ channels, we observed that exposure to 5 mM IAA completely abolished Zn^{2+} block and concomitantly modified STX binding with at least 20-fold reduction in affinity. These results lead us to propose a model in which Zn^{2+} binds to a subsite within or near the STX binding site of heart Na^+ channels. This site is also presumed to contain one or more cysteine sulfhydryl groups.

INTRODUCTION

Voltage-dependent Na^+ channels in excitable tissue of mammals can be classified into at least three pharmacological subtypes according to their affinity for tetrodotoxin (TTX), saxitoxin (STX), and μ-conotoxin peptides that bind at a common extracellular site (for reviews see Barchi, 1987; Trimmer and Agnew, 1989). TTX, STX, and derivatives of these toxins have been used to discriminate Na^+ channel subtypes with high toxin affinity from subtypes with low toxin affinity that are respectively found in brain and skeletal muscle vs. heart and denervated muscle of mammals. Similarly, μ-conotoxin peptides from *Conus geographus* snails have high affinity for Na^+ channel subtypes from skeletal muscle, but exhibit much lower affinity for neuronal and heart Na^+ channels (Cruz et al., 1985; Ohizumi et al., 1986; Moczydlowski et al., 1986). This pharmacological diversity is also reflected at the genetic level where Na^+ channel subtypes appear to be the product of at least five different genes in the rat as revealed by cDNA cloning (Noda et al., 1986; Kayano et al., 1988; Trimmer et al., 1989; Rogart et al., 1989).

The availability of such natural variants of Na^+ channels can be exploited in the analysis of function and mechanism. Using batrachotoxin (BTX) to prolong the open state of Na^+ channels incorporated into planar bilayers, we previously compared the characteristics of external block by divalent cations of a TTX-sensitive Na^+ channel from rat muscle with a TTX-insensitive subtype from dog heart (Ravindran et al., 1991). These studies showed that the two subtypes exhibited virtually the same blocking affinity for the divalent cations, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}, whereas the heart Na^+ channel displayed 50-fold higher affinity for Cd^{2+} and 100-fold higher affinity for Zn^{2+} than the muscle Na^+ channel. Whereas 10 mM external Zn^{2+} produced only an apparent reduction in conductance (fast block) in muscle Na^+ channels, 10 μM external Zn^{2+} induced discrete substate events of 15–25 ms duration in BTX-modified cardiac Na^+ channels (Schild et al., 1991). The high affinity of TTX-insensitive Na^+ channels for Cd^{2+} and Zn^{2+} has also been previously documented in a variety of preparations using electrophysiological (DiFancesco et al., 1984; Baumgarten and Fozzard, 1989; Backx et al., 1990) and $^{22}Na^+$ flux techniques (Frelin et al., 1986). This selectivity for the group IIB metals, Zn^{2+} and Cd^{2+}, suggests that the cardiac Na^+ channel subtype

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contains specific amino acid substitutions that enhance the binding affinity of these particular divalent metal ions.

Previous studies of the TTX/STX binding reaction have established that there is a competitive interaction between binding of these guanidinium toxins and alkali cations, with a high-field strength selectivity sequence of $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Reed and Raftery, 1976; Weigele and Barchi, 1978; Barchi and Weigele, 1979). Previous work also suggests that there may be a similar competitive binding interaction between certain divalent cations and guanidinium toxins. Using squid axons, Gilly and Armstrong (1982) found that Zn^{2+} slows the ON component of Na^+ channel-gating current and that TTX interferes with this action of Zn^{2+}. More recent studies of the effect of Zn^{2+} on the STX blocking kinetics of single canine brain Na^+ channels (BTX-modified) showed that Zn^{2+} lowers the affinity for STX by decreasing the apparent STX association rate without affecting the STX dissociation rate (Green et al., 1987). Similar results have been observed for the effect of Ca^{2+} on the STX blocking kinetics of rat brain Na^+ channels (Worley et al., 1986; Krueger et al., 1986). One interpretation of these latter observations is that there is a direct competitive interaction between the binding of these particular divalent cations and the binding of STX. However, because the TTX/STX binding reaction is also known to be influenced by the presence of negative surface charge (Hille et al., 1975; Strichartz et al., 1986; Green et al., 1987; Ravindran and Moczydlowski, 1989; Cai and Jordan, 1990), these results could also be due to a decrease in negative suface potential caused by the relatively high divalent cation concentrations used in these experiments. In other words, binding of Zn^{2+} and Ca^{2+} may not directly obstruct the binding of STX by direct competition, but could lower the effective association rate of the toxin by screening negative surface potential via a through-space mechanism (McLaughlin, 1989). Because the cardiac Na^+ channel exhibits much higher affinity for Zn^{2+} compared to other Na^+ channel subtypes, it is an ideal candidate for distinguishing these two mechanisms. This advantage results from the feasibility of using micromolar concentrations of Zn^{2+} that would not be expected to effectively screen the surface potential.

In this paper, we follow this strategy to examine the interaction of Zn^{2+} and STX binding as observed at the single-channel level using Na^+ channels from canine heart and calf heart that have been prolonged by BTX. We find that there is indeed a strictly competitive binding interaction between Zn^{2+} and STX at a site which displays equilibrium dissociation contants of ~ 30 μM for Zn^{2+} and 30 nM for STX at -50 mV in the presence of 0.2 M NaCl. Application of Gouy-Chapman theory suggests that the concentrations of Zn^{2+} required for this interaction are too low to produce significant screening of surface potential at this ionic strength. From examples of crystallographically determined Zn^{2+}-binding sites in various metalloenzymes, it is known that such sites often contain cysteine residues that are involved in coordination of this metal ion (Creighton, 1983; Brown et al., 1983). We found that the treatment of calf heart Na^+ channels with a cysteine-specific alkylating agent, iodoacetamide, reproducibly abolished the discrete blocking activity of Zn^{2+} and concomitantly modified the kinetics of STX binding. These results suggest that there is a unique cysteine group in mammalian cardiac Na^+ channels that is at least partially responsible for the high affinity of this particular Na^+ channel subtype for Zn^{2+}. Comparison of the primary amino acid sequences of a putative STX binding region of various Na^+ channel subtypes reveals a unique cysteine group in the rat cardiac subtype (Rogart et al., 1989) that is two residues away from a critical glutamate residue that has been implicated in STX binding in a rat brain Na^+ channel (Noda et al., 1989). This cys374 residue of the rat heart Na^+ channel is an attractive candidate for a liganding group involved in Zn^{2+} coordination in mammalian heart Na^+ channels.

METHODS

Membrane preparations and planar bilayers

Native plasma membrane vesicles were isolated from heart ventricular muscle and stored at -80°C as previously described (Guo et al., 1987; Ravindran et al., 1991). Dog hearts were salvaged from animals used for experimental surgery in the Department of Internal Medicine at Yale University School of Medicine as approved by the Yale Animal Care and Use Committee. Because the supply of such canine heart tissue became infrequent during the course of our experiments, we changed the source of mammalian tissue to calf hearts obtained from a local slaughterhouse. For this reason, most of the experiments in this paper have been duplicated for Na^+ channels from dog and calf heart. The results indicate that BTX-modified cardiac Na^+ channels from these two species are virtually indistinguishable with respect to blockade by STX and Zn^{2+}.

Planar bilayers were cast from a 25 mg/ml solution of phospholipids in decane over a 200 μm aperture in a polystyrene partition. Phospholipids were a mixture of 8:2 bovine brain phosphatidylethanolamine:1,2-diphytanoylphosphatidylcholine, obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). The solution on both sides of the bilayer was 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4. (MOPS is 3-(*N*-morpholino)propanesulfonic acid.) Experiments were carried out at ambient temperature (22–24°C). Na^+ channels were incorporated by addition of 5–50 μg/ml of heart membrane preparation and 0.2 μM BTX to one side of the bilayer with constant stirring. Incorporation of a single Na^+ channel was recognized by an abrupt increase in ~ 1 pA current at +50 mV. Only bilayers containing a single Na^+ channel were utilitzed for these experiments. Voltage signs are referenced to the physiological convention of extracellular (external) ground according to the orientation of each Na^+ channel determined at the beginning of

an experiment by observing rapid voltage-dependent gating at −90 mV (Krueger et al., 1983).

Single-channel recording and analysis

The recording system consisted of a List EPC-7 patch clamp amplifier (Medical Systems, Inc., Greenvale, NY), a 902LPF1 8-pole lowpass Bessel filter (Frequency Devices, Haverhill, MA) and a VR-10 digital data recorder (Instrutech, Elmont, NY) which uses a video cassette recorder. Probability and dwell time measurements of current records of single-channel fluctuations were carried out with the aid of user interactive mouse-driven software (Affolter and Sigworth, 1988) available for an Atari computer system (Instrutech). Single-channel records were digitized at a sampling rate of 2 kHz for data filtered at 100 Hz.

BTX-modified Na^+ channels from dog and calf heart are steeply activated by voltage in the range of −120 to −80 mV and exhibit a rather constant open-state probability near 0.9 at holding voltages more positive than −70 mV (Ravindran A., and E. Moczydlowski, unpublished results). This gating behavior is similar to that previously described for other Na^+ channel subtypes in planar bilayers (French et al., 1984; Moczydlowski et al., 1984*a*; Hartshorne et al., 1985; Recio-Pinto et al., 1987; Behrens et al., 1989). The competitive interaction between STX and Zn^{2+} binding was studied at −50 mV in order to focus on binding interactions with the open-channel conformation. Also, bilayers are relatively stable at this voltage which facilitates the collection of long records (1–2 h) of STX-blocking events from a single channel. In addition, the duration of STX-blocked states (τ = 3.6 s) at −50 mV is long enough to ensure good discrimination of such states from the long component of channel gating events (lifetime = 150–300 ms) and from more rapid Zn^{2+}-induced substate events (lifetime = 15–25 ms).

Analysis of the kinetics of STX-blocking events was carried out essentially as described previously (Moczydlowski et al., 1984*a*, *b*; Guo et al., 1987). Events attributed to the STX-blocked state were defined as complete closures lasting longer than 0.5 s in order to eliminate contamination by closing events due to channel gating. At the higher concentrations of Zn^{2+} used in these experiments, the lifetime of channel openings becomes so brief (e.g., Fig. 2 and Fig. 5 *C*) that automatic event detection using a 50% threshold criterion for STX-induced closing events would result in errors. Therefore, each computer-detected closing event was subjected to operator verification. In this procedure, only those closures longer than 0.5 s that definitely reside at the zero-current baseline were accepted as STX-blocked events. Because Zn^{2+} block is associated with a subconductance level which is ~12% of the open channel current (Schild et al., 1991), this procedure effectively eliminates dwell times in the Zn^{2+}-induced substate from the population of STX-blocked events.

Observed lifetimes of STX-blocked (τ_B) and STX-unblocked (τ_U) states were obtained by fitting populations of ~100 such events to a single exponential according to the method of Sigworth and Sine (1987) which uses logarithmically-binned frequency density histograms. The observed lifetime of the STX-unblocked state was corrected for a 15% overestimate of this parameter as a result of using the 0.5 s minimum cutoff on the shortest acceptable value of STX-blocked events (Moczydlowski et al., 1984*a*). The time-averaged probability of a single channel residing in the STX-blocked state, P_B, was estimated from the ratio of the total time in STX-blocked states to the total time of the analyzed record.

The equilibrium interaction of Zn^{2+} with the open conformation of Na^+ channels in the absence of STX was measured as the probability of being unblocked in the presence of Zn^{2+}, $P_{unblocked}$, which was evaluated by two different methods. In the first method the unconditional probability of residing in the open-current level was measured by a standard automatic event detection using a 50% threshold criterion. To correct for an ~10% probability of channel closing in the absence of Zn^{2+}, $P_{unblocked}$ is computed as a normalized probability, P_{norm}, which is defined as the ratio, $P_o(+Zn)/P_o(-Zn)$, of the unconditional open-state probability in the presence of a given Zn^{2+} concentration to the unconditional open-state probability of the same channel measured in the absence of Zn^{2+}. In the second method, $P_{unblocked}$ is a conditional probability defined as the open-state probability within a burst of channel activity that is entered upon departure from a complete channel closure lasting longer than 0.1 s and is terminated by entry to a complete closure lasting longer than 0.1 s. Within such selected bursts, the open-state probability, P_{burst}, is automatically computed using a 50% threshhold. The value of P_{burst} for a given record is taken as the mean of at least five such individual P_{burst} measurements within the record. Standard deviations of such samples were within ±10% of the mean.

RESULTS

Blockade of single cardiac Na^+ channels by STX and Zn^{2+}

Fig. 1 illustrates the effect of 100 nM STX added to the external side of single BTX-modified Na^+ channels from dog heart and calf heart. At −50 mV, BTX-modified Na^+ channels incorporated into planar bilayers from heart ventricular membranes of these species exhibit an open-state probability of 0.91 ± 0.03 (± SD, n = 10). In the absence of STX, most of the brief closures due to channel gating are <0.5 s in duration. After the addition of 100 nM STX, longer duration blocking events are observed which correspond to residence times of individ-

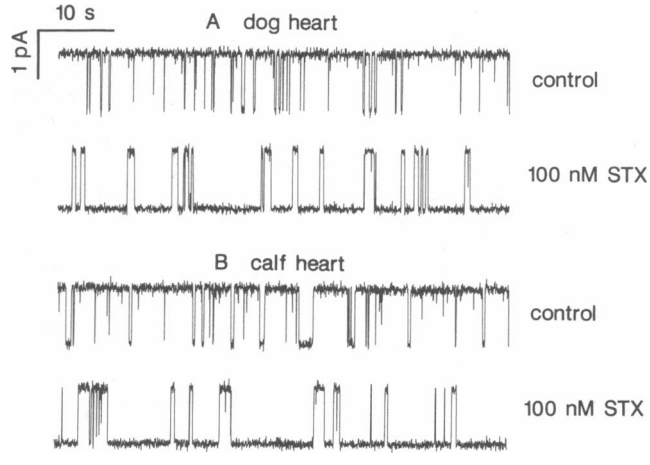

FIGURE 1 Effect of STX on BTX-modified Na^+ channels from mammalian heart. Planar bilayer recordings of a single Na^+ channel from dog heart (*A*) or calf heart (*B*) are shown before (control) or after addition of 100 nM STX to the external chamber. The holding voltage is −50 mV and 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4 is present on both sides of the bilayer. Channel opening is upward in this and all subsequent current records. Each current trace is 60 s in duration.

ual STX molecules on the channel (French et al., 1984; Moczydlowski et al., 1984*b*; Green et al., 1987; Guo et al., 1987). These blocking events can be analyzed according to a one-site binding reaction:

$$\text{(STX-unblocked) O} + \text{STX} \underset{k_{off}}{\overset{k_{on}[\text{STX}]}{\rightleftharpoons}} \text{O} \cdot \text{STX (STX-blocked)}, \quad (1)$$

where the equilibrium dissociation constant for STX is given by the ratio of the rate constants, $K_{STX} = k_{off}/k_{on}$. The above scheme predicts that dwell time histograms of the block and unblocked events are exponentially distributed with time constants, τ_B, for the STX-blocked state and, τ_U, for the STX-unblocked state given by:

$$\tau_B = k_{off}^{-1} \quad (2)$$

$$\tau_U = (k_{on}[\text{STX}])^{-1}. \quad (3)$$

Such lifetime measurements of blocked events fit to a single exponential (e.g., Fig. 6) resulted in values of τ_B = 3.7 ± 0.5 s (± SE, n = 3 channels) and τ_B = 3.6 ± 0.3 s (± SE, n = 6) for dog heart and calf heart Na^+ channels, respectively, in the presence of 100 nM STX. Corresponding values of the lifetime of the unblocked state are τ_U = 1.2 ± 0.2 s (± SE, n = 3) and τ_U = 1.1 ± 0.1 s (± SE, n = 6) for dog and calf, respectively. This comparison of the two mammalian species indicates that STX blocking kinetics are virtually indistinguishable for Na^+ channels from dog and calf heart. Eqs. 2 and 3 predict that if the STX concentration is doubled, the lifetime of the blocked state should be unaffected, whereas the lifetime of the unblocked state should be reduced by one-half. Such an experiment for the calf heart channel at 200 nM STX yielded values of τ_B = 3.5 ± 0.2 s (± SE, n = 3) and τ_U = 0.57 ± 0.17 s (± SE, n = 3) in good agreement with the kinetic predictions. Eqs. 2 and 3 may be used to calculate k_{off} and k_{on} rate constants for the STX blocking reaction which are summarized in Table 1. These results indicate that the equilibrium dissociation constant ($K_{STX} = k_{off}/k_{on}$) for STX is 32 nM for dog and 29 nM for calf under the conditions of Fig. 1. A similar analysis of STX blocking kinetics for rat brain and rat skeletal muscle Na^+ channels previously gave values of K_{STX} equal to 0.8 nM for brain and 1.4 nM for muscle at −50 mV (Guo et al., 1987). This comparison of Na^+ channels from different mammalian tissues confirms the lower STX affinity of heart Na^+ channels in comparison to TTX/STX-sensitive subtypes.

TABLE 1 STX blocking kinetics before and after IAA modification*

Na^+ channel	k_{off}	k_{on}	K_d
	s^{-1}	$s^{-1}M^{-1}$	nM
Dog heart (3)	0.28 ± 0.06	0.89 ± 0.23 × 10^7	32 ± 8
Calf heart (9)	0.29 ± 0.05	1.0 ± 0.3 × 10^7	29 ± 10
Calf heart (4) after IAA	1.5 ± 0.4	0.27 ± 0.01 × 10^7	570 ± 140

*Rate and equilibrium constants for STX blocking kinetics of BTX-modified Na^+ channels at −50 mV are listed as the mean and standard deviation for the number of single-channel bilayers in parentheses.

Another distinctive characteristic of cardiac Na^+ channels is illustrated in Fig. 2, namely sensitivity to block by micromolar concentrations of external Zn^{2+}. Fig. 2 shows records from an experiment where a single BTX-modified calf heart Na^+ channel was recorded in symmetrical 0.2 M NaCl in the presence of increasing concentrations of Zn^{2+} in the range of 10–160 μM at a constant holding voltage of −50 mV. This experiment shows that Zn^{2+} induces brief flickering events in a concentration-dependent fashion. Close inspection of the records of Fig. 2 also indicates that the brief Zn^{2+}-induced closures do not transit to the fully closed current level but close to

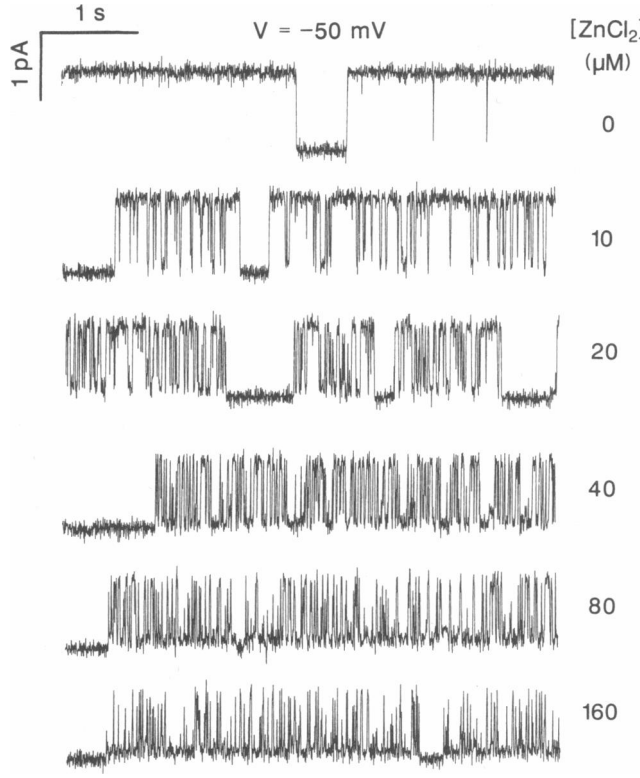

FIGURE 2 Effect of external Zn^{2+} on a BTX-modified Na^+ channel from calf heart. Current traces from a single-channel bilayer are shown in the absence and presence of 10–160 μM external $ZnCl_2$. This bilayer was not exposed to STX. Conditions are otherwise the same as in Fig. 1. Note the faster time scale than Fig. 1; each current trace is 5 s in duration.

a subconductance current level that is ~12% of the open-state current under these conditions. A more detailed analysis of this phenomenon for dog heart Na^+ channels by Schild et al. (1991) showed that the kinetics of the Zn^{2+}-dependent substate process could be approximated by a kinetic scheme involving the binding of Zn^{2+} to a site which induces a conformational transition to a subconductance state of the channel. This model involved a four-state scheme similar to one introduced by Pietrobon et al. (1989) for H^+-induced substate behavior in L-type Ca^{2+} channels and predicts a dependence on $[Zn^{2+}]$ that is formally equivalent to a one-site binding equilibrium. Here, we further examine this equilibrium behavior.

To assess the inhibition of a single channel by Zn^{2+} we require a probability measurement that is proportional to the occupancy of the channel by Zn^{2+}. Analysis of the closed-state behavior of records such as those in Fig. 2 is complicated by the fact that single channels normally exhibit brief closures in the absence of Zn^{2+} which cannot easily be distinguished from the brief substate closures induced by Zn^{2+}. However, the relative time the channel spends in the open state provides a valid measurement of the probability, $P_{unblocked}$, that a channel is not blocked by Zn^{2+}, which can be related to the blocking probability by $P_{unblocked} + P_{blocked} = 1.0$. As described in Methods, we measured $P_{unblocked}$ in two different ways. The first method uses a parameter called P_{norm}, which is defined as $P_o(+Zn)/P_o(-Zn)$, the ratio of the unconditional time-averaged open-state probability in the presence of Zn^{2+} to that in the absence of Zn^{2+}. This ratio is a normalized open-state probability which corrects for a small probability ($P \sim 0.1$) of channel closing in the absence of Zn^{2+}. The second method involves a conditional probability called P_{burst}, which is defined as the measured open-state probability for individual bursts of Zn^{2+} blocking events. Such bursts are identified by a period of flickering Zn^{2+}-induced activity that is begun upon departure from a well-resolved channel closure at least 0.1 s and is terminated by entry to such a long closure. In this way, the conditional P_{burst} measurement excludes long closures from the record and provides an independent measurement of the fast-flickering substate process. This P_{burst} analysis was undertaken to assess the possibility that binding of Zn^{2+} to normal closed states of the channel might lengthen such closed states via an interaction between Zn^{2+} and channel gating. Such an interaction could potentially alter the closed-open equilibrium of the channel in the presence of Zn^{2+}. If Zn^{2+} binding were to significantly lengthen channel closures, P_{norm} values should be substantially lower than P_{burst} measurements for the same experimental conditions.

The open symbols in the Zn^{2+} titrations of Fig. 3, *A* and *B*, illustrate P_{norm} measurements for dog and calf Na^+ channels at −50 mV and also show a comparison with P_{burst} measurements for the calf heart channel (Fig. 3 *B*). These results show that such titrations of the Zn^{2+} blocking effect approximate a one-site binding equilibrium as indicated by the solid-line theoretical curves which are drawn according to an inverted Langmuir isotherm, $P_{unblocked} = K_{Zn}/\{[Zn^{2+}] + K_{Zn}\}$, with K_{Zn} = 31 μM (Fig. 3 *A*, dog) or 34 μM (Fig. 3 *B*, calf). We have analyzed such titrations according to the corresponding Hill equation for the fractional vacancy of a system that involves the binding of n Zn^{2+} ligands to n interacting sites:

$$P_{unblocked} = K_{Zn}^n/\{[Zn^{2+}]^n + K_{Zn}^n\}. \quad (4)$$

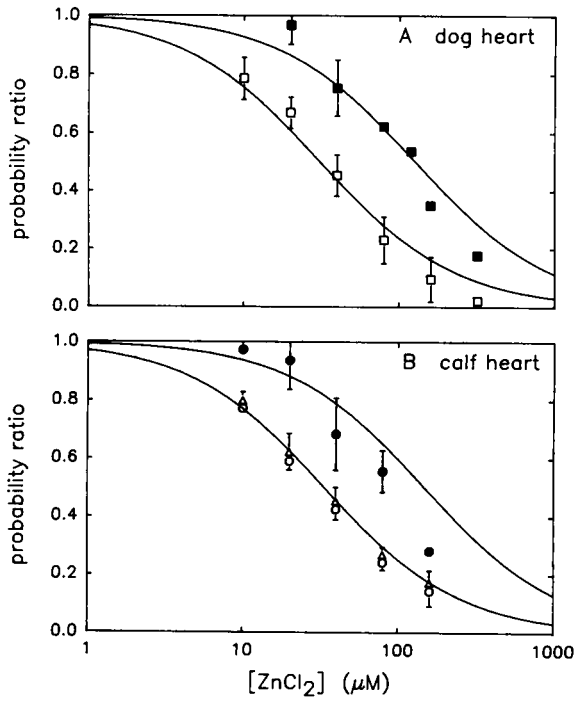

FIGURE 3 Binding competition between Zn^{2+} and STX as demonstrated by equilibrium Zn^{2+} titrations. The holding voltage was −50 mV for experiments with dog (*A*) and calf (*B*) Na^+ channels. Open symbols refer to Zn^{2+}-titration experiments in the absence of STX where the probability that channels are not occupied by Zn^{2+} ($P_{unblocked}$) was computed in two different ways described in Methods: (□, ○) P_{norm}; (△) P_{burst}. Solid symbols (■, ●) refer to Zn^{2+} titrations in the presence of 100 nM STX where the relief of STX block by Zn^{2+} was measured as the probability ratio, $P_B(+Zn)/P_B(-Zn)$, of being blocked by STX in the presence of Zn^{2+} to that in the absence of Zn^{2+}. Parts *A* and *B* compare actual data with theoretical behavior (*solid lines*) predicted according to a one-site, direct competition model described by Eqs. 4, 7, 8, and parameters for the simulation given in the text. Error bars denoting standard deviations from the mean are shown for samples of data including at least three bilayers.

The following equation is a log-linearized form of Eq. 4 that is often used to evaluate the Hill coefficient, n:

$$\log\{(1 - P_{unblocked})/P_{unblocked}\} = n \log[Zn^{2+}] - n \log K_{Zn}. \quad (5)$$

Fig. 4 shows Hill plots according to Eq. 5 of P_{norm} and P_{burst} measurements for Zn^{2+} titrations of dog heart Na^+ channels at −70 mV and calf heart Na^+ channels at −50 mV. The results indicate that the two methods for measuring $P_{unblocked}$ are nearly identical except that the P_{burst} method consistently results in slightly higher probability values than the P_{norm} method. This implies that the interaction of Zn^{2+} with closed states of the channel does not markedly affect the closed-open gating equilibria under the present conditions. Linear regression fits of the data in Fig. 4 to Eq. 5 yields values of $n = 1.17$, $K_{Zn} = 18$ μM for dog (□) and $n = 1.09$, $K_{Zn} = 29$ μM for calf (○) using the P_{norm} data, and $n = 1.08$, $K_{Zn} = 22$ μM for dog (■) and $n = 1.07$, $K_{Zn} = 24$ μM for calf (●) using the P_{burst} data. Because the measured values of Hill coefficients are very close to 1.0, we may proceed under the reasonable assumption that this process is approximated by the following one-site equilibrium:

$$\text{(Zn-unblocked) O} + Zn \overset{K_{Zn}}{\rightleftharpoons} \text{S} \cdot Zn \text{ (Zn-blocked substate)}, \quad (6)$$

where K_{Zn} is the equilibrium dissociation constant for Zn^{2+}

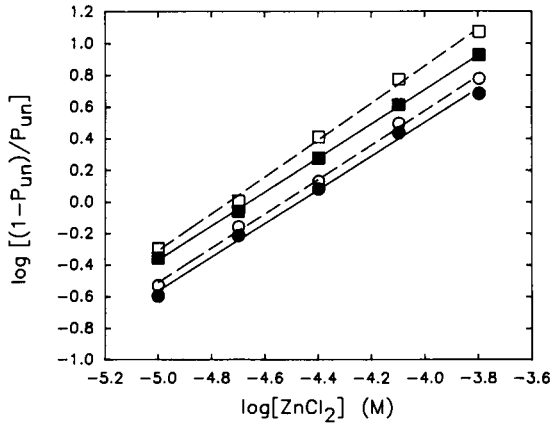

FIGURE 4. Hill plots of Zn^{2+} titrations in the absence of STX. The probability that a channel is not occupied by Zn^{2+}, $P_{unblocked}$, is plotted according to a linearized version of the Hill equation (Eq. 5). (□, ■) dog heart, $V = -70$ mV; (○, ●) calf heart, $V = -50$ mV. Two different methods were used to calculate $P_{unblocked}$ as discussed in the text: (□, ○) P_{norm}; (■, ●) P_{burst}. Parameters for linear regression fits to Eq. 5 are given in the text. The same data at −50 mV for the calf Na^+ channel are also plotted in nonlinearized form in Fig. 3 *B*.

Zn^{2+} relieves blockade by STX in a competitive fashion

The equilibria of Eqs. 1 and 6 describe the binding of STX and Zn^{2+} to a site on the open or unblocked channel that results either in a complete closure (Eq. 1) or a substate closure (Eq. 6). We now address the question of whether the binding of STX and Zn^{2+} is competitive; i.e., whether the binding of one ligand mutually excludes binding of the other ligand. Such a competitive interaction between two blocking molecules has been previously described for charybdotoxin and TEA^+ block of a large conductance Ca^{2+}-activated K^+ channel (Miller, 1988), where both the charybdotoxin peptide and TEA^+ are believed to block by binding to overlapping external sites in the outer mouth of this K^+ channel. Analysis of this latter interaction was facilitated by the ability to identify longed blocked periods induced by charybdotoxin as residence times of the toxin and the ability to easily quantitate the reduction in current amplitude associated with the fast-blocking effect of TEA^+. This type of analysis is somewhat more complicated in the present case because both STX and Zn^{2+} induce discrete closing events. However, at −50 mV, the two blocking processes have well separated time constants, with the lifetime of the Zn^{2+}-induced substrate in the range of 15–25 ms (Schild et al., 1991) and the lifetime of STX-induced closures equal to 3.6 s. Thus, if there is a competitive binding interaction between Zn^{2+} and STX, the addition of increasing concentrations of Zn^{2+} to a channel exposed to a fixed concentration of STX should result in a decreased frequency of longer duration STX-blocked events with a corresponding increase in the proportion of time spent in Zn^{2+}-dependent flickering.

Examples of single-channel records from such an experiment are shown in Fig. 5 where traces of a single calf heart Na^+ channel exposed to 100 nM STX are shown in the absence of Zn^{2+} and after the addition of 40 μM and 160 μM $ZnCl_2$. In these records, STX-blocked events are defined as dwell times in the fully closed state which are >0.5 s. STX-unblocked events denoted by solid lines above the traces, are thus identified as opening bursts between such STX-blocked events. These records show that the frequency of STX-blocking events diminishes and the average duration of STX-unblocked events dramatically lengthens as the Zn^{2+} concentration is raised. To quantitate this relief of STX blockade by Zn^{2+}, we measured the time-averaged probability, P_B, that a channel resides in STX-blocked states. The solid symbols in Fig. 3, *A* and *B*, correspond to the ratio $P_B(+Zn)/P_B(-Zn)$, which is the probability of being in the STX-blocked state in the presence of Zn^{2+} normalized to that in the absence of Zn^{2+}. This analysis shows

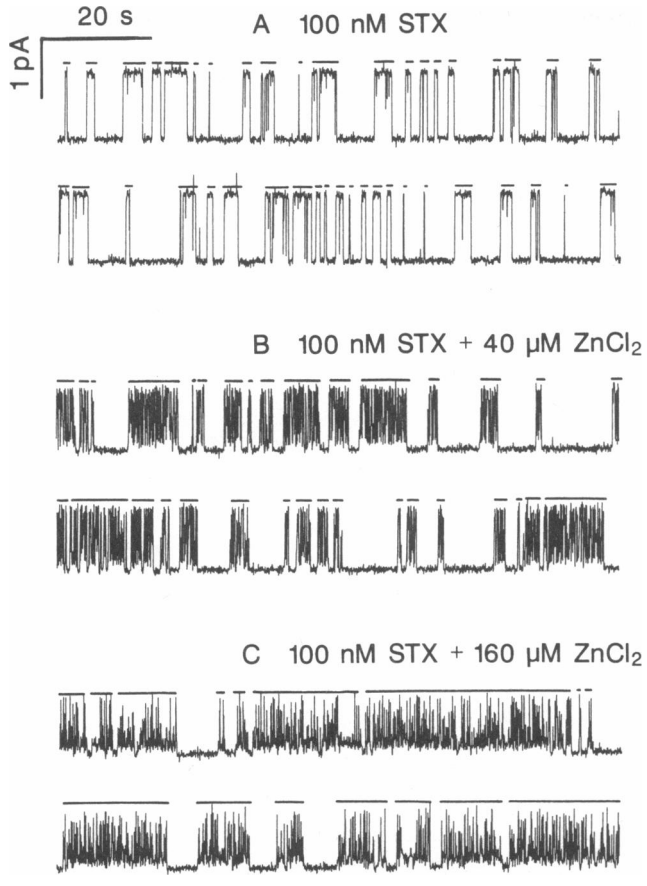

FIGURE 5. Effect of Zn^{2+} on STX block of a single BTX-modified Na^+ channel. Parts *A*, *B*, and *C* each show a pair of continuous current records from the same calf heart Na^+ channel at $V = -50$ mV in the presence of 100 nM external STX and 0 (*A*), 40 μM (*B*), and 160 μM (*C*) external $ZnCl_2$. Solid lines drawn above the records identify burst dwell times when the channel is not occupied by STX defined by full closures lasting longer that 0.5 s as described in the text. The records illustrate that the average dwell time in the STX-unblocked state lengthens with increasing $[Zn^{2+}]$, whereas the average dwell time in the STX-blocked state remains essentially constant.

that the addition of increasing Zn^{2+} essentially results in the complete relief of STX block.

To examine the quantitative implications of simple competition predicted by the simultaneous equilibria of Eqs. 1 and 6, we derived the theoretical dependence of $P_B(+Zn)/P_B(-Zn)$ on $[Zn^{2+}]$ by algebraic manipulation of equilibrium mass action expressions for this probability ratio:

$$P_B(+Zn)/P_B(-Zn) = K_{app}/([Zn^{2+}] + K_{app}), \quad (7)$$

where K_{app} is an apparent equilibrium dissociation constant for Zn^{2+} which is equal to:

$$K_{app} = K_{Zn}(1 + [STX]/K_{STX}). \quad (8)$$

Eq. 8 is the familiar expression for the apparent dissociation constant of a ligand (Zn^{2+}) in the presence of a competitive inhibitor (STX) that is commonly encountered in enzyme kinetics and Scatchard plot analysis of one-site binding reactions. Because we previously obtained values for K_{STX} and K_{Zn} for both dog and calf Na^+ channels from direct titration of their independent blocking activity, we may compare the theoretical predictions of Eqs. 7 and 8 with the measured values of $P_B(+Zn)/P_B(-Zn)$. This comparison is indicated by the right-most solid line curves in Fig. 3, *A* and *B*, which are in reasonable agreement with the actual data, given the experimental uncertainty. Most significantly, there is a definite shift of the titration for Zn^{2+}-relief of STX block to higher Zn^{2+} concentrations than the titration of Zn^{2+} block in the absence of STX. This shift of the equilibrium dose-response relationship is strong evidence of binding competition between Zn^{2+} and STX as predicted by Eqs. 1 and 6.

Kinetic predictions of binding competition at a single site

As previously noted by Miller (1988), Eqs. 1 and 6 make certain predictions regarding the dependence of the blocking kinetics of the slowly binding toxin, STX, on the concentration of the faster blocking ligand, Zn^{2+}. Probability histograms of STX-blocked and STX-unblocked dwell times are expected to be exponential with the following lifetime relationships for STX-blocked (τ_B) and STX-unblocked (τ_U) states expressed as ratios of the respective lifetimes in the presence of Zn^{2+} to that in the absence of Zn^{2+}:

$$\tau_B(+Zn)/\tau_B(-Zn) = 1 \quad (9)$$

$$\tau_U(+Zn)/\tau_U(-Zn) = 1 + [Zn]/K_{Zn}. \quad (10)$$

Eq. 9 reflects the fact that if Zn^{2+} and STX are mutually exclusive, then Zn^{2+} cannot affect the STX-dissociation rate, because both ligands cannot be bound simultaneously. Eq. 10 indicates that the average waiting time for a channel to be occupied by STX linearly increases as a function of the competitor concentration.

Fig. 6 shows cumulative dwell time histograms of STX-blocking events collected from a single calf heart Na^+ channel in the presence of 100 nM STX before and after the addition of 80 μM $ZnCl_2$. This figure illustrates our general finding of exponential populations of STX blocked and unblocked dwell times in the absence and presence of Zn^{2+}. The experiment of Fig. 6 also shows that the lifetime of the STX-blocked state was essen-

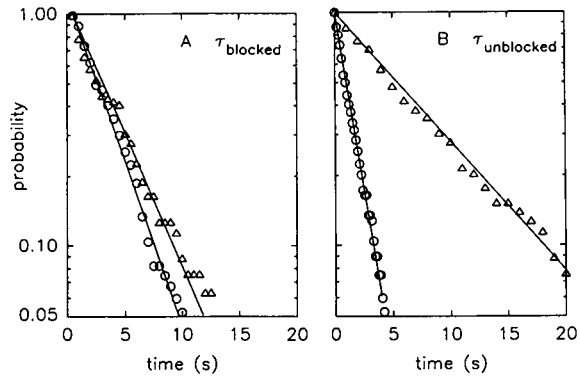

FIGURE 6. Cumulative dwell-time histograms of STX blocking events in the absence and presence of Zn^{2+}. Data were collected from a single BTX-modified calf heart Na^+ channel at −50 mV in the presence of 100 nM external STX without $ZnCl_2$ (○) or with 80 μM external $ZnCl_2$ (△). The ordinate is the probability that an event is longer than or equal to a duration on the abscissa. Populations of STX-blocked (*A*) or STX-unblocked (*B*) dwell times contained 79–134 events. Solid lines are drawn according to single time constants of: (*A*) ○, 3.1 s; △, 3.8 s; (*B*) ○, 1.4 s; △, 7.9 s.

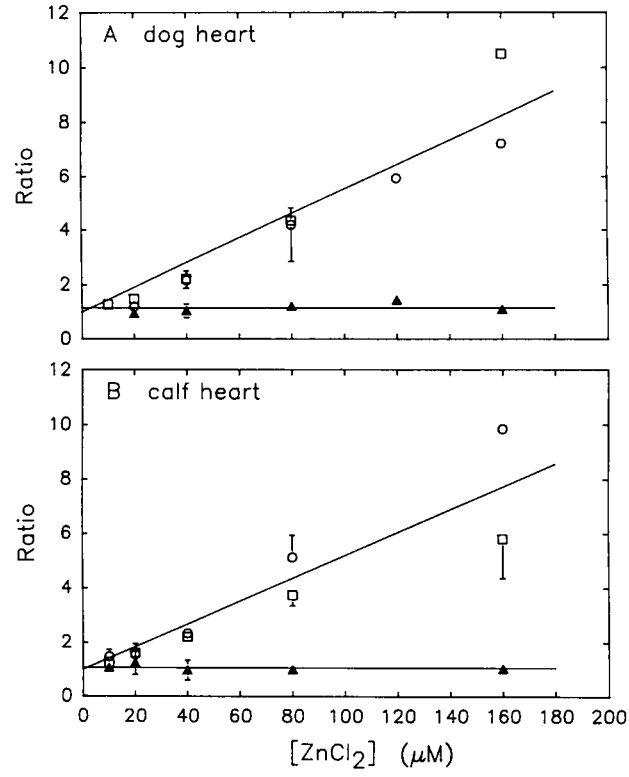

FIGURE 7 Binding competition between Zn^{2+} and STX demonstrated by kinetic measurements. Mean dwell times of STX-blocked or STX-unblocked events were measured in the presence of 100 or 200 nM STX in the absence and presence of various Zn^{2+} concentrations. The results are plotted as the ratio of STX-blocked or STX-unblocked dwell times in the presence of Zn^{2+} to that measured for the same channel in the absence of Zn^{2+}: (▲), $\tau_B(+Zn)/\tau_B(-Zn)$; (○), $\tau_U(+Zn)/\tau_U(-Zn)$. The reciprocal of the equilibrium probability of being unoccupied by Zn^{2+}, $1/P_{unblocked}$ (□) is also plotted for the same experiments. The solid lines represent expected behavior of a one-site binding competition model described by Eq. 9 (▲) and Eqs. 10 and 11 (○, □) with parameters given in the text. Data points with error bars represent means and standard deviations for three or four bilayers, where the error was larger than the symbol.

tially unaffected by the addition of 80 μM Zn^{2+} (Fig. 6*A*), whereas the lifetime of the STX-unblocked state increased 5.5-fold in the presence of Zn^{2+} (Fig. 6*B*).

Results from many such experiments are summarized in Fig. 7 where the experimental lifetime ratios of Eqs. 9 and 10 are plotted as a function of $[Zn^{2+}]$ for both calf and dog Na^+ channels. These results show that the observed lifetime ratio of the STX-blocked state is independent of Zn^{2+} and is very close to 1.0 for both Na^+ channels, with mean $\tau_B(+Zn)/\tau_B(-Zn)$ values of 1.1 ± 0.2 (± SD, $n = 5$) for dog (Fig. 7*A*) and 1.1 ± 0.1 (± SD, $n = 5$) for calf (Fig. 7*B*). Fig. 7 also shows that the corresponding lifetime ratio of the STX-unblocked state, $\tau_U(+Zn)/\tau_U(-Zn)$, closely follows the predicted linear relationship of Eq. 10. Because Eq. 10 has the same $[Zn^{2+}]$-dependence as the Zn^{2+} blocking reaction in the absence of STX, Fig. 7 also plots the reciprocal of the probability that a channel is unblocked by Zn^{2+} in the absence of STX ($1/P_{norm}$ or $1/P_{burst}$), which is simply the reciprocal of Eq. 4 with $n = 1$:

$$1/P_{unblocked} = 1 + [Zn^{2+}]/K_{Zn}. \quad (11)$$

This comparison shows that the equilibrium probability, $1/P_{unblocked}$, and the lifetime ratio, $\tau_U(+Zn)/\tau_U(-Zn)$, agree within experimental error and appear to follow a similar relationship. Linear regression fits of the combined data in Fig. 7 to Eqs. 10 and 11 give K_{Zn} values of 22 μM for dog and 24 μM for calf.

Iodoacetamide treatment abolishes Zn^{2+} block and modifies the blocking kinetics of STX

Results of the preceding analysis are compatible with the idea that binding of Zn^{2+} to BTX-modified heart Na^+ channels in a substate-inducing mode involves the contribution of certain residues in common with those involved in STX-binding and block. Experiments of Worley et al. (1986) previously suggested that binding of Ca^{2+} and STX involved common aspartate or glutamate residues, because treatment of brain Na^+ channels with the carboxyl methylating reagent, trimethyloxonium, was shown to abolish STX block and greatly reduce the

blocking effect of Ca^{2+}. The unique selectivity of TTX-insensitive Na^+ channel subtypes for the group IIB metals, Cd^{2+} and Zn^{2+}, suggests that there is a substitution of certain functional groups within the divalent cation binding site that gives rise to the enhanced affinity for these particular metal ions. A review of the crystal structures of known Zn^{2+}-binding sites in various proteins reveals that Zn^{2+} ions are usually liganded to the functional groups of aspartate, glutamate, histidine, and cysteine in a distorted four- or five-coordinate geometry (Creighton, 1983; Brown et al., 1983). Because carboxyl groups of aspartate and glutamate residues are also widely found to be involved in binding of nongroup IIB metals such as Ca^{2+}, it appears that the imidazole group of histidine and the sulfhydryl group of cysteine are responsible for conferring selectivity toward Cd^{2+} and Zn^{2+}. On this basis, specific chemical modifications of these latter residues is a possible approach toward identification of the Zn^{2+} binding site in cardiac Na^+ channels.

During the course of our work, Rogart et al. (1989) reported the primary sequence of a putative STX-insensitive Na^+ channel subtype from rat heart and Noda et al. (1989) identified a critical glutamate residue (glu387) in the primary sequence of the rat brain type II Na^+ channel, which abolished TTX and STX blockade when changed to a glutamine. Using this information, we examined the primary sequence of various Na^+ channel subtypes in the region of glu387, to see whether there is a histidine or cysteine residue(s) unique to the heart subtype, which may partially account for high-affinity Zn^{2+} binding.

Fig. 8 shows an alignment of 23 residues of primary sequence of various cloned Na^+ channels centered around the glu387 residue implicated in STX binding in rat brain type II Na^+ channels (Noda et al., 1989). The boxed identical residues indicate a high degree of absolute conservation in this region, with substitutions observed at only six positions. The sequence of the rat heart channel (Rogart et al., 1989) includes a unique cysteine group at position 385 in Fig. 8 (position 374 using the rat heart numbering sequence) which is only two residues away from glu387. This coincidence prompted us to focus on sulfhydryl modifying reagents, to determine whether such reagents would affect the ability of Zn^{2+} to induce flickering substate behavior in calf heart Na^+ channels.

Fig. 9 shows such an experiment, where we tested the ability of iodoacetamide (IAA) to modify Zn^{2+}-blocking behavior. At neutral pH and 22°C, IAA is a highly selective alkylating reagent for cysteine thiol groups although alkylation of other residues is also possible under harsher conditions (Strauss, 1984). Fig. 9 *A* and *B*,

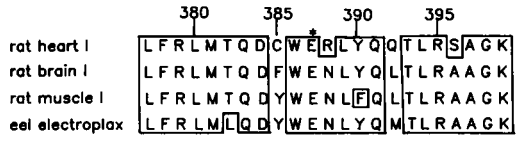

FIGURE 8 Comparison of the primary sequence of various cloned Na^+ channel subtypes in the putative region proposed to be involved in TTX and STX binding. The numbering sequence of residues is based on that for the type II Na^+ channel from rat brain as reported by Noda et al. (1986). The compared sequences are taken from: rat heart I (Rogart et al., 1989), rat brain I (Noda et al., 1986), rat muscle I (Trimmer et al., 1989) and eel electroplax (Noda et al., 1984). The rat heart sequence is also identical to a putative TTX-insensitve Na^+ channel subtype isolated from denervated rat skeletal muscle (Kallen et al., 1990). The rat brain I sequence in this region is identical to the type II subtype from rat brain (Noda et al., 1986). A type III subtype from rat brain has also been reported which differs in this region from rat brain type I and II by a tyrosine (Y) at position 385 (Kayano et al., 1988). Boxed residues indicate regions of identity among the various subtypes and the starred glutamate (E) at position 387 indicates the position where a mutation to glutamine has been shown to abolish TTX and STX sensitivity (Noda et al., 1989).

show records from the same calf heart Na^+ channel before and after the addition of 40 μM external Zn^{2+}. After flickering block was induced by addition of Zn^{2+}, 5 mM IAA was added to the external chamber and the channel was continuously monitored for any observable changes. At ~20 min after the addition of IAA, the fast-flickering behavior abruptly ceased (Fig. 9 *C*) and the channel activity appeared similar to that of control records in the absence of Zn^{2+} with a slightly reduced conductance, 19 pS instead of 23 pS measured for the control channel. We have successfully observed such an effect of IAA for six different calf heart Na^+ channels. This effect was observed in every attempt, except in cases where the bilayer broke during the IAA treatment. In each successful experiment, once the IAA-effect occurred, continued monitoring of the channel for up to one hour did not reveal any resumption of Zn^{2+} blocking activity suggesting that an irreversible modification of the channel had occurred.

To test whether abolition of Zn^{2+} block by IAA was also accompanied by modification of STX-blocking kinetics, 400 nM STX was added to the external chamber of six different IAA-modified calf Na^+ channels. Fig. 9 *D* shows that addition of STX induced blocking behavior in the IAA-modified channel of this experiment; however, the channel was less sensitive to STX block than control channels. For example, the IAA-modified channel of Fig. 9 *D* exhibited a probability of 0.38 of residing in the STX-blocked state in the presence of 400 nM STX compared to an expected blocking probability of 0.93 at this STX concentration for a site that normally exhibits a

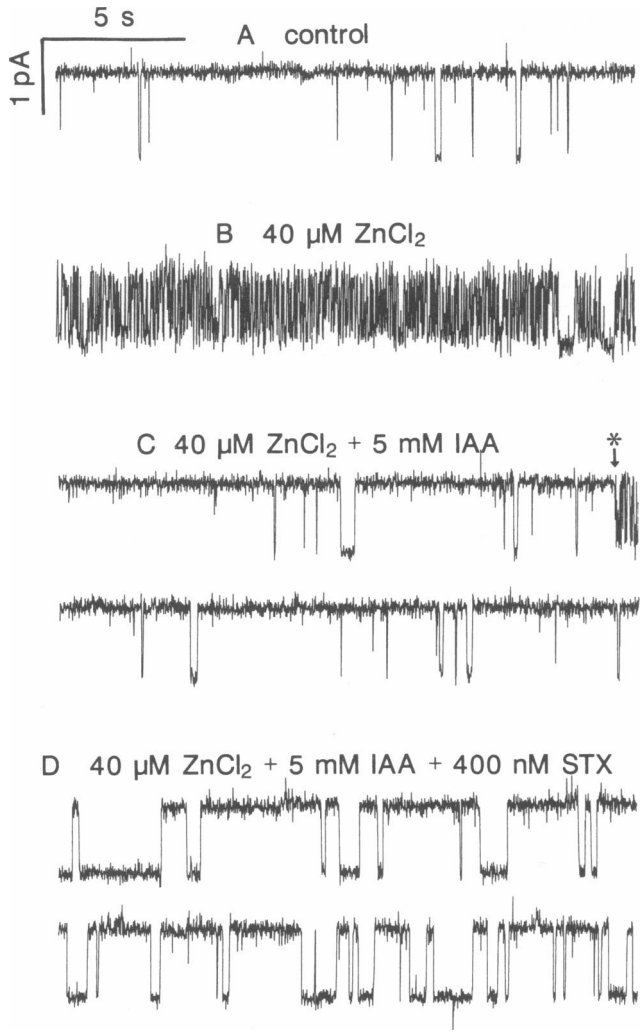

FIGURE 9 Abolition of Zn^{2+} block and modification of STX blocking kinetics by iodoacetamide. Current records from the same calf heart Na^+ channel are shown under various sequential conditions. Real time proceeds from right to left in these records. (*A*) Control trace at $V = -50$ mV in the presence of symmetrical 0.2 M NaCl, 10 mM MOPS-NaOH, pH 7.4. (*B*) Record of flickering block activity after the addition of 40 μM external $ZnCl_2$. (*C*) 5 mM iodoacetamide (IAA) was added to the external chamber in the presence of 40 μM $ZnCl_2$ and the channel record was monitored for observable changes. ~20 min after the addition of IAA, the Zn^{2+}-dependent flickering activity abruptly ceased at the starrred arrow and the channel exhibited behavior similar to that in the control record of *A* with a slight reduction in unitary conductance. (*D*) After the IAA effect had occurred, 400 nM STX was added to the external chamber to record STX blocking activity.

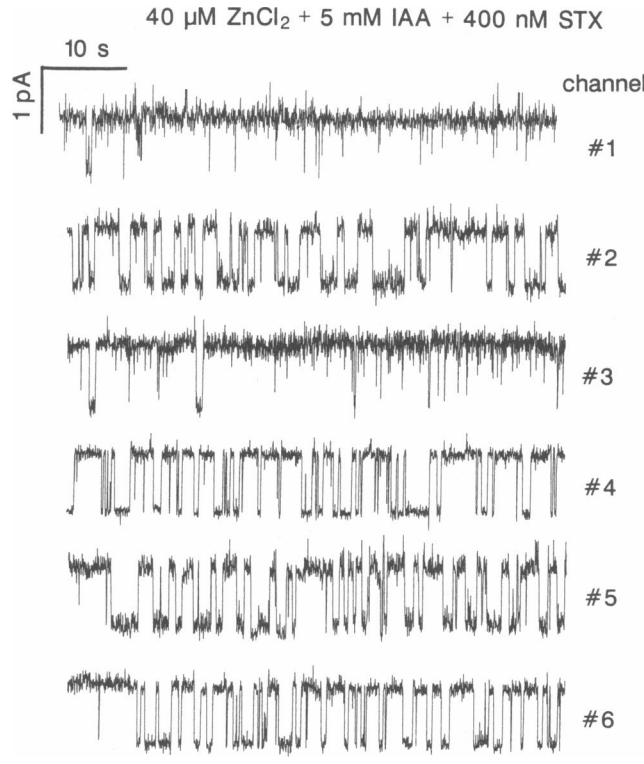

FIGURE 10 Iodoacetamide-modified Na^+ channels exhibit two types of behavior in the presence of STX. Records of six different calf heart Na^+ channels (#1–#6) are shown after Zn^{2+}-blocking activity was abolished by 5 mM IAA and 400 nM STX was added to monitor STX blocking activity. Channels #1 and #3 appeared to be completely insensitive to STX whereas channels #2, #4, #5, and #6 exhibited similar STX-induced blocking activity with low affinity for STX.

dissociation constant of 29 nM for STX (Table 1). For the six IAA-modified channels we examined, two distinct types of behavior were observed with respect to STX as illustrated in Fig. 10. The records of channels #1 and #3 in this figure, indicate that 400 nM STX failed to produce any significant blocking activity, as if these channels were completely STX-resistant. For the other channels, #2, #4, #5, and #6 in Fig. 10, 400 nM STX produced a similar pattern of discrete but unusually brief blocking events (mean blocked time = 0.70 ± 0.09 s [±SE, $n = 4$] for IAA-modified vs. 3.6 ± 0.3 s [±SE, $n = 6$] for control). Quantitative analysis of STX blocking kinetics in these latter four channels showed that the dwell-time histograms of blocked and unblocked events were monoexponential as predicted by Eq. 1 (not shown); however both the STX-association and dissociation rate constants were altered as summarized in Table 1. These results indicate that for the four channels that were still blocked by STX after IAA-modification, the STX dissociation rate was increased 5.2-fold and the STX association rate constant was reduced 3.8-fold, resulting in a 20-fold lower affinity of STX (K_{STX} = 570 nM for IAA-treated vs. 29 nM for control).

DISCUSSION

Competitive nature of the Zn^{2+}/STX^{2+} interaction

Our results with BTX-modified Na^+ channels from dog and calf heart provide two independent lines of evidence suggesting that Zn^{2+} binds to a subsite of the TTX/STX receptor site. The first piece of evidence, developed from kinetic and equilibrium analysis, is the competitive nature of the binding interaction between STX and Zn^{2+}. The blocking behavior of both the guanidinium toxin, STX, and the divalent cation, Zn^{2+}, are individually well-described by a single-site process. This behavior, confirmed here for STX block of calf heart Na^+ channels, has been previously documented for Na^+ channels from rat brain, dog brain, rat muscle, rat heart and dog heart by various laboratories using TTX, STX, and STX derivatives (French et al., 1984; Moczydlowski et al., 1984*a*, *b*; Green et al., 1987; Guo et al., 1987; Dugas et al., 1989). Substate block of BTX-modified Na^+ channels from dog heart external Zn^{2+} in the micromolar range has also been previously analyzed in terms of a one-site process (Schild et al., 1991). The present comparison of equilibrium titrations of flickering Zn^{2+} block of dog and calf heart Na^+ channels shows that these two species has very similar blocking affinity for Zn^{2+} at −50 mV: K_{Zn} = 18–22 μM for dog and K_{Zn} = 27–34 μM for calf, with the range of values given for several different methods of analysis as discussed in the text. When external Zn^{2+} was added to a single channel undergoing reversible block by STX, we observed a dramatic relief of STX block as measured by a decrease in the frequency of characteristic STX-blocking events (Fig. 5). Our kinetic analysis of this behavior is consistent with the predictions of a scheme involving the binding of two ligands at a common site. For the dog heart Na^+ channel, we obtained a value of K_{Zn} = 26 μM from the lifetime increase of the STX-unblocked state; the analogous result for the calf heart Na^+ channel is K_{Zn} = 19 μM. Because the two processes of Zn^{2+} block and Zn^{2+}-dependent lengthening of the STX-unblocked state exhibit a very similar dissociation constant for Zn^{2+}, it is likely that the same Zn^{2+} binding site that mediates flickering substate block also mediates binding competition with STX.

Similar results have been obtained for BTX-modified Na^+ channels by two other laboratories in the case of Zn^{2+} competition with STX at low ionic strength (20 mM NaCl) in dog brain Na^+ channels (Green et al., 1987) and Ca^{2+} competition with STX block at a higher ionic strength (125 mM NaCl) in rat brain Na^+ channels (Worley et al., 1986; Krueger et al., 1986). A unique aspect of our experiments is the correlation of Zn^{2+}/STX competition with Zn^{2+} binding to a site that induces a subconductance conformation. Such mechanistic detail is not presently available in other Na^+ channel subtypes where divalent cations induce a fast block appearing as a lower conductance due to lack of resolution of brief events. A second unique aspect of our results is the use of micromolar concentrations of the divalent cation Zn^{2+} at a relatively high ionic strength of 0.2 M NaCl. Mammalian heart Na^{2+} channels have the advantage that binding competition between Zn^{2+} and STX can be observed without significantly affecting negative surface potential by the screening of negative surface charges accompanied by large changes in ionic strength. This is especially relevant to STX/TTX binding because much evidence indicates that a negative surface potential markedly influences binding of these toxins to Na^+ channels (Henderson et al., 1974; Hille et al., 1975; Grissmer, 1984; Strichartz et al., 1986; Green et al., 1987; Ravindran and Moczydlowski, 1989; Cai and Jordan, 1990). The effect of divalent cations on the negative surface potential can be assessed by the Gouy-Chapman theory of surface charge (McLaughlin et al., 1989) which has been successfully used to model the effect of NaCl on TTX and STX binding (Green et al., 1987; Ravindran and Moczydlowski. 1989; Cai and Jordan, 1990). If the negative surface charge density in the vicinity of the STX binding site is on the order of $1e^-/400\ A^2$ as estimated previously (Green et al., 1987; Ravindran and Moczydlowski, 1989), the Grahame equation (Grahame, 1947) predicts that the addition of 1 mM XCl_2 at an ionic strength of 0.2 M NaCl only reduces the surface potential from −36 to −35 mV. From this model of the surface charge effect, it is therefore clear that the addition of 40–80 μM Zn^{2+} (which produced a significant relief of STX block) is too low to have any significant screening effect. On this basis, our results provide strong support for a mechanism involving direct binding competition between STX and Zn^{2+}.

The second line of evidence for Zn^{2+} binding to a subsite of the STX-receptor site in heart Na^+ channels involves chemical modification experiments with the sulfhydryl reagent, IAA. Treatment of single BTX-modified Na^+ channels with this reagent resulted in virtually complete elimination of discrete Zn^{2+}-induced blocking activity accompanied by modification of STX blocking kinetics. This result suggests that a reduced sulfhydryl group is involved in coordination of Zn^{2+} and that alkylation of this sulfhydryl group with IAA sterically interferes with the normal mode of STX binding. In four out of six IAA-modified channels, the STX binding affinity was reduced 20-fold. However, in two IAA-treated Na^+ channels, the blocking action of STX

appeared to be completely eliminated. A possible interpretation of this latter observation is that alkylation of a second sulfhydryl group by IAA completely obstructs STX binding and renders the heart Na^+ channel toxin-insensitive. Alternatively, it is also possible that alkylation of a single sulfhydryl group could result in two different conformations of the STX-binding site which would either be capable of binding STX weakly or not at all. The precedent for this latter possibility is the fact that sulfhydryl group modification has sometimes been observed to result in the exposure of previously buried residues, suggesting partial protein unfolding (Strauss, 1984).

Although we interpret the chemical modification experiment with IAA as evidence for overlapping locations of the STX and Zn^{2+} binding sites, one might argue that the effects of IAA are the result of an indirect conformational change and that mutually exclusive binding of Zn^{2+} and STX^{2+} is due to electrostatic repulsion between two doubly charged cations. For such a mechanism involving coulombic repulsion, one can estimate the minimum distance between a bound Zn^{2+} ion and STX^{2+} necessary to result in an immeasurably low affinity for STX^{2+}. This calculation can be modeled after the work of Russel and Fersht (1987) who measured the change in pK_a of the histidine-64 group of subtilisin caused by the introduction of neighboring charged residues via site-specific mutagenesis. For such an interaction between two charged species on a protein surface, Coulomb's law and the standard relationship between free energy and an equilibrium constant can be used to derive the following expression for the change in the negative logarithm (base 10) of the dissociation binding constant, ΔpK_d, for STX:

$$\Delta pK_d = z_1 z_2 e^2 / 2.3026(4\pi k T \epsilon_0 \epsilon d) \qquad (12)$$

where z_1 in our case is the valence of $Zn^{2+} = +2$, z_2 is the valence of STX^{2+} assumed to be equivalent to a +2 point charge, e is the elementary charge, k is Boltzmann's constant, T is absolute temperature, ϵ_0 is the permittivity constant, ϵ is the dielectric constant, and d is the distance between Zn^{2+} and STX^{2+}. For the interaction of two monovalent groups on the surface of subtilisin, Russell and Fersht (1987) found that the apparent dielectric constant for this type of interaction was in the range of 40–90 with a mean value of 60, approximating a waterlike environment. Assuming a value of $\epsilon = 60$, we find that Eq. 12 predicts that a bound Zn^{2+} ion located at 4.1–5.5 Å from bound STX^{2+} would decrease the affinity of STX^{2+} by a factor of 10^3–10^4, placing it beyond an easily detectible range of measurement. We consider that 5.5 Å is the minimum possible separation for such a repulsive interaction, because negatively charged groups in the binding site of Zn^{2+} and STX^{2+} are likely to reduce the strength of the repulsion. Thus, even if one assumes a noncompetitive binding interaction between Zn^{2+} and STX^{2+}, in which the two charged ligands are structurally capable of binding simultaneously, but cannot readily do so because of mutual repulsion, one is forced to conclude that the binding sites for these two ligands must be physically close to one another, at a separation distance certainly on the order of the size of STX molecule.

A descriptive model of toxin block

In summary, we will present our current working model of the guanidinium toxin binding site of Na^+ channels elucidated by the present results and previous functional studies of the TTX/STX binding reaction in various Na^+ channel subtypes. It is known that this site mediates specific binding of three rather different molecules, TTX, STX, and in muscle, μ-conotoxin, with a high degree of recognition of subtle chemical changes in these molecules as observed in studies of toxin derivatives (Strichartz, 1984; Kao, 1986; Guo et al., 1987). Although various structural similarities in these three toxins can be identified, such as guanidinium and hydroxyl groups, it seems likely that the binding site must involve an extended domain that is large enough to bind each of these molecules and that allows for partial mutual overlap expected on the basis of three-way binding competition between TTX, STX, and μ-conotoxin. To achieve chemical specificity and high affinity, we expect that the binding of each of these molecules involves a large number of hydrogen bonds ($\sim$10) formed between the toxin and numerous functional groups of amino acid side chains or the peptide bond backbone. This is expected by analogy to that documented in three-dimensional structures of ligand-protein complexes of bacterial binding proteins for small neutral and charged molecules such as arabinose, galactose, leucine/valine, and sulfate (Quiocho et al., 1987). Evidence for such hydrogen bond interactions has previously been obtained from structure-activity studies of derivatives of TTX and STX that involve small changes of polar functional groups. This picture would predict that substitutions of many different amino acid residues in this site via mutagenesis should lower the affinity for toxin binding by disruption of one or more of these participating hydrogen bonds.

The site must also contain a number of negatively charged aspartate and glutamate groups within or adjacent to the site, to account for the negative surface charge that electrostatically influences the toxin binding reaction (Green et al., 1987; Ravindran and Moczydlowski, 1989) and to also account for the results of carboxyl group modification (Worley et al., 1986; Cha-

bala et al., 1986). (It is possible that sialic acid residues on attached carbohydrate groups [Miller et al., 1983] could comprise some of the local negative surface charge, although there is no available evidence to indicate that sialic acid directly affects TTX/STX binding.) One or several negatively charged groups could also be absolutely required for toxin binding. For example, one might imagine that a critical glutamate residue such as glu387 in the rat II brain Na^+ channel could form an obligate salt linkage with guanidinium groups in TTX, STX, or μ-conotoxin (arginine residues). This might explain why changing this particular group to a neutral glutamine group would completely abolish toxin sensitivity (Noda et al., 1989). In contrast, some of the neighboring carboxylate groups that enhance the toxin association rate or the channel conductance via a long-range surface charge effect are not absolutely essential for toxin binding because Chabala et al. (1986) found that it is possible to modify some of these carboxyl groups with retention of toxin block.

Much previous discussion has focused on the question of whether guanidinium toxin binding directly occludes ion flux or induces a conformational change that closes the channel. Whereas the evidence for direct occlusion by TTX or STX is rather weak, there is a strong likelihood that both of these mechanisms play a role. The fact that alkali cations that permeate the channel (particularly Na^+) and divalent cations that block the channel (e.g., Zn^{2+}), also compete for TTX/STX binding suggests to us that the toxin binding site is somewhere in the vestibule of the channel, such that the competing Na^+ site normally participates in permeation, perhaps in an early ion dehydration step as proposed by Strichartz (1984). Evidence that the bound toxin does not deeply extend into the narrow part of the channel pore includes the lack of interaction with internally added permeant or blocking ions and the lack of a direct interaction of the transmembrane electric field with the net charge of the toxin (Moczydlowski et al., 1984*b*; Green et al., 1987). Our model would also place the competitive Zn^{2+}-blocking site in the vestibule where it could mediate the subconductance effect (Schild et al., 1991) and compete with STX binding without completely occluding Na^+ flux.

Previous studies have rejected the notion that divalent cation blockers occupy a subsite of the TTX/STX binding site for several reasons. Krueger et al. (1986) argued that it is unlikely that the complex bonding interactions that occur between the toxin and the channel could also occur between a Ca^{2+} ion and the channel. We basically agree with this statement but propose that extensive sharing of bonding interactions is not necessary for direct Zn^{2+}/STX competition. In our view, all that is required is sharing of at least one common residue, such as a critical glutamate carboxyl group, as a ligand that participates in both Zn^{2+} or Ca^{2+} binding and STX binding. Thus, when either the inorganic cation or the toxin is bound, the other is excluded from binding. Using canine brain Na^+ channels, Green et al. (1987) found that there was kinetic competition between Zn^{2+} and STX, but placed the Zn^{2+} site at least 15 Å away from the STX site because there was no observable change in the voltage dependence of TTX/STX binding. We have not yet directly examined the effect of Zn^{2+} on the voltage dependence of STX block in heart Na^+ channels, so we do not know whether heart Na^+ channels differ from brain Na^+ channels in this respect. However, because only cardiac Na^+ channels have been thus far found to exhibit the flickering substate phenomenon induced by micromolar Zn^{2+}, it is possible that the Zn^{2+}-blocking site investigated in our work is structurally and/or spatially distinct from the Zn^{2+}-blocking site studied by Green et al. (1987).

Much evidence now supports the notion that TTX/STX binding results in conformational changes of the channel. For example, in BTX-modified Na^+ channels, the TTX/STX-binding site appears to be allosterically linked to voltage-sensing regions of the protein as revealed by voltage-dependent toxin binding (Moczydlowski, 1984*b*). There also appears to be an allosteric interaction between the BTX binding site (which is coupled to channel gating) and the TTX/STX binding site as observed by enhanced dissociation of BTX (Brown, 1986). In some Na^+ channels there is also a use-dependent effect of channel gating on TTX and STX binding (Cohen et al., 1981; Carmeliet, 1987; Huang et al., 1987; Lonnendonker, 1989) and an effect of STX on slow inactivation (Strichartz et al., 1986). These phenomena are indicative of allosteric interactions between the toxin-binding site and channel domains that are involved in gating. Because of these allosteric interactions, it seems quite reasonable to propose that binding to the guanidinium toxin receptor is also able to induce closing of the channel itself. This proposal is in harmony with the subconductance block by Zn^{2+} in heart Na^+ channels, which we have characterized as a Zn^{2+}-induced conformational change that affects the permeation process (Schild et al., 1991). Aside from the new evidence for a sulfhydryl group in the STX/Zn^{2+} site of a particular heart Na^+ channel, we feel that the most important implication of our current results is the correlation of STX binding with a Zn^{2+} site that mediates substate production. We believe that this correlation is the strongest evidence yet found that STX induces closure of the channel via a conformational change, an idea strongly embraced by Green et al. (1987).

In the preceding discussion, we have chosen to present

a narrow interpretation of many results obtained in experiments directed toward the mechanism of TTX/STX block. Critical readers will be quick to realize that many of our conclusions, such as direct Zn^{2+}/STX binding competition, would be refuted if the experimental pertubations induce conformational changes that closely mimic direct competition. For this reason, we acknowledge that the present mechanistic studies do not prove this speculative model of guanidinium toxin block, as neither will any single experiment involving site-specific mutagenesis. Rather, our knowledge of this fascinating toxin-channel interaction will continue to progress with the availability of increasingly refined functional and structural information.

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