Divalent Cation Competition with [³H]Saxitoxin Binding to Tetrodotoxin-resistant and -sensitive Sodium Channels

A Two-Site Structural Model of Ion/Toxin Interaction

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ABSTRACT Monovalent and divalent cations competitively displace tetrodotoxin and saxitoxin (STX) from their binding sites on nerve and skeletal muscle Na channels. Recent studies of cloned cardiac (toxin-resistant) and brain (toxin-sensitive) Na channels suggest important structural differences in their toxin and divalent cation binding sites. We used a partially purified preparation of sheep cardiac Na channels to compare monovalent and divalent cation competition and pH dependence of binding of [³H]STX between these toxin-resistant channels and toxin-sensitive channels in membranes prepared from rat brain. The effects of several chemical modifiers of amino acid groups were also compared. Toxin competition curves for Na^+ in heart and Cd^{2+} in brain yielded similar K_D values to measurements of equilibrium binding curves. The monovalent cation sequence for effectiveness of [³H]STX competition is the same for cardiac and brain Na channels, with similar K_I values for each ion and slopes of -1. The effectiveness sequence corresponds to unhydrated ion radii. For seven divalent cations tested (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Zn^{2+}) the sequence for [³H]STX competition was also similar. However, whereas all ions displaced [³H]STX from cardiac Na channels at lower concentrations, Cd^{2+} and Zn^{2+} did so at much lower concentrations. In addition, and by way of explication, the divalent ion competition curves for both brain and cardiac channels (except for Cd^{2+} and Zn^{2+} in heart and Zn^{2+} in brain) had slopes of less than -1, consistent with more than one interaction site. Two-site curves had statistically better fits than one-site curves. The derived values of K_I for the higher affinity sites were similar between the channel types, but the lower affinity K_I's were larger for heart. On the other hand, the slopes of competition curves for

Cd^{2+} and Zn^{2+} were close to -1, as if the cardiac Na channel had one dominant site of interaction or more than one site with similar values for K_I. pH titration of [^{3}H]STX binding to cardiac channels showed a pK_a of 5.5 and a slope of 0.6–0.9, compared with a pK_a of 5.1 and slope of 1 for brain channels. Tetramethyloxonium (TMO) treatment abolished [^{3}H]STX binding to cardiac and brain channels and STX protected channels, but the TMO effect was less dramatic for cardiac channels. Trinitrobenzene sulfonate preferentially abolished [^{3}H]STX binding to brain channels by action at an STX protected site. On the other hand n-ethylmaleimide (NEM) preferentially reduced the affinity of [^{3}H]STX binding to cardiac channels, and this was prevented by coincubation with STX. The NEM treatment also reduced the competition of Cd^{2+} for STX binding to cardiac channels. Diethylpyrocarbonate and phenylglyoxyl abolished binding to both channel types. We conclude that divalent ions act at two sites on the Na channel that affect STX binding. Although both channel types contain one or more carboxyl groups, the charged regions are not identical. The STX binding region in cardiac Na channels contains a unique site, probably a cysteine, that binds Cd^{2+} and Zn^{2+} with high affinity.

INTRODUCTION

Tetrodotoxin (TTX) and saxitoxin (STX) are naturally occurring, competitive, high-affinity ligands that block Na^+ channels. The toxins have been useful in determining the number and distribution of Na^+ channels in excitable cells (Ritchie and Rogart, 1977) and assisting in purification of the minute quantities of channel protein from cell membrane (Hille, 1992). Channel subtypes also have been classified according to their toxin affinity levels (Rogart, 1986). Because the toxins appear to bind and block at the outer mouth of the channel (Hille, 1992), they are valuable as probes of channel structure.

The toxin binding locus has several functional properties. A titrable carboxyl group with a pK_a of 5–6 is important, and this site was originally suggested to be the selectivity filter of the channel (Hille, 1975a). Toxins are displaced competitively by monovalent and divalent ions, implying overlapping sites for the ions and the toxins. Several chemical modifiers of amino acids, such as tetramethyloxonium (TMO) alter the binding of, and blockade by, the toxins (Shrager and Profera, 1973; Reed and Raftery, 1976; Spalding, 1980; Rack and Woll, 1984; Gülden and Vogel, 1985; Worley, French, and Krueger, 1986).

Several channel subtypes have been cloned (Noda, Ikeda, Kayano, Suzuki, Takeshima, Kurasaki, Takahashi, and Numa, 1986; Rogart, Cribbs, Muglia, Kephart, and Kaiser, 1989; Kallen, Sheng, Yang, Chen, Rogart, and Barchi, 1990). The channels include ~2,000 amino acids and form four homologous transmembrane regions, each containing six transmembrane segments. This structural information offers some insight into the toxin and ionic interactions. For each of the four homologous regions, the connecting sequences between transmembrane segments 5 and 6 are thought to fold into the membrane to create an outer channel vestibule and a pore-forming region (P region). When the amino acids of the P region are aligned according to the model of Guy and Conti (1990), three rings of charged residues can be identified. Studies using cloned Na channel α subunits expressed in *Xenopus* oocytes have shown that neutralization of several of the charged residues in these

rings can reduce or abolish toxin block (Terlau, Heinemann, Stuhmer, Pusch, Conti, Imoto, and Numa, 1991). A cysteine near the inner charged ring appears to contribute appreciably to the lower cardiac Na channel toxin affinity (Schild and Moczydlowski, 1991; Satin, Kyle, Chen, Bell, Cribbs, Fozzard, and Rogart, 1992a), while simultaneously participating in a high-affinity site for Cd^{2+}. The intermediate ring of the brain Na channel also has one more net negative charge than the cardiac channel because of a nonconserved arginine adjacent to the ring in the cardiac channel.

Toxin binding to cardiac channels has not been explored in as much detail as in nerve, partly because the lower affinity for toxin has made comparable studies difficult or impossible. We have developed a method of partial purification of cardiac Na^+ channels (Doyle and Winter, 1989) that permits binding studies to be done. We show evidence that the toxin binding to cardiac and brain channels is pH titrable, displaceable by cations, and modifiable by chemical reagents. However, significant differences exist in all three of these properties between the channel types: (a) the titrable carboxyl site shows a different pK_a; (b) Cd^{2+} and Zn^{2+} displace toxin at much lower concentrations; and (c) the cardiac channel does not respond to channel modifiers of lysine and cysteine side chains the same as nerve channels. The latter is consistent with the suggestion that a cysteine residue in the P region of repeat I is an important determinant of the differences in toxin and divalent ion interactions with the channels (Schild and Moczydlowski, 1991; Satin et al., 1992a).

In contrast to the differences between these channel subtypes, a similarity is revealed. In both cardiac and brain channels divalent cations exhibit two-site behavior in competition with toxin binding. We conclude that both channel types contain a site with affinity for divalent cations on the order of 100 μM. Both channel types contain a second site, with affinity on the order of 1 mM in brain and 10 mM in heart. Physical interpretations of the competitive interaction of divalent cations with [^{3}H]STX binding are presented in terms of possible steric and allosteric mechanisms. A structural model is presented in which two divalent cation binding sites correspond to two of three rings of charge in the channel vestibule/pore.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]STX was purchased from Amersham Corp. (Arlington Heights, IL) and unlabeled STX from Calbiochem Corp. (La Jolla, CA). TMO was from Fluka Chemical Corp. (Ronkonkoma, NY). Other protein modifying agents were from Sigma Chemical Co. (St. Louis, MO). The chloride salts of metal ions used were of reagent grade. Sheep hearts were obtained fresh from a local slaughterhouse.

Membrane Preparation

Sheep heart. Surface and t-tubular membranes from sheep heart ventricle highly enriched in TTX-resistant (TTX-R) STX binding receptors and depleted of TTX-sensitive (TTX-S) STX binding receptors were prepared as described in detail previously (Doyle and Winter, 1989), with some modification. The features of the preparative procedure are (a) extensive predissection of the heart, (b) controlled homogenization at moderate speeds and washing of the

dissected heart muscle, and (c) vigorous homogenization and production of sarcolemmal membranes by conventional methods.

(a) The interior (working) portion of wall of the left ventricle was the source of myocardial cells used for further processing. All processing was performed at 0–4°C.

(b) The material was ground twice with an electric meat grinder with 0.4-mm holes. The resulting paste was homogenized at an intermediate setting with a shaft homogenizer (model T185, 50% maximal rpm; Tekmar Co., Cincinnati, OH) for 30 s in 130 mM KCl, 30 mM HEPES-Tris, pH 7.4, and an array of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 mM EGTA, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, and 0.1 μg/ml pepstatin), which were used throughout the procedure. The homogenate was centrifuged at 600 g_{max} for 10 min. This step was repeated twice.

(c) The resulting pellet was homogenized vigorously (100% maximal rpm) three times for 30 s each in 300 mM sucrose, 10 mM HEPES-Tris, pH 7.4. The supernatant from a 10,000 g_{max} centrifugation was saved and the pellet was rehomogenized and recentrifuged in the same way. Both supernatants were then centrifuged at 27,500 or 54,000 g_{max} for 1 or 2 h to produce a pellet of crude microsomal membranes.

This pellet was washed once with 0.6 M KCl, and the resulting pellet was suspended in 42% sucrose, 400 mM KCl, 20 mM sodium pyrophosphate, 1 mM $MgCl_2$, and 10 mM HEPES-Tris, pH 7.4, and layered under a discontinuous sucrose density gradient with layers of 14, 28, and 34% sucrose in the same buffer. The gradient was centrifuged for 3 h at 141,000 g_{max}. Fractions above the 28 and 34% sucrose layers were collected, diluted fourfold with water, pelleted, raised in 300 mM sucrose, 10 mM HEPES-Tris to a concentration of 5–10 mg protein/ml, aliquotted, and stored at −70°C.

To produce membranes that were enriched in surface sarcolemma and were predominantly rightside-out, we used our previously reported approach (Doyle, Kamp, Palfrey, Miller, and Page, 1986), which was based on the method of Jones (1988). As a modification, to prepare membranes that contained a reduced amount of high affinity [^{3}H]STX binding sites, an initial high salt (0.6 M KCl) homogenization with a T185 shaft (Tekmar Co.) was used at low speed (15–20% maximal rpm) three times for 1 min each and followed by a low speed centrifugation (600 g for 10 min). The procedure was then continued as previously reported (Doyle et al., 1986).

Rat brain. A P$_3$ membrane fraction was prepared by modification of the method of Catterall, Morrow, and Hartshorne (1979). The brain was removed from a Sprague-Dawley rat and homogenized in 10 vol sucrose buffer (0.32 M sucrose, 10 mM HEPES-Tris, pH 7.4, 0.1 mM phenylmethanesulfonyl chloride, 1 mM EGTA, and 1 mM EDTA) by three 5-s bursts with a T185 ultra-turax (Tekmar Co.) at half-maximal speed. The resulting homogenate was sedimented at 1,000 g for 10 min. The supernatant was saved and the sediment was resuspended in 10 vol sucrose buffer with one 5-s half-maximal burst of the homogenizer and again sedimented at 1,000 g for 10 min. The two supernatants were combined and sedimented at 17,000 g for 60 min. The pellet was washed by resuspension with 10 strokes of a hand-held teflon/glass homogenizer in 5 vol sucrose buffer without EGTA or EDTA and sedimentation at 17,000 g for 60 min. The pellet was resuspended in sucrose buffer without EGTA or EDTA with 10 strokes of the hand-held homogenizer to a concentration of 5–10 mg protein/ml, aliquotted, and stored frozen at −70°C.

Lysis of membrane vesicles. Frozen membranes were thawed at room temperature. Sheep heart membranes were sedimented at 16,000 g for 30 min in an Eppendorf benchtop centrifuge. The pelleted membranes were raised in ice-cold 5 mM HEPES-Tris buffer, pH 7.4, to a concentration of 5–10 mg protein/ml and incubated on ice for 30 min before use in assay medium. Rat brain membranes were diluted 50-fold with ice-cold 5 mM HEPES-Tris buffer and incubated on ice for 30 min.

Binding of [³H]STX to membrane vesicles. The binding of [³H]STX to membrane vesicles was measured by filtration using Whatman GF/F glass fiber filters (Doyle, Brill, Wasserstrom, Karrison, and Page, 1985). The standard binding buffer in a volume of 0.2 ml for 100 µg of heart membrane protein and 1.0 ml for 10–20 µg of brain membrane protein was 200 mM choline chloride and 10 mM HEPES adjusted to pH 7.4 with Tris unless otherwise noted. The chloride salts of the mono- and divalent cations tested were substituted for choline chloride so that the ionic strength of the medium was kept constant. For heart membranes, saturation binding experiments were done over an [³H]STX concentration range of 0.5–50 nM. Displacement experiments performed at a single soaking concentration were done at ∼20 nM. Samples were incubated for 2 h in an ice bath. For brain membranes, the concentration range used for saturation binding experiments was 0.1–10 nM, and displacement experiments were done at ∼1.0 nM unless otherwise noted. Incubation time was 1 h on ice. Displacement experiments were designed so that the ratios [[³H]STX]/K_D for both heart and brain channels could be as near to one another as possible, given the added constraints that (*a*) to keep [[³H]STX] relatively constant from sample to sample, <2% of the total [³H]STX in the sample should be bound, and (*b*) the concentration of toxin receptor in the sample should be ≪ the K_D of the toxin for the receptor (Weiland and Molinoff, 1981). Nonspecific binding of [³H]STX was determined in the presence of 2 µM unlabeled STX for heart membranes and 1 µM for brain (cold wash). For equilibrium binding experiments cold washes were measured at four soaking concentrations. For competition experiments cold washes were measured at each point. All points were measured in duplicate.

Modification of membrane protein by chemical agents. Modification of membrane protein by the chemical agents TMO, phenylglyoxyl (PG), *p*-chloromercuribenzoate (PCMB), *n*-ethylmaleimide (NEM), trinitrobenzenesulfonate (TNBS), and diethylpyrocarbonate (DEPC) was performed for time periods and at temperatures, concentrations, and volumes, and in buffered media, as indicated in the figure legends and in the text. Reaction of membranes with TMO was allowed to go to completion in all cases since TMO is rapidly hydrolyzed in aqueous media (within minutes). Otherwise, after reaction by modifiers or when unlabeled STX was used in conjunction with a modifying agent as a means of demonstrating protection against modification at the [³H]STX binding site, reactants were removed from the medium before assay of [³H]STX binding by three dilutions to 28 ml followed by centrifugations to collect the membrane material. When time of reaction was a measured parameter, modification by NEM and PCMB was stopped by addition of an excess of D,L-cysteine, and modification by DEPC was stopped by an excess of histidine.

Data analysis. Saturation binding curves were fit by nonlinear regression analysis to the rectangular hyperbolic equation:

$$STX_{bound} = \frac{S_0[STX]}{K_D + [STX]} \qquad (1)$$

where S_0 is the maximum STX specifically bound and K_D is the equilibrium dissociation constant. In some instances, for graphic purposes, data were portrayed in Scatchard or double-reciprocal representations, with curves generated using the values of S_0 and K_D generated by the hyperbolic fits.

The value of a cation inhibition constant, K_I, using saturation binding analysis was determined as the mean of the values of the apparent K_D for STX in a set of saturation curves at various ionic concentrations using the relationship:

$$K_D^{apparent} = K_D \left(1 + \frac{[I]}{K_I} \right) \qquad (2)$$

where [I] is the concentration of the test cation and K_D is the equilibrium dissociation constant of STX in the absence of test cation.

Data from experiments measuring the displacement of labeled STX by cations or other agents were fit by nonlinear regression analysis to the equation:

$$\% \text{STX}_{\text{bound}} = \frac{1}{100}\left[\frac{A}{1 + \left(\frac{[I]}{EC_{50}^{(1)}}\right)} + \frac{100 - A}{1 + \left(\frac{[I]}{EC_{50}^{(2)}}\right)}\right] \quad (3)$$

where A is expressed in %. 100% bound was defined to be specifically bound toxin (total amount bound − amount bound in the presence of excess unlabeled toxin) in the absence of the test competitive agent. For single-site fits, $A = 100$. For cation–toxin interactions a model of simple competition between reversible ligands was assumed (i.e., the curve asymptotes to 0% occupancy of specific toxin sites at infinite cation concentration) and gave the best fits. In the case of inhibition of toxin binding by the irreversible (at least on the time scale of the assay system) protein modifying agents NEM and PCMB, best fits were obtained by allowing the % occupancy at infinite agent concentration to be variable.

Values of K_I were determined from the values of EC_{50} for single- and dual-site fits by the relation:

$$K_I = \left(\frac{EC_{50}}{1 + \frac{[\text{STX}]}{K_D}}\right) \quad (4)$$

where K_D is the equilibrium dissociation constant for STX in the absence of test competitor.

The slopes of displacement curves were determined as best fits of the relation:

$$\% \text{STX}_{\text{bound}} = \frac{1}{1 + \left(\frac{[I]}{EC_{50}}\right)^n} \quad (5)$$

where n = slope = pseudo Hill coefficient.

RESULTS

Previous investigators have used one of two approaches to measure the competition of various cations for the binding of [^{3}H]STX or [^{3}H]TTX to sodium channels. Several investigators (Reed and Raftery, 1976; Weigele and Barchi, 1978; Barchi and Weigele, 1979) produced a set of saturation curves, i.e., equilibrium binding curves at various concentrations of competing ion, and calculated the K_I for the ion from the shift in the apparent K_D that the ion produced. In contrast, other investigators (Henderson, Ritchie, and Strichartz, 1974; Lombet, Renaud, Chicheportiche, and Lazdunski, 1981) produced displacement curves, i.e., the reduction in toxin binding at a (nearly) constant concentration of free toxin as a function of increasing concentration of the ion of interest. If cation competition for toxin binding is simple (i.e., both agents compete for occupancy of a single identical binding site on a receptor), then the two approaches should give the same result. However, even if the interaction is more complex, both approaches may still give the same result, and either approach, depending upon the range of ligand concentrations and the analysis

used, may appear to divulge a simple competitive interaction (Klotz, 1985; Tomlinson and Hnatowich, 1988).

Since our membrane fractions from heart have a density of TTX-R [^{3}H]STX binding sites (~ 1 pmol/mg protein), that is on the order of 10-fold less than the density of TTX-S [^{3}H]STX sites in the nerve and muscle preparations, we found it expedient to measure ion competition by the latter method. However, to compare values for cation competition for toxin binding in heart with those in other tissues, to determine whether or not the competition was of a simple nature, and to determine values for those cations (e.g., Cd^{2+}) not previously reported for certain preparations, we measured competition both ways for several specific ions.
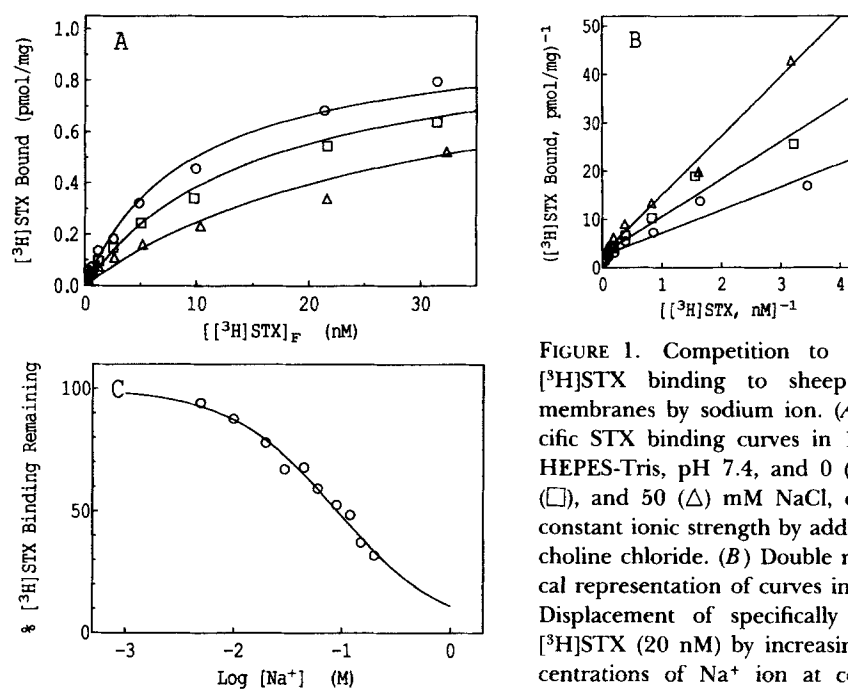

FIGURE 1. Competition to specific [^{3}H]STX binding to sheep heart membranes by sodium ion. (*A*) Specific STX binding curves in 10 mM HEPES-Tris, pH 7.4, and 0 (O), 25 (□), and 50 (△) mM NaCl, each at constant ionic strength by addition of choline chloride. (*B*) Double reciprocal representation of curves in *A*. (*C*) Displacement of specifically bound [^{3}H]STX (20 nM) by increasing concentrations of Na$^+$ ion at constant ionic strength (adjusted to 200 mM with choline chloride).

In Fig. 1 we show the result of measuring the apparent K_D of [^{3}H]STX binding to sheep heart membranes in the presence of 0, 25, and 50 mM NaCl. The value of the K_D is reduced with increasing Na$^+$ but the maximal binding is unchanged, indicating simple competition. The value of the K_I for sodium we calculate from these curves is 29.9 ± 4.5 mM. By comparison, when we measured the reduction in [^{3}H]STX binding to these membranes at a constant [^{3}H]STX equilibrium soaking concentration as a function of increasing Na concentration, we calculate a value of K_I for sodium of 34.3 ± 5.0 mM. A similar analysis of the competition of Na$^+$ for [^{3}H]STX binding to rat brain membranes gave values of 34.2 ± 1.8 mM by the former method and 26.0 ± 2.3 mM by the latter (data not shown).

In Fig. 2 we show the results of a similar analysis and comparison for the competition of Cd^{2+} for the binding of $[^3H]STX$ to rat brain synaptosomal membranes. From the values of the apparent K_D for $[^3H]STX$ binding in the presence of 0, 1, 2.5, and 3.75 mM Cd^{2+}, the K_I of Cd^{2+} competition was 619 ± 48 μM (Fig. 2 A). Reduction of $[^3H]STX$ binding as a function of increasing Cd^{2+} concentration at constant $[^3H]STX$ equilibrium soaking concentrations of either 0.87 or 9.8 nM gave a value of 510 ± 30 μM (Fig. 2 C).

pH Titration

Of primary interest is whether TTX-R $[^3H]STX$ binding sites such as those found in mammalian heart contain a pH-titrable, negatively charged residue as has been
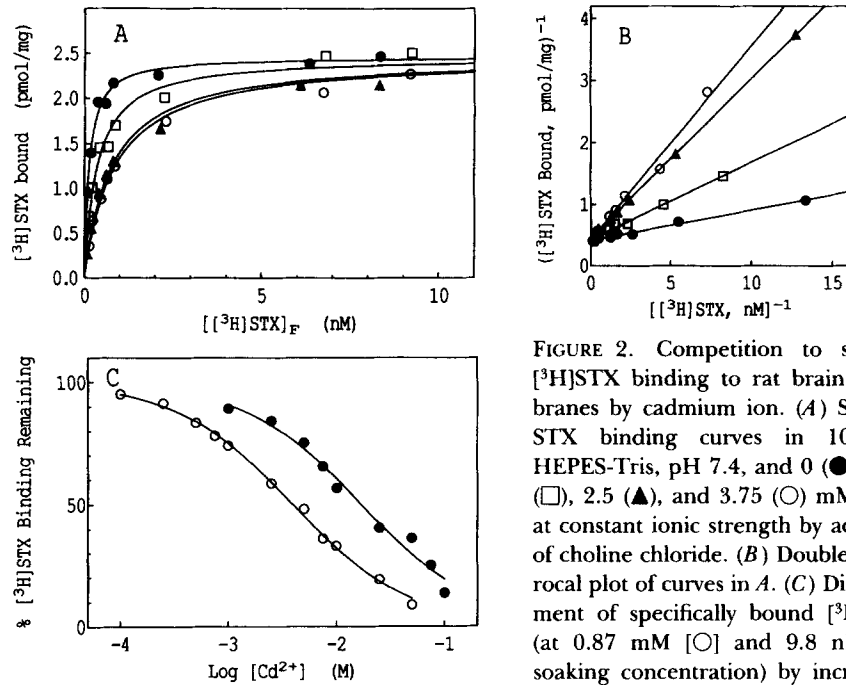

FIGURE 2. Competition to specific $[^3H]STX$ binding to rat brain membranes by cadmium ion. (A) Specific STX binding curves in 10 mM HEPES-Tris, pH 7.4, and 0 (●), 1.25 (□), 2.5 (▲), and 3.75 (○) mM Cd^{2+} at constant ionic strength by addition of choline chloride. (B) Double reciprocal plot of curves in A. (C) Displacement of specifically bound $[^3H]STX$ (at 0.87 mM [○] and 9.8 nM [●] soaking concentration) by increasing concentrations of Cd^{2+} ion at constant ionic strength.

demonstrated for TTX-S $[^3H]STX$ binding sites in other tissues (Hille, 1975a; Weigele and Barchi, 1978; Barchi and Weigele, 1979). When we compared the amount of $[^3H]STX$ binding with our heart and brain preparations as a function of pH we found that both types of binding sites contained a pH-titrable group with a pK_a in the range of 5.0–6.0 (Fig. 3). However, quantitative differences in the curves for the two subtypes were apparent. Whereas the pK_a value for the rat brain synaptosomes was 5.1, the pK_a value for sheep heart ventricle membranes was 5.5. In addition, the slopes of pH titration curves for rat brain membranes were reproducibly
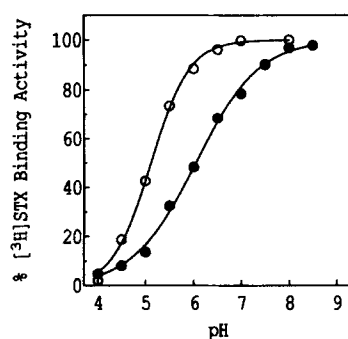
FIGURE 3. Effect of pH on specific binding of [³H]STX to sheep heart and rat brain membranes. (●) Specific binding of [³H]STX at 20 nM concentration to 0.5 mg/ml sheep heart membranes in 200 μl incubation buffer. Buffer contained 200 mM choline chloride, 25 mM citric acid, 25 mM maleic acid, 25 mM 3(N-morpholino)propane sulfonic acid (MOPS), and 25 mM Tris adjusted to various pH levels with HCl or TMA·OH. (○) Specific binding of[³H]STX at 0.9 nM concentration to 0.025 mg/ml rat brain membranes in 1 ml incubation buffer.

in the range of 1.1–1.3 ($n = 4$), whereas for heart membranes the slope values ranged from 0.6 to 0.9 ($n = 4$).

Effect of Cations on [³H]STX Binding

For the monovalent metal cations studied in this report, the selectivity sequence for competition with [³H]STX binding in heart muscle membranes was the same as that reported for brain and skeletal muscle: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Weigele and Barchi, 1978; Barchi and Weigele, 1979) (Table I). This sequence follows that of the dehydrated ionic radii of these ions (Hille, 1975b). The values of K_I for the ions in heart muscle differ from those of brain in all cases by less than a factor of two. The values of the slopes of the curves were in all cases not significantly different from -1.

The inhibiting effect of divalent metal cations on current through sodium channels has long been known (Woodhull, 1973; Århem, 1980). In addition, it has been observed that Ca^{2+} and Zn^{2+} cause a reduction in channel current concomitantly with a reduction in STX binding affinity (Worley et al., 1986; Schild and Moczydlowski,

TABLE I

Competition to [³H]STX Binding to Sheep Heart Membranes by Monovalent Cations

		Sheep heart		Rat brain*
	n	K_I	n_H	K_I
		mM		mM
Li	2	14.0 ± 3.5	1.10 ± 0.05	22.6 ± 2.8
Na	2	29.4 ± 4.5	1.01 ± 0.15	34.3 ± 4.5
K	2	54.8 ± 9.5	0.96 ± 0.10	53.6 ± 14.0
Rb	4	79.1 ± 6.6	1.05 ± 0.03	89.0 ± 31
Cs	2	93.6 ± 16.4	1.05 ± 0.04	147.0 ± 19

n, number of independent measurements; n_H, slope of the curve, the pseudo Hill coefficient.

*Barchi and Weigele (1979).

1991). Other observers, however, have reported that block of current by Cd^{2+} in native heart channels (DiFrancesco, Ferroni, Visentin, and Zaza, 1985) and Zn^{2+} in BTX-modified brain channels (Green, Weiss, and Andersen, 1987a) does not occur with simultaneous relief of STX block. Of the metal divalent cations studied to date, those of group IIB in the periodic table, Zn^{2+}, Cd^{2+}, and Hg^{2+}, are additionally interesting. Cd^{2+} blocks sodium current of native TTX-R cardiac channels (DiFrancesco et al., 1985; Baumgarten and Fozzard, 1989) and of the cloned cardiac Na channel (Satin, Kyle, Chen, Rogart, and Fozzard, 1992b), and Zn^{2+} and Cd^{2+} block sodium flux and current of BTX-modified TTX-R channels from heart (and other tissues) (Frelin, Cognard, Vigne, and Lazdunski, 1986; Ravindran, Schild, and
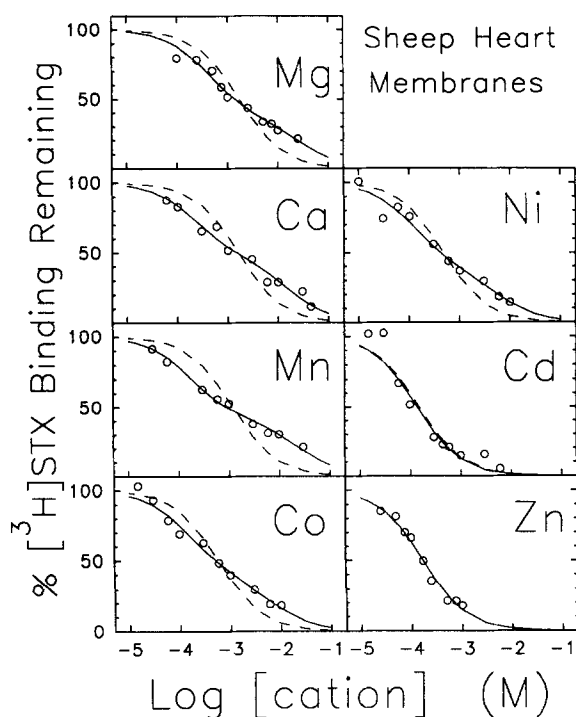

FIGURE 4. Displacement of [³H]STX specifically bound to sheep heart membranes by increasing concentrations of divalent cations. 50 μg sheep heart membrane was incubated in 200 μl containing 10 mM HEPES-Tris, pH 7.4, various concentrations of cations and choline chloride such that the ionic strength was equivalent to 200 mM choline chloride, and a final soaking concentration of ~20 nM [³H]STX. Dashed lines are best single-site fits to the data. Solid lines are the best two-site fits to the data. Values of the inhibition constants for the ions are given in Table II.

Moczydlowski, 1991) with 10–50-fold greater potency than for TTX-S channels in nerve and skeletal muscle.

With this in mind, we measured the competitive effect of Zn^{2+} and Cd^{2+} on [³H]STX binding in our sheep heart and rat brain membrane preparations (Figs. 4 and 5). In addition, we measured the competitive effect of Ca^{2+} and Mg^{2+} from group IIA, Mn^{2+} from group VIIA, and Co^{2+} and Ni^{2+} from group VIII. For heart membranes, Zn^{2+} and Cd^{2+} produced the lowest values of K_I of all ions tested and the data were well fit by curves that assumed one dominant site of cation/toxin interaction (i.e., single-site fits). In contrast, the data for the other divalent ions tested in heart were fit significantly better by two-site rather than single-site curves (Table II). For these ions a site (here designated as site 1) exists with a K_I on the order of 100
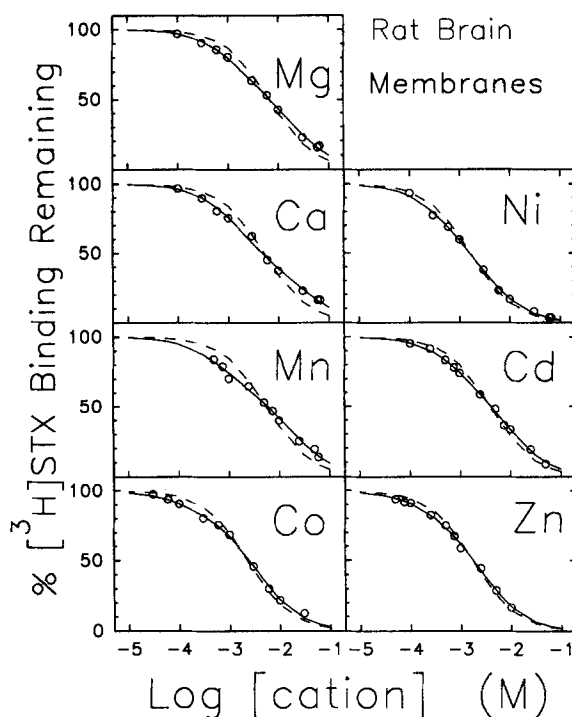
FIGURE 5. Displacement of [^{3}H]STX specifically bound to rat brain membrane by increasing concentrations of divalent cations. 10 μg of rat brain membrane was incubated in 1.0 ml containing 10 mM HEPES-Tris, pH 7.4, with various concentrations of cations and choline chloride such that the ionic strength of the medium was equivalent to 200 nM choline chloride, and a final soaking concentrataion of ~1 nM [^{3}H]STX. Dashed lines are best single-site fits to the data. Solid lines are best two-site fits to the data. Values of the inhibition constants for the ions are given in Table III.

μM, with a range of 90–340 μM. A second site (site 2) is resolvable that has K_I values on the order of 10 mM, with a range of 5–20 mM. If two sites exist for Cd^{2+} and Zn^{2+}, these sites are unresolvable and both would have K_I values of ~75 μM.

When we compared single-versus dual-site fits to cation competition of toxin binding for the brain membrane data (Table III) we found that dual site fits were also

TABLE II

Competition to [^{3}H]STX Binding to Sheep Heart Membranes by Divalent Cations

	n	K_I	K_I^1	K_I^2	% K_I^1	P
		mM	mM	mM		
Mg	2	0.992 ± 0.059	0.338 ± 0.068	18.9 ± 7.9	64.8, 57.8	0.0003, 0.036
Ca	5	0.794 ± 0.058	0.341 ± 0.094	11.8 ± 9.5	36.8, 65.9, 71.2, 53.4, 34.1	0.005, 0.003, 0.015, 0.009, 0.02
Mn	3	0.551 ± 0.061	0.129 ± 0.026	7.58 ± 2.27	57.9, 65.7, 42.9	0.00001, 0.003, 0.003
Co	4	0.261 ± 0.022	0.125 ± 0.060	7.67 ± 4.16	84.7, 70.7, 88.0, 61.1	0.009, 0.00001, 0.151, 0.003
Ni	2	0.216 ± 0.022	0.091 ± 0.002	4.7 ± 0.60	61.0, 75.4	0.008, 0.018
Cd	2	0.074 ± 0.012	ND	ND	ND	ND
Zn	2	0.071 ± 0.002	ND	ND	ND	ND

%K_I^1, the percentage of the sites measured at the [[^{3}H]STX] used (~20 nM) that were of the higher-affinity type in the best fit, for each of the n curves produced and analyzed. P, the probability, determined by F test analysis, that a two-site fit is significantly better (P < 0.05) than a one-site fit for each of the n curves produced and analyzed.

ND, not determined. Values for $K_I^1 = K_I^2 = K_I$.

significantly better for all ions tested except Zn^{2+} (Table III). As with the heart membranes, site 1 exists with values of K_I on the order of 100 μM, with a range of 13–340 μM. Site 2 has K_I values on the order of 1 mM, with a range of 0.3–5.3 mM. For the brain data dual-site fits more closely resemble single-site fits than for the heart data, since the difference in the affinity of the two sites in the brain is less than the difference in the heart. However, as a consequence of the more favorable high-affinity (~0.2 nM) binding of the toxin to brain channels, the precision of the data allows for the discrimination of fit significance as well as, or better than, for the heart.

The heterogeneity of competitive cation binding sites could arise from a heterogeneity of Na channel receptors or from a heterogeneity of cation binding sites that affect a single population of receptors. The binding sites would not be interactive in the former case. In the latter case the sites would be negatively cooperative (Weiland and Molinoff, 1981) (see Discussion).

TABLE III

Competition to [^3H]STX Binding to Rat Brain Membranes by Divalent Cations

	n	K_I	K_I^1	K_I^2	$\% K_I^1$	P
		mM	mM	mM		
Mg	2	1.68 ± 0.20	0.338 ± 0.038	5.29 ± 1.25	44.3, 41.7	0.00008, 0.00005
Ca	5	1.07 ± 0.11	0.337 ± 0.087	4.98 ± 1.14	88.0, 25.5, 63.4, 58.2, 45.3	0.0093, 0.0022, 0.0003, 0.0006, 0.011
Mn	2	0.94 ± 0.13	0.080 ± 0.025	1.93 ± 0.59	10.9, 38.9	0.015, 0.0009
Co	2	0.50 ± 0.03	0.039 ± 0.009	0.83 ± 0.08	20.6, 17.0	0.003, 0.0007
Ni	2	0.33 ± 0.01	0.077 ± 0.022	0.57 ± 0.13	40.1, 13.3	0.001, 0.064
Cd	3	0.48 ± 0.03	0.155 ± 0.016	1.77 ± 0.40	61.1, 37.7, 54.2	0.001, 0.0001, 0.067
Zn	4	0.21 ± 0.02	0.018 ± 0.010	0.263 ± 0.025	19.0, 4.2, N.D., 19.5	0.001, 0.273, 0.951, 0.210

$\% K_I^1$, the percentage of the sites measured at the [[^3H]STX] used (~1 nM) that were of the higher-affinity type in the best fit, for each of the n curves produced and analyzed.

P, the probability, determined by F test analysis, that a two-site fit is significantly better ($P < 0.05$) than a one-site fit for each of the n curves produced and analyzed.

ND, not determined. Values for $K_I^1 = K_I^2 = K_I$.

As shown in Table II, the sequence of K_I values for both sets of sites in heart is consistent with the Irving-Williams series ($Mg^{2+} < Ca^{2+} < Mn^{2+} < Co^{2+} < Ni^{2+} < Cd^{2+} < Zn^{2+}$) in which the ions are ordered according to their stabilization constants (Fiabane and Williams, 1977). For brain, Ni^{2+}, Cd^{2+}, and Zn^{2+} at site 1 are out of sequence with heart, and at site 2 Zn^{2+} and Cd^{2+} are out of sequence. At site 2 heart has four to eight times less affinity than brain for all the ions with the exception of Zn^{2+} and Cd^{2+}, for which it has 4-fold and 24-fold greater affinity.

Modification of [^3H]STX Binding by Modifiers of Carboxyl, Arginyl, Sulfhydryl, Histidyl, and Lysyl Residues

We sought modification of binding in TTX-R channels by the methylating agent TMO (Shrager and Profera, 1973; Reed and Raftery, 1976; Barchi and Weigele, 1979; Spalding, 1980; Rack and Woll, 1984; Gülden and Vogel, 1985; Freeman, Dawson, Bladen, and Gray, 1986) because pH titration of [^3H]STX binding to heart
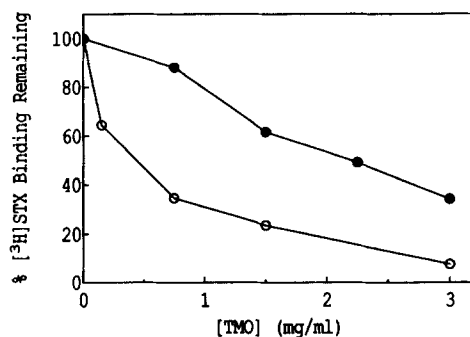
FIGURE 6. Inactivation of specific [^{3}H]STX binding to sheep heart and rat brain membranes by increasing concentrations of TMO. 600 μg of sheep heart (●) or rat brain membrane (○) in 10 ml 200 mM Na bicarbonate, pH 8.5, was treated with TMO for 10 min at room temperature. The final solution pH after treatment was 7.4. Membranes were pelleted by centrifugation and assayed for protein content and [^{3}H]STX binding as described in Experimental Procedures.

TTX-R channel is qualitatively similar to that of rat brain and skeletal muscle and eel electroplax TTX-S channels. Fig. 6 shows that TMO produces a concentration-dependent inhibition of specific [^{3}H]STX binding in heart as well as brain membranes at room temperature. However, the dose response of heart toxin receptors was less than that of the brain receptors when the two were incubated with the modifier under equivalent ionic conditions and protein concentrations.

Inhibition of [^{3}H]STX binding by treatment of 2 mg/ml TMO to brain membranes at 0°C was equivalent to that produced at room temperature, and this inhibition was almost entirely prevented when the membranes were preequilibrated with 100 nM STX before addition of TMO (Fig. 7 B). Inhibition by TMO of [^{3}H]STX binding to heart membranes was greater at 0°C than at room temperature. Some protection against TMO modification of [^{3}H]STX binding in sheep heart membranes was accomplished by preequilibration of unlabeled STX and occurred in a dose-dependent manner, although protection was not as effective as for brain (Fig. 7 A). The reduced effect of TMO on [^{3}H]STX in heart is not surprising since the off rate of [^{3}H]STX from its receptor in the sheep heart membranes is < 1 min at 0°C (Doyle et
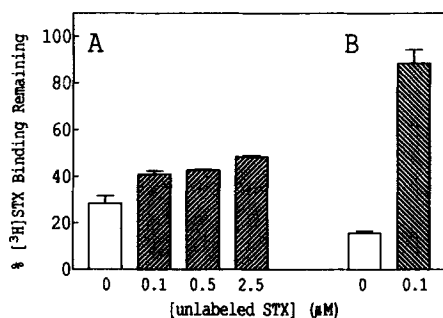

FIGURE 7. Protection against TMO inactivation of [^{3}H]STX binding to sheep heart and rat brain membranes by unlabeled STX (A) 1.2 mg of sheep heart membrane in 10 ml 200 mM Na bicarbonate buffer, pH 8.4, was treated with 7.5 mg/ml TMO at 0°C in the presence of varying concentrations of unlabeled STX. Unlabeled STX was removed from the membranes by repeated centrifugation washes before specific [^{3}H]STX binding was determined. (B) 1.0 mg rat brain membranes in 10 ml 200 mM Na bicarbonate buffer, pH 8.4, treated with 2 mg/ml TMO at 0°C.

al., 1985), 10 times faster than in brain (Barchi and Weigele, 1979). The half life of TMO before hydrolysis in aqueous media is of the same order of magnitude as the off rate of toxin in the heart (Spalding, 1980). The results do indicate that a TMO-modifiable group is part of the [³H]STX binding site of the heart TTX-R channel as well as the brain TTX-S channel.

Our observation that the heart TTX-R channels were less sensitive to equivalent doses of TMO than were the brain TTX-S channels could have arisen for one of two reasons. The geometry of the heart membranes, which were primarily t-tubular in origin (Doyle and Winter, 1989) and were mostly inside out (although leaky), could have slowed access of the short-lived TMO to the vicinity of the channel mouth. Alternatively, the local environment of the TMO-modifiable site, by virtue of different geometry or charge distribution, could confer less affinity for TMO to it than to the brain site. A candidate for such a difference is the unique presence in heart channels of the arginine residue adjacent to RHI glu376 (Rogart et al., 1989), the carboxylic acid conserved in all known sodium channels that has been shown by point mutation experiments to be an absolute determinant of STX binding (Noda, Suzuki, Numa, and Stühmer, 1989). We therefore determined whether PG, a member of the α dicarbonyl group of reagents known for their specificity for arginyl side chains in proteins (Meves, Rubly, and Stampfli, 1988) would discriminate between heart and brain channels at their STX binding sites. However, 50 mM PG administered to membranes at room temperature for 1 h in either 125 mM bicarbonate or lutidine buffer, pH 9.0, abolished 72% of specific [³H]STX binding to heart membranes and 97% to brain membranes (data not shown).

Group IIb metals Zn^{2+} and Cd^{2+} are well known to have strong affinity (K_I on the order of 10–100 μM) for binding sites in soluble, membrane, and DNA binding proteins that contain some combination and juxtaposition of carboxyl groups and/or side chains of cysteine and histidine (Morgan, 1981; Lee, Gippert, Soman, Case, and Wright, 1989; Cunningham, Bass, Fuh, and Wells, 1990; He and Quiocho, 1991). Since both brain TTX-S channels and heart TTX-R channels apparently are not readily distinguishable on the basis of carboxyl residues, we were interested to see if the differential affinity of Cd^{2+} for the two channel subtypes would correlate with differential sensitivity of the two subtypes to modification by sulfhydryl and histidyl modifiers. NEM is an essentially irreversible covalent modifier of protein side chains that is fairly specific for sulfhydryl side chains when administered at neutral pH. PCMB is a mono-functional mercurial that binds noncovalently and reversibly to sulfhydryl groups (Smyth, Blumenfeld, and Konigsberg, 1964; Strauss, 1984). When administered at room temperature, both of these agents had a strongly inhibiting effect on [³H]STX binding to sheep heart membranes, but much less effect on binding to rat brain membranes (Fig. 8). The covalent modification reaction by NEM was temperature dependent, showing no effect at 0°C. At room temperature the inhibitory effect of NEM at 33 mM had a half time of ~25 min (data not shown). Despite the fact that the rate of dissociation of [³H]STX from heart channels is on the order of seconds, 10 μM unlabeled STX was able to fully protect against the effect of 10 mM NEM and to partially protect against the effect of 50 mM NEM administered for 1 h at room temperature (Fig. 9).

Two additional aspects of the dose response of our membranes to NEM treatment
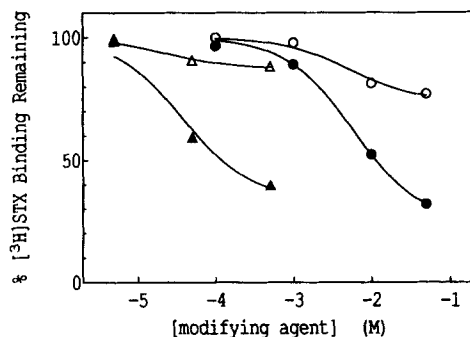
FIGURE 8. PCMB and NEM inhibition of [³H]STX binding to sheep heart and rat brain membranes. 500 μg of sheep heart (●) or rat brain (○) membrane in 1 ml was treated for 1 h at room temperature with NEM in 100 mM HEPES-Tris buffer, pH 7.2. Reactions were stopped by addition of 250 mM cysteine. 1 mg of sheep heart (▲) or rat brain (△) membrane in 1 ml was treated for 1 h at 4°C with PCMB in 100 mM HEPES-Tris buffer, pH 7.2. Reactants were washed by repeated centrifugation as described in Experimental Procedures. [³H]STX binding to treated membranes was measured at 20 nM for sheep heart and 0.3 nM for rat brain, ~1.5 times the K_D for unmodified membranes in each case.

(Fig. 8) were of particular interest. The displacement data were best fit by curves that assumed, despite the fact that NEM modification is irreversible, that modification of channels did not lead to abolition of STX binding. This was borne out by experiments (Fig. 10) in which equilibrium binding curves of NEM-treated and untreated membranes were compared. For the rat brain membranes there was a modest (1.5-fold) reduction in the K_D without a substantial change in the number of sites present. For the sheep heart membranes resolution of equilibrium binding parameters for NEM-modified membranes was difficult. Even the K_D values of our unmodified membrane low-affinity [³H]STX binding sites were at the limit of resolution (~10 nM) of the straight filtration measurement. Nevertheless, best fits of either the binding isotherms or Scatchard transformations were consistent with the conclusion that the major effect of NEM was to reduce the K_D approximately fivefold, with little if any effect on the number of available sites. Second, the effect of NEM on rat brain membranes was substantially greater than that of PCMB. Indeed, 50 mM NEM administered to rat brain membranes at 37°C for 2 h caused the abolition of more than half the number of sites available in the untreated control (data not shown).

NEM is known to modify groups other than sulfhydryls under some conditions. Lysine has been reported to be a primary alternative (Means and Feeney, 1971). Previous observations (Strichartz, 1984; Jaimovich, Liberona, and Hidalgo, 1985)
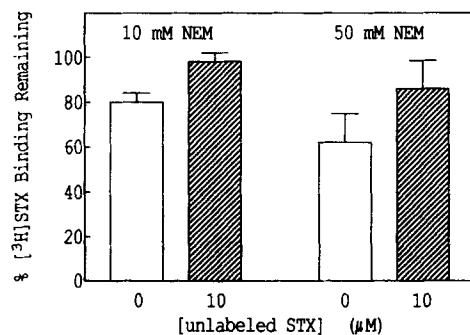

FIGURE 9. Protection against NEM inhibition of [³H]STX binding to sheep heart membranes by unlabeled STX. 800 μg of sheep heart membrane in 1 ml was treated with 10 or 50 mM NEM in the presence of 0 or 10 μM unlabeled STX at room temperature for 1 h. Unlabeled STX and NEM were removed by repeated centrifugation washes before specific [³H]STX binding was determined at 20 nM soaking concentration.

have reported that treatment of membranes from rat brain and frog skeletal muscle with TNBS resulted in abolition of [^{3}H]STX binding to TTX-sensitive sites therein. TNBS is most selective for the ε-amino group of lysine when administered at pH 8.5–9.5. Our treatment of rat brain membranes with 10 mM TNBS at pH 8.5 at room
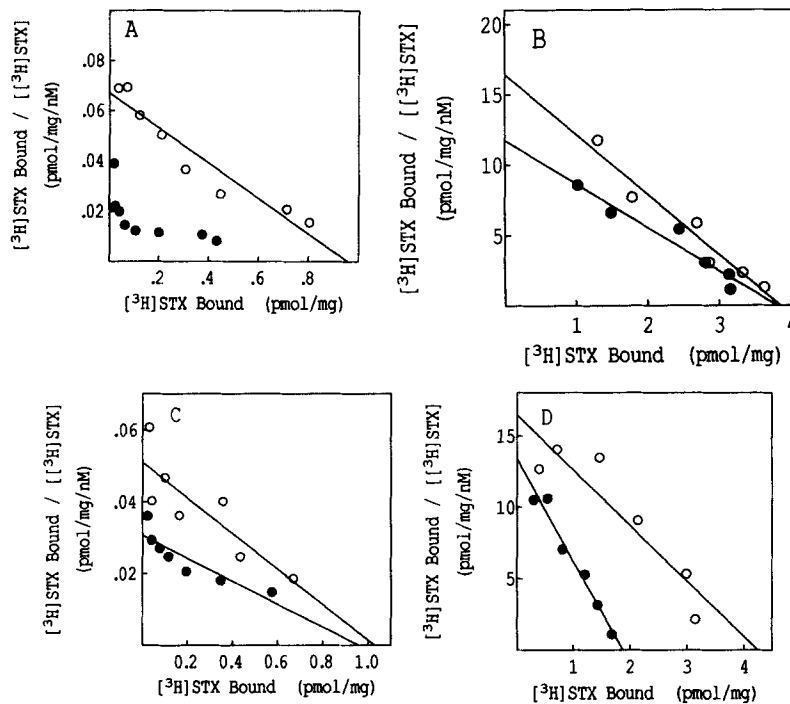

FIGURE 10. Scatchard representation of equilibrium binding isotherms of [^{3}H]STX binding to sheep heart (A) and rat brain membranes (B), which were (●) and were not (○) treated with 50 mM NEM at room temperature for 2 h in 100 mM HEPES-Tris buffer, pH 7.2. Binding parameters for sheep heart were K_D = 14.2 nM, SO = 0.96 pmol/mg protein for untreated membranes, and 0.25 nM and 0.008 pmol/mg and 70.4 nM and 1.03 pmol/mg for treated membranes. Binding parameters for rat brain were 0.20 nM and 3.8 pmol/mg for untreated, and 0.27 nM and 3.75 pmol/mg for treated membranes. (C) Sheep heart and (D) rat brain membranes which were (●) and were not (○) treated with 10 mM TNBS at room temperature for 2 h in 50 mM MOPS adjusted to pH 8.5 with trimethylammonium hydroxide. Binding parameters for sheep heart were K_D = 20.1 nM, SO = 1.03 pmol/mg protein for untreated membranes, and 31.8 nM and 0.96 pmol/mg for treated membranes. Binding parameters for rat brain were 0.25 nM and 4.1 pmol/mg for untreated, and 0.14 nM and 1.9 pmol/mg for treated membranes.

temperature for 2 h resulted in abolition of half the available [^{3}H]STX binding sites along with a modest increase in the K_D of the remaining sites (Fig. 10), consistent with these previous reports. TNBS activity was slowed but measurable at 0°C, and we found that unlabeled STX could protect against TNBS abolition of [^{3}H]STX binding (data not shown), thus localizing modification to the [^{3}H]STX binding site itself.

Interestingly, when sheep t-tubular membranes were similarly treated with TNBS, there was no apparent reduction in the number of available TTX-R [³H]STX binding sites. Although these t-tubular membranes are leaky to molecules the size of STX, TMO, PCMB, and NEM, they are primarily inside out. To address the possibility that TNBS did not have access to the [³H]STX binding site, we prepared sheep heart membranes enriched in surface sarcolemma, which have been previously characterized as primarily rightside-out (Doyle et al., 1986). Fig. 10 shows that even in these membranes TNBS did not cause a significant reduction in the number of TTX-R [³H]STX binding sites, although it did produce a small reduction in the K_D by a factor of ~1.5.

Since we demonstrated that an NEM-modifiable site (or sites) was intimately associated with [³H]STX binding, especially to cardiac Na channels, it was important to determine if NEM modification affected Cd^{2+} competition to [³H]STX binding (Fig. 11). 2-h treatment of sheep heart membranes with 50 mM NEM reduced the amount of [³H]STX bound at 20 nM toxin concentration to ~41% of that bound in
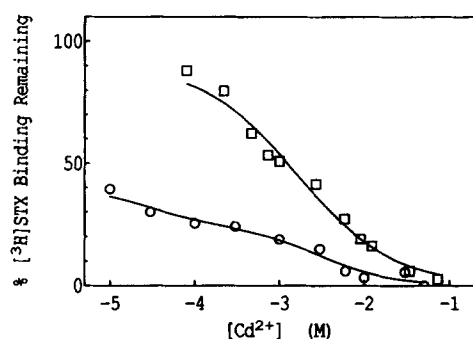

FIGURE 11. Competition by Cd^{2+} ion to [³H]STX binding to sheep heart (O) and rat brain (□) membranes treated with 50 mM NEM at room temperature for 2 h. The percent of binding is related to the amount of binding measured in untreated control samples. STX binding was measured at 4°C at a soaking concentration of 20 nM for sheep heart and 0.75 nM for rat brain. K_I values derived from two-site fits to the data were 0.021 and 2.40 mM for sheep heart membranes, and 0.102 mM and 1.32 mM for rat brain membranes. These values are to be compared with the mean values of Cd^{2+} competition to untreated membranes in Tables II and III.

an untreated control, consistent with a fivefold reduction in K_D as shown in Fig. 10. The NEM treatment had a dual effect on Cd^{2+} competition to [³H]STX binding. The first was to shift the K_I value (single-site fit) by a factor of 6.4 to the right, indicating that the competitive effect of Cd^{2+} was substantially reduced. Second, the slope of the competition curve was substantially reduced, indicating that negative cooperativity between two sites, not apparent in untreated samples, was induced. In contrast, NEM treatment did not significantly alter the K_I values of Cd^{2+} competition to [³H]STX binding to rat brain membranes.

Although there are no known agents that exclusively modify histidine side chains, DEPC is considered to be a useful agent under particular conditions (Miles, 1977). Although DEPC has been shown to ethoxylate nitrogens covalently in a variety of amino acid side chains including histidine, lysine, and arginine, as well as the phenolic oxygen of tyrosine, reactivity with histidine is favorably although not exclusively selected at mildly acid pH (5.5–6.0). In addition, ethoxylation of histidine and tyrosine residues can be singled out since for them the modification can be

reversed by hydroxylamine. 10 mM DEPC administered at room temperature for 1 h at either pH 5.5 or 7.5 abolished >50% of the specific [^{3}H]STX binding to membranes from both rat brain and sheep heart membranes with a modest change in K_D (less than a factor of 1.5 for both; data not shown). Reversal of the DEPC effect by hydroxylamine was modest. Incubation in 1 M hydroxylamine, pH 5.5, overnight at 4°C restored in DEPC-treated membranes only 18% of the binding measured in untreated membranes. Hydroxylamine incubation alone had no effect itself on the binding. Thus DEPC did not serve as a discriminator of the relative disposition of histidine side chains at the [^{3}H]STX binding sites of the two channel subtypes.

DISCUSSION

Nature of the STX Interaction

The mammalian heart TTX-R Na channels have a pH-titrable, TMO-modifiable amino acid residue as a component of the STX binding site, as do the better characterized TTX-S channel subtypes from nerve and skeletal muscle. The pH dependency of [^{3}H]STX binding to heart TTX-R channels is qualitatively similar to that of brain TTX-S channels; an ionizable group with a pK_a in the range of 5.0–6.0 is evident in both, but the reduced slope and measurable shift of the heart channel pH titration curve implies that the carboxyl site is different or an additional ionizable group or groups play a role in modulating STX binding. All of the cloned Na channels, including RBII and μl representing TTX-S isoforms and RHI (SkM2) representing TTX-R isoforms, contain a number of carboxy-amino acids that are important for toxin binding (Terlau, Heinemann, Stuhmer, Pusch, Conti, Imoto, and Numa, 1992), and the channels are almost identical in the P region.

Monovalent and Divalent Ion Competition with STX Binding

The competitive effect of monovalent cations upon [^{3}H]STX binding to sheep heart TTX-R sites is similar to that reported for rat heart TTX-R sites (Lombet et al., 1981) and is not different from the competitive effect of monovalent cations on rat brain and skeletal muscle TTX-S sites (Weigele and Barchi, 1978; Barchi and Weigele, 1979). The apparent affinity constant of each ion for TTX-R sites is within a factor of two of that for TTX-S sites. The affinity sequence for the TTX-R and TTX-S channels is the same. It fits the Irving-Williams series, implying that the array of coordination sites of these ions is similar to that with free ligands in solution; i.e., the sites are not constrained by any special geometric factors. The slopes of the monovalent cation competition curves for heart membranes are in all cases not significantly different from one, implying a single binding site (or a number of sites with similar binding affinities). Implicit also from these slopes is that there is no evidence of screening or alteration of free (or "active") cation or toxin concentration due to cation accumulation or nonspecific binding.

Divalent ions also competed with [^{3}H]STX binding to cardiac channels. Two properties of divalent ion competition require discussion. First, [^{3}H]STX was displaced from cardiac Na channels by Cd^{2+} and Zn^{2+} at substantially lower concentrations than for the brain channels. Second, the displacement curves of most of the divalent ions were not well fit by a single-site binding curve. Both the TTX-S and

TTX-R channels showed better fit with a two-site model, except for Cd^{2+} and Zn^{2+} displacement with cardiac channels and Zn^{2+} with brain channels. Although other interpretations are possible, it is reasonable to consider that there are two divalent cation binding sites on Na channels, both of which can affect STX binding.

The Case for Specific Interactions between STX and Ions

In general, there are three ways for inorganic cations to interact with [³H]STX binding to sodium channels in our membrane preparations. The first and more interesting interaction is by binding to specific sites on the Na channel protein itself, and we will subsequently interpret our results assuming this type of interaction. Second, however, a change in the concentration of a particular ionic species can alter surface charge, indirectly affecting the local concentration of toxin (which is itself a divalent cation) as well as other ionic species (including the particular ion itself) near the membrane surface. This effect of cations we will call screening. Third, cations can bind directly to nonspecific negative surface charges: either on phospholipids, on other proteins, or on the Na channel peptide itself or sugar groups attached to it. Cations binding to these nonspecific sites could act similar to screening by altering local potential gradients, and thereby the affinity of toxin for its binding site or other ions (including themselves) for their binding sites. In addition, they could be effectively immobilized and thus alter the free (or "active") divalent cation concentration or displace nonspecifically bound toxin and thus alter the free (or active) toxin concentration. We sought to avoid these specific and nonspecific shielding effects by maintaining constant ionic strength with choline, but if some ionic species (e.g., a particular divalent cation) either accumulates near or binds to the membrane surface differently than choline, constant ionic strength in the bulk solution does not necessarily lead to constant field strength or constant nonspecific binding (McLaughlin, 1989).

The effect that we observe with increasing divalent cation concentration for all but Cd^{2+} and Zn^{2+} in heart is an attenuation of the competitive effect of the ions toward [³H]STX binding, which is opposite in direction from what one would predict to result from the modulation of the near-surface [³H]STX concentration by the screening of nonspecific negative charges on the surface of the membranes. Since the experiments reported in Table II were performed at toxin concentrations below the saturation level, a decrease in toxin concentration would result in a decrease in the amount of toxin specifically bound. Screening of divalent cation binding by accumulation of cation near the surface can also be ruled out. In the case of simple accumulation the effect is a consequence only of ionic charge, and the various ions tested would give rise to similar results (McLaughlin, 1977).

An opposite effect can occur for cation binding to negative charges on the surface of the membranes, however. On the one hand, increasing divalent cation concentrations can increasingly displace nonspecifically bound toxin from the membrane, thus increasing the concentration of free toxin near the membrane surface. The magnitude of this effect depends on the degree to which nonspecifically bound toxin is immobilized such that its free (or active) concentration is altered. Our observation that measuring the competitive effect of Cd^{2+} on [³H]STX binding to rat brain membranes at nominal free concentrations of 10 and 0.87 nM, at which concentra-

tions ~98 and 83% of sites would be bound in the absence of Cd^{2+} (Fig. 2 C), gave significantly indistinguishable values is evidence that toxin immobilization by nonspecific negative surface charges is not of major consequence in our assays. Alternatively, nonspecific negative surface charges could serve to immobilize divalent cation, thus decreasing the free (or active) cation concentration. Such an effect could produce a deviation of the toxin displacement curve from a single-site relation by lowering the active divalent ion concentration in solutions of divalent ion >1 mM. There would still be two sites, but only one on the protein itself. We observed, however (data not shown), that Mn^{2+} produced the same slope in competition with [^{3}H]STX binding to sheep heart membranes even when we increased the ratio of total Mn^{2+} to membrane surface area fivefold in experiments in which we increased the incubation volume from 0.2 to 1.0 ml. This is evidence that immobilized cation is not responsible for the attenuation of competition with increasing cation concentration.

It would appear, then, that the overriding effect of the metal ions used in these experiments is to modulate specifically bound [^{3}H]STX at specific cation binding sites that are localized to the STX-receptor protein itself. This view is supported by the observation that toxin binding to batrachotoxin-modified Na^+ channels incorporated into planar lipid bilayers was independent of the charge state of the bilayer lipids (Green, Weiss, and Andersen, 1987b; Ravindran and Moczydlowski, 1989; Worley, French, Pailthorpe, and Krueger, 1992).

Physical Interpretations of the Two-Site Binding Competition between Divalent Cations and STX

We have used the simplest multi-site equation (Eq. 3) to demonstrate that a two-site model for the divalent cations is statistically favored over a one-site model. The physical interpretation of Eq. 3 corresponds to there being two distinct binding sites for [^{3}H]STX, at each of which competitive binding with divalent cations occurs in a simple manner (defined in Results). The differentiation of these two sites occurs because the ratio K_D/K_I differs at these two sites. We believe this is the least likely physical mechanism based on the homogeneity of [^{3}H]STX receptor sites observed in the direct binding curves. Conversely, homogeneous [^{3}H]STX receptors could show heterogeneous binding properties for the divalent cations. However, we see no evidence of heterogeneity of channel types with regard to monovalent cation binding. In addition, a model of at least two cation sites per channel is consistent with plausible analytical models of charge distribution on the Na channel extracellular surface (Cai and Jordan, 1990).

We consider, then, the class of models in which both cation binding sites reside on a single channel. In this situation the sites cannot be both simply competitive and independent because binding of cation to the higher-affinity site would progressively displace all of the toxin and the lower-affinity site would display no effect or an additive effect. The equations for alternative models are more complex (Klotz, 1985; Tomlinson and Hnatowich, 1988), and further experiments are required to demonstrate their validity, including analysis of binding kinetics. As a result, we have not used these other formalisms in this initial work describing divalent cation and [^{3}H]STX interaction. The best fits to our binding data occurred using the assumption of 0% occupancy of specific toxin sites at infinite cation concentration, suggesting

that at least one of the cation binding sites might be simply competitive. Thus, we will provide the next most simple physical interpretations from those alternative models that require a nonsimple interaction to occur upon cation binding to one of the sites.

In the first case the cation sites may be independent. Binding of cation to its high-affinity site results in nonsimple competition (e.g., cation binding competes for some but not all of the STX coordination sites; Schild and Moczydlowski, 1991). Cation binding to the low-affinity site may then compete simply with toxin and give rise to the complex curves we observe. In the second case binding of cation to its high-affinity site results in simple competition with toxin and subsequent binding to the low-affinity site results in a nonsimple interaction. One of two possibilities would be required to produce the biphasic curves we observe. Binding of cation to its low-affinity site could compete for binding of cation to the high-affinity site, thus reducing its competitive effect on [³H]STX binding. This negative cooperativity could result from cations sharing some but not all coordination bonds (Schild and Moczydlowski, 1991), or from electrostatic repulsion between the cations (Salgado, Zeh, and Narahashi, 1986). Cooperativity could also occur allosterically by changes in protein conformation upon cation binding (Klotz, 1985). Conversely, binding of cation to its lower-affinity site could lead to a conformational change which results in an increase in affinity for [³H]STX to its binding site. On the basis of equilibrium binding measurements as performed here, we cannot distinquish between the above possibilities (Weiland and Molinoff, 1981; Tomlinson and Hnatowich, 1988). Indeed, as a consequence of the potential for superposition of these several possible interactions between sites and protein conformations, simple predictions or interpretations are unlikely to be complete.

Variation of Divalent Cation Effects

Because divalent ions are potent blockers of the Na channel and potent competitors for toxin, their different modes of interaction can give us some insight into the channel structure. Divalent metal cations and small ligands such as amino acids in solution form complex chelations composed of two to as many as nine bonds, each of which is of a combined ionic and covalent nature (Gurd and Wilcox, 1956). Depending on the amino acid groups available to be bound (e.g., carboxyl or amino), each ion has a preferred coordination number (e.g., 6 for Mg^{2+}, 7 or 8 for Ca^{2+}) and a preferred coordination geometry (e.g., octahedral for Mg^{2+}, less demand on geometry for Ca^{2+} [Martin, 1990]). The relative complexing ability of divalent metal ions for ligands in solution depends on their ligand field stabilization energies in addition to their charge to radius ratio (Fiabane and Williams, 1977; Århem, 1980). For a particular ion and ligand the stabilization energy can be loosely predicted on the basis of the categorization of ions into three groups: hard acids (e.g., Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}) that form primarily ionic bonds and prefer hard bases (e.g., H_2O, OH^-, ROH, OR^-, NH_3), soft acids (e.g., Cd^{2+}) that form primarily covalent bonds and prefer soft bases (e.g., RSH, RS^-, $-CN^-$, $-SCN^-$), and borderline acids (e.g., Zn^{2+}, Ni^{2+}, and Co^{2+}) that prefer borderline bases (e.g., RNH_2) but are amenable to hard and soft bases. Ions can be ordered for a wide range of ligands in solution according to the Irving-Williams series: $K^+ < Na^+ < Li^+ \ll Mg^{2+} > Ca^{2+} < Mn^{2+} < Co^{2+} < Ni^{2+} < Zn^{2+}$. In proteins, where amino acids are arranged in a

relatively fixed array, the ultimate affinity of a divalent cation for a site is dependent additionally on the geometry of the site, which determines how well a particular ion can form an optimum number of coordination bonds (usually five or six) and coordination geometry, as well as on desolvation energy, rate of reaction, and redox properties (Nieboer, Maxwell, and Stafford, 1984). Implicit in this description is that for a given binding site two divalent ions (e.g., Ca^{2+} and Ni^{2+}) may not share precisely the same array of coordinations nor the same bond strengths.

The selectivity sequence of divalent cation effect is similar to that for free ligands in solution, indicating that there are no major geometrical constraints for most of the ions at the site(s) of primary effect. We do, however, see two exceptions to the Irving-Williams series. One is the different position of Cd^{2+} in the two sequences. As will be discussed further below, the differential affinity of Zn^{2+} and Cd^{2+} for the two channel types probably follows from the presence of a sulfhydryl group in the pore of the cardiac channel (Schild and Moczydlowski, 1991; Satin et al., 1992a; Terlau et al., 1992). In the classification of transition metals as hard and soft acids it can be seen that Cd^{2+} is a more purely soft acid than Zn^{2+}, which is considered to be borderline between the two categories. The effect of the loss of a sulfhydryl group as a coordination site would appear to have a greater effect on Cd^{2+} than Zn^{2+}, which has a greater array of ligands that it prefers. Another distinction is that for both heart and brain channels we observe that Ca^{2+} is a more efficacious competitor than Mg^{2+}. Other investigators have observed that Mg^{2+} was more competitive than Ca^{2+} (Barchi and Weigele, 1979; Lombet et al., 1981). Of the divalents tested here, Ca^{2+} and Mg^{2+} have the most pronounced ionic character in their bonding arrangements. A feature of Mg^{2+} that might account for its less than predicted affinity in our experiments is its considerably smaller size than Ca^{2+}, which leads to a 1,000-fold slower rate of substitution of water than for Ca^{2+} (Martin, 1990). Solvation energy thus might play a governing role in the relative binding affinities of these two ions. Alternatively, Mg^{2+} has a more restricted preference for complex geometries and bond lengths, whereas the larger Ca^{2+} more freely fits a wide variety of coordinations and bond lengths (Martin, 1990).

Structural Model for Cation/Toxin Sites

There has been dramatic progress in resolving the structure of the Na channel in the last six to seven years, beginning with the publication of a suggested membrane topology by Noda, Shimizu, Tanabe, Takai, Kayano, Ikeda, Takahashi, Nakayama, Kanaoha, Minamino et al. (1984) from the amino acid sequences of two rat brain Na channels. A combination of analyses of the molecule's physical chemical properties (Cai and Jordan, 1990), functional expression of chimeras and proteins with point mutations, and application of molecular modeling (Guy and Conti, 1990) has led us to propose a structural framework for the interpretation of our experiments (Fig. 12). The outer vestibule and pore of the channel are thought to be formed by folding partly into the membrane of the connecting segment between S5 and S6 in each of the four repeats. This results in a pore composed of eight strands, one hairpin from each repeat, as in the proposal of Durell and Guy (1992) for voltage-dependent K channels. The amino acids in this region include several charged residues that can be arranged so as to form three negatively charged rings (Fig. 12), analogous to the

charged rings of the muscle ACh channel (Konno, Busch, Vonkitzing, Imoto, Wang, Nakai, Mishina, Numa, and Sakmann, 1991). Mutation of the charged amino acids of the Na channel intermediate and inner rings of the rat brain II α subunit dramatically reduces or abolishes toxin block of the mutated Na channels (Terlau et al., 1991). These amino acids are also identified as part of the pore structure because their modification additionally changes single channel conductance. There is also evidence that the intermediate ring is involved in Ca^{2+} binding. Replacement of the glutamic acid by glutamine (neutralization) in repeat 1 of the intermediate ring of the rat brain

A   **B**
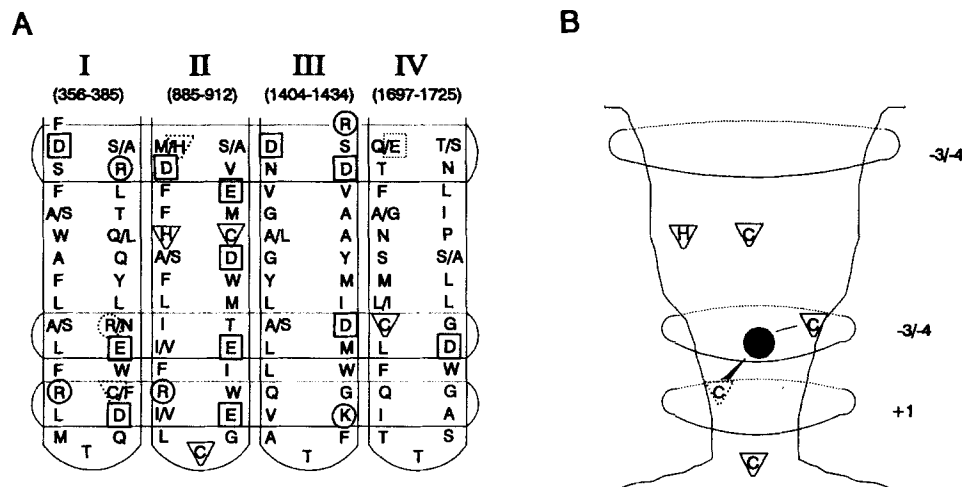

FIGURE 12. A folding scheme for the sodium channel pore region. (*A*) Single letter amino acid code identifies the primary sequence for the four repeats of the Na channel in the pore region (Guy and Conti, 1990). Residues in squares are negatively charged and those in circles carry a net +1 charge at physiological pH. Histidines and cysteines are enclosed in triangles. Nonconserved His, Cys, and charged residues are outlined with a dashed line with the cardiac-specific residue listed first. The domain number and the amino acid position corresponding to the cloned cardiac channel (RHI) are listed above each segment. (*B*) Cartoon illustrating a cross-sectional view of the putative relation of the rings of charged residues to the pore of the channel. The net charge of each of the rings is listed to the right with that of the cardiac channel preceding that of the toxin-sensitive isoforms. The dot indicates a possible location of a group IIB divalent cation. The diagram is not meant to suggest that group IIB cations completely occlude the pore.

II α subunit alters Ca^{2+} block of the expressed Na current (Pusch, Noda, Stuhmer, Numa, and Conti, 1991). It is also of interest that an increase in the negativity of the inner ring results in a channel permeable to Ca^{2+}, suggesting that it is part of the selectivity filter (Heinemann, Terlau, Stuhmer, Imoto, and Numa, 1992).

The charged amino acids in these three rings are conserved in the brain, skeletal muscle, and cardiac channels, so the proposed ring structure is inadequate to explain the relatively low toxin affinity of the cardiac channel and its sensitivity to Cd^{2+} and Zn^{2+}. However, adjacent to the repeat I charged residues of the intermediate and inner rings the cardiac Na channel has significant sequence differences. We recently

demonstrated that a conservative mutation of the cardiac cysteine at position 374 to an aromatic acid (tyrosine or phenylalanine, such as is native to high-affinity toxin binding channel types) led to a dramatic increase in the toxin affinity of the cardiac channels (Satin et al., 1992a). This result implies that the charged amino acids identified by Terlau et al. (1991) as necessary for the toxin block were not sufficient to explain the different toxin affinities. Furthermore, the decreases in affinity seen by Terlau et al. (1991) were accompanied by changes in single channel conductance, and they might have been partly the result of a nonspecific distortion of the complex toxin binding region, rather than specific alteration of the toxin interaction sites. The most likely interpretation of these mutation studies is that the toxin binding locus is complex, and that the negatively charged rings and an aromatic amino acid corresponding to position 374 in the cardiac α subunit are all necessary for the high affinity seen in the TTX-S channels.

Satin et al. (1992a) also demonstrated that removal of the cysteine at 374 concomitantly reduced Cd^{2+} affinity. This offered clear evidence that the toxin and Cd^{2+} sites overlapped in some fashion. That this cysteine is directly involved in divalent binding is supported by the recent report of Backx, Yue, Lawrence, Marban, and Toaselli (1992) that insertion of a cysteine (in place of a tyrosine) at this site in the adult skeletal muscle α subunit increased the affinity of the channel for Zn^{2+} block.

Since both the intermediate and inner rings are demonstrably important for toxin binding, and the size of the toxin molecules would make the outer ring also a potential toxin interaction site, perhaps as suggested by Hu and Kao (1991), it is interesting to speculate in what manner the two divalent ion sites identified in our [^{3}H]STX displacement studies could be related to the ring structures. We suggest that our two divalent cation binding sites are the intermediate ring (site 2) and the outer ring (site 1). The cysteine that is essential for the high-affinity binding of Cd^{2+} and Zn^{2+} in cardiac channels is located between the intermediate and inner rings. The unique arginine in heart is adjacent to the intermediate ring and might be responsible for the lower affinity of site 2 cation sites in heart than in brain. This aspect of the model is consistent with the observation that mutation of arg377 to asp in RHI increases the efficacy of Cd^{2+} toward current block and reduces STX current block (Satin et al., 1992a). In this model the inner ring would not be a divalent binding site, but would serve as part of the selectivity filter. This discussion emphasizes the complexity inherent in the involvement of various ligands with the Na channel mouth and that our model is tentative. The observation by Pusch et al. (1991) that neutralization of a positively charged lysine in the inner portion of S4 of region I enhances extracellular voltage-dependent Ca^{2+} block of the Na channel is an example of the complex interactions that can occur. In addition, Ravindran et al. (1991) demonstrated that Zn^{2+} could produce a subconductance state in BTX-treated channels, whereas other investigators did not see a subconductance state in non-BTX-treated channels (Baumgarten and Fozzard, 1989; Backx et al., 1992).

Relation between Divalent Cation Competition to STX Binding and Block of Sodium Conductance

For Na channels divalent cation block of sodium current has been observed to occur over two different ranges of affinity. Voltage-dependent sites have $K_B(0)$ values (the

blocking affinity at 0 mV) for Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} on the order of 10 mM in heart (Ravindran et al., 1991; Sheets and Hanck, 1992), nerve (Yamamoto, Yeh, and Narahashi, 1984; Worley et al., 1986), and skeletal muscle (Ravindran et al., 1991). They are reported to be located 30% into the transmembrane electric field. Voltage-dependent block by Cd^{2+} and Zn^{2+} with $K_B(0)$ on the order of 100 μM has been observed in BTX-modified cardiac channels in planar lipid bilayers (Ravindran et al., 1991) and by Cd^{2+} in DPI 201-106 treated channels under patch clamp (Backx, Marban, and Yue, 1990). A second recently described site of relatively voltage-independent block of conductance in heart Purkinje fiber cells by Zn^{2+} and Cd^{2+} (DiFrancesco et al., 1985) as well as Ni^{2+} (Sheets and Hanck, 1992) occurs with a $K_B(0)$ on the order of 100 μM and is located at an electrical distance of ~ 0.1.

It is appealing, on the basis of such correlations, to list the voltage-dependent block site as a candidate for site 2. The values for $K_B(0)$ for current block and K_I for toxin competition are well matched, the selectivity sequences are similar, and the marked differential between the effect of Cd^{2+} and Zn^{2+} and that of the other ions obtains. It must be noted that involvement of site 2 in STX binding implies that the toxin binding site is partially within the membrane electrostatic field. However, the question of the voltage dependence of toxin block is controversial and caution should be exercised when correlating equivalent electrical distance with depth into the pore because of uncertainty in knowing the local voltage profile. In addition, Green et al. (1987a) have concluded that in rat brain BTX-modified Na^+ channels incorporated into planar lipid bilayers there is no electrostatic interaction between Zn^{2+} at its block site and STX at its binding site. Since BTX modification of Na channels might cause the displacement of portions of the protein that play a role in the cationic/STX interactions observed here, it is difficult to compare the results of BTX-modified channels and unmodified channels.

Amino Acid Modifying Agents

The effects of the amino acid modifying agents may also be discussed in terms of this structural model. TMO has many potential targets for interaction (Gülden and Vogel, 1985; our Fig. 12). It is attractive to suggest that the repeat I intermediate ring glutamic acid is a TMO target in TTX-S channels observed here, but this site may be less accessible in cardiac channels because of the presence of the cardiac-specific adjacent arginine. Application of 50 mM TMO on rabbit ventricular cells (Dudley and Baumgarten, 1990) failed to relieve Ca^{2+} block, although application of 50 mM TMO in this study shows that [^{3}H]STX binding is affected. It may be that TMO modification of a group(s) other than in the intermediate ring alters [^{3}H]STX binding in cardiac channels.

The effect of NEM modification on [^{3}H]STX binding and Cd^{2+} competition to [^{3}H]STX binding in heart channels together with the absence of significant NEM modification of brain channels is entirely consistent with the point mutation experiments described above and can be ascribed at least in part to the cysteine at 374. It should be noted that cys374 could be one of several cys residues that coordinate with group IIb divalent cations in heart. On the other hand, NEM modification does not completely abolish either [^{3}H]STX binding or the competitive effect of Cd^{2+} in heart. This aspect of the result underscores the multicoordinate nature of the binding of

both of these ligands. The two-component nature of the Cd^{2+} competition to toxin binding to NEM-treated heart membranes also reveals the presence of more than one site of action of Cd^{2+} (and by analogy Zn^{2+} as well) not apparent in Cd^{2+} and Zn^{2+} competition curves with untreated heart.

Our observation that TNBS abolishes STX binding to rat brain channels and has minimal effect on sheep heart channels is novel. Strichartz (1984) has postulated that a lysine in rat brain plays a role in the modulation of STX binding by pH. Our observations reveal that the pH dependence of STX binding in sheep heart is quantitatively different than that for rat brain. In their model of STX and TTX binding to rat brain channels, Green et al. (1987a) proposed that a conformational change of the protein involving the motion of a positively charged group such as an arginine or a lysine was responsible for toxin conductance block and high affinity binding. Inspection of the amino acid sequences for sodium channels reveals several candidates for the differential TNBS effect observed here. At positions relative to RBII 908 located in the connecting region between repeat IIS5 and SS1 all sequences have a lysine except RHI, which has serine. Also, at RBII 1687, between IV S5 and SS1, RBIII, SkMI, and eel have lysine and RBI and II have arginine, whereas RHI has tryptophan. Despite the unique presence of an arginine residue adjacent to RHI glu376 (i.e., RBII glu387) in heart, glyoxal family modifiers did not distinguish between heart and brain channels in our studies. This suggests a role in toxin binding for other arginine residues in Na channels.

Conclusion

Our proposed structural model is only one of many possible alignments of the amino acids composing the outer vestibule-pore region, and the evidence supporting this model is incomplete. However, it offers a useful framework for the interpretation of our experimental results on STX binding properties and ionic interaction, and it provides a basis for determining the multiple coordination sites of the toxins and divalent ions through expression of channels with appropriate point mutations. It is reasonable to expect that functional studies of selectively changed Na channels will lead to a clarification of the ion and toxin interaction sites.

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