Batrachotoxin-modified Sodium Channels in Planar Lipid Bilayers

Characterization of Saxitoxin- and Tetrodotoxin-induced Channel Closures

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ABSTRACT The guanidinium toxin-induced inhibition of the current through voltage-dependent sodium channels was examined for batrachotoxin-modified channels incorporated into planar lipid bilayers that carry no net charge. To ascertain whether a net negative charge exists in the vicinity of the toxin-binding site, we studied the channel closures induced by tetrodotoxin (TTX) and saxitoxin (STX) over a wide range of [Na⁺]. These toxins carry charges of +1 and +2, respectively. The frequency and duration of the toxin-induced closures are voltage dependent. The voltage dependence was similar for STX and TTX, independent of [Na⁺], which indicates that the binding site is located superficially at the extracellular surface of the sodium channel. The toxin dissociation constant, K_D, and the rate constant for the toxin-induced closures, k_c, varied as a function of [Na⁺]. The Na⁺ dependence was larger for STX than for TTX. Similarly, the addition of tetraethylammonium (TEA⁺) or Zn²⁺ increased K_D and decreased k_c more for STX than for TTX. These differential effects are interpreted to arise from changes in the electrostatic potential near the toxin-binding site. The charges giving rise to this potential must reside on the channel since the bilayers had no net charge. The Na⁺ dependence of the ratios K_D/STX/K_D/TTX and k_c/STX/k_c/TTX was used to estimate an apparent charge density near the toxin-binding site of about -0.33 e·nm⁻². Zn²⁺ causes a voltage-dependent block of the single-channel current, as if Zn²⁺ bound at a site within the permeation path, thereby blocking Na⁺ movement. There was no measurable interaction between Zn²⁺ at its blocking site and STX or TTX at their binding site, which suggests that the toxin-binding site is separate from the channel entrance. The separation between the toxin-binding site and the Zn²⁺ blocking site was estimated to be at least 1.5 nm. A model for toxin-induced channel closures is proposed, based on conformational changes in the channel subsequent to toxin binding.

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INTRODUCTION

The guanidinium toxins saxitoxin (STX) and tetrodotoxin (TTX) have played a critical role in the identification, characterization, and purification of voltage-dependent sodium channels (see reviews by Barchi, 1982; Agnew, 1984; Catterall, 1984). These toxins specifically and reversibly inhibit sodium channel currents (Narahashi et al., 1964; Hille, 1968), and appear to bind to a common extracellular site (Colquhoun et al., 1972; Wagner and Ulbricht, 1975). Although the molecular mechanism underlying the current inhibition is unknown, it has been proposed that it occurs when a toxin molecule binds and physically occludes the channel (Kao and Nishiyama, 1965; Hille, 1971, 1975; Kao and Walker, 1982). Despite the wide acceptance of this scheme, there is no direct experimental evidence supporting it.

Little is known about the structure and surface chemistry of the binding site, but comparative studies on STX- and TTX-induced closures indicate that there is a net negative charge near (or at) the binding site. At physiological pH, TTX has a charge of +1 (Woodward, 1964), while STX has a charge of +2 (Shimizu et al., 1981). The addition of Ca" has a larger depressant effect on STX binding and current inhibition than on TTX binding and current inhibition (Henderson et al., 1974; Hille et al., 1975a), which indicates that Ca" changed an electrostatic potential at the binding site. The reduction of toxin binding and current inhibition by protons suggests that the toxins bind to a group with an apparent pK, of ~5.4 (Colquhoun et al., 1972; Ulbricht and Wagner, 1975a, b). Further, since toxin binding and current inhibition are removed by carboxylate-modifying reagents (Shrager and Profera, 1973; Baker and Rubinson, 1975; Reed and Raftery, 1976; Spalding, 1980), there is considerable evidence for a functionally important carboxylate group or groups at the toxin-binding site.

We have examined the STX- and TTX-induced channel closures of batrachotoxin (BTX)-modified Na channels incorporated into planar lipid bilayers to address three questions: Is there a net negative charge on the channel protein near the toxin-binding site? If so, how will this affect the kinetics of toxin binding and the interpretation of toxin-cation interactions at the binding site? What is the relation between the toxin-binding site and the permeation path? The various versions of the occlusion model place the toxin-binding site in or adjacent to the permeation path. The question of how the toxin molecule could close the channel can thus be addressed indirectly from information about the location of the toxin-binding site relative to the permeation path. We conclude that the toxin-binding site is distant from the permeation path, and propose a model for toxin-induced closures based on conformational changes in the channel. Preliminary reports of some of the work reported here have been presented elsewhere (Green et al., 1984a–c, 1986).

MATERIALS AND METHODS

Experimental Procedures and Materials

Voltage-dependent, BTX-modified sodium channels from canine forebrain were incorporated into planar lipid bilayers as described in the preceding article (Green et al., 1987).
All experiments were done at room temperature (22–25°C) at pH 7.4. Except where noted, the experiments were done with symmetrical solutions having Na⁺ as the only cation and Cl⁻ as the major anion. In the text, the solutions are identified by their total Na⁺ concentration and the concentration of the buffer (phosphate or HEPES). The makeup of the electrolyte solutions and other experimental procedures and materials were as described in Green et al. (1987). The orientation of the channels was established from their voltage-activation characteristics, and STX or TTX was added to the “extracellular” solution. Membrane potential differences are expressed relative to the extracellular solution.

Data Analysis

In the presence of the guanidinium toxins, BTX-modified sodium channels undergo spontaneous as well as toxin-induced closures that have very different average durations (cf. Figs. 1 and 2). Similar to what was found by French et al. (1984) and Moczydlowski et al. (1984a), little error is introduced if only closures lasting >600 ms are assigned to the toxin-induced category.

The toxin-induced closures were quantified as the fractional closed time, \( f_c \):

\[
f_c = \frac{\sum_{i=0}^{n} i \cdot T_i}{n \cdot T_{\text{obs}}},
\]

where \( n \) is the total number of channels in the membrane, \( T_{\text{obs}} \) is the total observation time for the experiment, \( i \) is an index parameter (0 ≤ \( i \) ≤ \( n \)), and \( T_i \) is the total time \( i \) closed channels were observed, counting only closing events lasting >600 ms.

The average duration of the toxin-induced closures, \( \tau_c \), was estimated as:

\[
\tau_c = f_c \cdot T_{\text{obs}} / n_{\text{t}}.
\]

Estimates of \( \tau_c \) and \( \tau_o \) were also obtained from dwell-time histograms, using a maximum-likelihood estimator (Hall and Sellinger, 1981). Statistical estimates of parameters in Eqs. 4, 5, 9, and 10 were obtained by fitting the equations to the data using a nonlinear least-squares fitting routine based on a combination of the grid search and Marquardt-Levenberg algorithms (e.g., Bevington, 1969, pp. 204–246), with inclusion of the “jackknife” procedure (e.g., R. G. Miller, 1974). Fits to Eqs. 11, 12, 14, or 15 together with Eq. 13 were done interactively.

RESULTS

Toxin-induced Closing Events

At membrane potentials positive to −60 mV, BTX-modified sodium channels are open most of the time in the absence of STX or TTX (see Fig. 1). Most spontaneous channel closures last <600 ms, and their closed-time distribution (Fig. 1B) cannot be described by a single-exponential decay, which indicates that there is more than one closed state of the channel (see also Moczydlowski et al., 1984a). In contrast, the open-time distribution (Fig. 1C) is reasonably well described by a single-exponential decay, which is consistent with a single open state.
The addition of nanomolar amounts of either STX or TTX to the extracellular solution resulted in very long-lived channel closures (Krueger et al., 1983; Green et al., 1984c; Moczydlowski et al., 1984a, b) (see Fig. 2A). (This figure depicts data obtained with TTX; similar results were obtained with STX.) The toxin-induced closures are complete; that is, the amplitude of the current transitions corresponds to the amplitude of the channels' unitary current steps (Green et al., 1987). The toxin action is reversible; it was abolished by perfusing the extracellular solution with a toxin-free, but otherwise identical, solution (data not shown). Toxin-induced closures were not observed when the toxins were added to the intracellular solution. (Subconductance events [Green et al., 1987] may last substantially >600 ms. They are, however, easily recognized and cannot be mistaken for toxin-induced closures. There are no discernible differences between toxin binding to channels in the fully open state and that to channels in the subconductance or flickerly states.)

**Figure 1.** Spontaneous channel closures. The current traces in A illustrate spontaneous current transitions in a single BTX-modified sodium channel. The channel is open most of the time, but has occasional closing transitions to the fully closed state. The arrow points to a transition to a subconductance state. Experiment 850415: 0.5 M Na', 0.01 M HEPES, ΔV = +63 mV, cutoff frequency = 40 Hz. (B) Survivor plot of spontaneous channel closures. The histogram is based on 304 transitions from a single channel. The subpopulation of channel closures that last >0.2 s were analyzed as a single-exponential decay: \( N(t) = N \cdot \exp(-t/\tau) \), where \( N(t) \) is the number of closed events of duration \( \geq t \), \( N \) is the number of events in the population at \( t = 0 \), and \( \tau \) is the mean closed time. There is an excess number of short-lived closures. \( \tau = 0.42 \) s, \( N = 115 \). Experiment 830810: 0.5 M Na', 0.01 M HEPES, ΔV = +60 mV. (C) Survivor plot of the open-time distribution for the same channel. The distribution is described by a single-exponential decay. \( N = 292 \), \( \tau = 0.78 \) s.
The dwell-time histograms change after toxin addition, consistent with the appearance of a single population of long-lived closing events (Fig. 2, B and C). The frequency and mean duration of the short-lived closings are unaffected by the toxin addition (cf. Figs. 1 and 2); that is, the toxins induce a new type of closing event and have no effect on the frequency or duration of the events seen in the absence of toxin. The lifetime distribution of long-lived, toxin-induced channel closures can be described by a single-exponential decay (Fig. 2B). Similar estimates for \( \tau_c \) were obtained from an exponential fit to this subpopulation and from Eq. 2 (3.6 vs. 3.9 s).

The open-time distribution is also well described by a single-exponential decay (Fig. 2C). The interpretation of the data is complicated by the existence of several kinetically distinct closed states, and the time constant of the decay is not simply related to the rate of toxin binding. If the analysis is restricted to open times between closures lasting >0.6 s, the distribution is also described by a single-exponential decay (Fig. 2D). The time constant of this distribution repre-
sents the mean time between toxin-induced channel closures and is related to the rate of toxin binding. Again, very similar estimates for $\tau_o$ were obtained from the exponential fit and from Eq. 3 (4.6 vs. 4.7 s). The single-exponential decay of the toxin-associated dwell-time distributions indicates that the toxins bind and produce reversible transitions between a single open and a single toxin-induced closed state.

**Equilibrium Toxin Binding and Its Na$^+$ and Potential Dependence**

The dose-response characteristics of toxin-induced channel closures can be quantified by the relation between the fractional closed time, $f_c$ (Eq. 1), and $[T]$, where T denotes the toxin, either STX or TTX. The data for STX and TTX in 0.05 M Na$^+$ are illustrated in Fig. 3. At high toxin concentrations, $f_c$ approaches

$$f_c = f_{c,max} - [T] / (K + [T]),$$  

where $f_{c,max}$ is the maximum $f_c$, and $K$ is the $[T]$ at which $f_c = 0.5 \cdot f_{c,max}$. Because $f_{c,max}$ for both STX and TTX is indistinguishable from 1.0, binding-site occupancy and channel closure are indistinguishable within the limits set by the resolution. Therefore, $f_c$ is interpreted to be the probability that the binding site is occupied by toxin, while $K$ is interpreted to be the toxin's apparent dissociation constant, $K_D$. Given Eq. 4, and that $f_{c,max} \approx 1.0$, $K_D$ can be estimated based on a single determination of $f_c$ at a given $[T]$.

![Figure 3. Dose-response curves for toxin block. (A) A two-channel current trace at high [STX]; both channels are closed most of the time. Occasional brief (upward) transitions to the open state are seen; $f_c$ is very close to 1. [STX] is 60 times the magnitude of dissociation constant determined in B. Experiment 840706: 0.05 M Na$^+$, 0.01 M HEPES, 43.0 nM STX, $\Delta V = +60$ mV. (B) $f_c$ vs. toxin concentration for STX (▲) and for TTX (●). The curves denote the best fits of Eq. 4 to the data. For STX: $f_{c,max} = 1.01 \pm 0.03$, $K = 0.699 \pm 0.034$ nM; for TTX: $f_c = 1.01 \pm 0.03$, $K = 12.4 \pm 1.5$ nM. The arrows denote the $K_D$ values. 0.05 M Na$^+$, 0.01 M HEPES, $\Delta V = 60$ mV.](image-url)
$K_D$ for both STX and TTX varies as a function of the membrane potential difference, $\Delta V$ (French et al., 1984; Green et al., 1984c; Moczydlowski et al., 1984a, b) (see Fig. 4). The voltage dependence of $K_D(\Delta V)$ can be described by:

$$K_D(\Delta V) = K_D(0) \cdot \exp(\delta \cdot \Delta V \cdot e/kT), \tag{5}$$

where $K_D(0)$ is $K_D$ at 0 mV, $\delta$ is an equivalent valence that quantifies how the applied potential affects toxin binding, $e$ is the elementary charge, $k$ is Boltzmann's constant, and $T$ is the temperature in Kelvin. When Eq. 5 is fitted to the data in Fig. 4, the $\delta$ estimates are similar, 0.63 ± 0.08 for STX and 0.71 ± 0.10 for TTX. The $K_D(0)$ estimates differ 10-fold, however: 1.03 ± 0.15 nM for STX and 10.6 ± 1.6 nM for TTX.

The results from experiments at other $[\text{Na}^+]$ (0.02 ≤ $[\text{Na}^+]$ ≤ 3.5 M) are summarized in Table I. The $K_D(0)$ values vary as functions of $[\text{Na}^+]$. The variation is more pronounced for the divalent STX than for the monovalent TTX. Estimates for $\delta$ vary by a factor of <2. At each $[\text{Na}^+]$, the difference between the $\delta$ estimates for STX and for TTX is within our experimental error. STX and TTX bind and close the channel with the same voltage dependence.
Kinetics of Toxin-induced Channel Closures

On the basis of the equilibrium experiments, the simplest kinetic scheme for the action of STX and TTX is that they induce transitions between two distinguishable channel states: an open, toxin-free state, O, and a closed, toxin-bound state, CT:

\[ O + T \stackrel{k_c}{\rightleftharpoons} CT, \]  

(Scheme I)

where \( k_c \) is the rate constant for toxin association with the channel and \( k_o \) is the rate constant for toxin dissociation from the channel. The rate constants are related to the average duration of the toxin-induced open and closed times (e.g., Fig. 2):

\[ k_c = \frac{1}{(\tau_o \cdot [T])}, \]  

(6)

\[ k_o = \frac{1}{\tau_c}, \]  

(7)

and the toxin dissociation constant is:

\[ K_D = k_o/k_c. \]  

(8)

Fig. 5 depicts open-time distributions at different \([TTX]\). These data, along with similar information for STX, and the associated closed-time data for both toxins are analyzed further in Fig. 6. Consistent with Scheme I, \( 1/\tau_c \) for STX and TTX increases in proportion to \([T]\), while \( 1/\tau_c \) is almost independent of \([T]\).

The voltage dependence of \( K_D \) results from voltage-dependent changes in both \( k_c \) and \( k_o \). The rate constants for toxin-induced channel closures are plotted as a function of \( \Delta V \) in Fig. 7. The voltage dependence of the rate constants can be expressed as

\[ k_c(\Delta V) = k_c(0) \cdot \exp(-\delta_c \cdot \Delta V \cdot e/kt), \]  

(9)

and

\[ k_o(\Delta V) = k_o(0) \cdot \exp(\delta_o \cdot \Delta V \cdot e/kt), \]  

(10)

\[ n \] is the number of determinations for \( K_o (80 \leq \Delta V \leq +120 \text{ mV}) \).
where $k_c(0)$ and $k_o(0)$ denote the rate constants at 0 mV, and $\delta_c$ and $\delta_o$ are equivalent valences that quantify how the applied potential affects the corresponding rate constants. When Eqs. 9 and 10 are fitted to the data in Fig. 7, the estimates for $k_o$, $\delta_o$, and $\delta_c$ are similar for STX and TTX, while the estimates for $k_c(0)$ differ 10-fold. The ratio $k_c(0)/k_o(0)$ is 1.41 nM for STX and 13.2 nM for TTX, and the sum $\delta_o + \delta_c$ is 0.58 and 0.63, in reasonable agreement with the $K_D$ and $\delta$ estimates obtained from the $f_c$ data.

![Image of graph showing open-time survivor plots as a function of [TTX].](image)

**Figure 5.** Open-time survivor plots as a function of [TTX]. The data are displayed as $\ln[N(t)/N]$ vs. $t$. Only openings occurring between closures $\geq 0.6$ s were included in the distributions. The lines denote fits of single-exponential decays to the data. [TTX] = 3.05 nM: $\tau_o = 37.0$ s and $N = 79$; for 6.1 nM: $\tau_o = 17.2$ s and $N = 61$; for 12.2 nM: $\tau_o = 10.8$ s and $N = 57$; for 24.4 nM: $\tau_o = 6.4$ s and $N = 63$; for 51.0 nM: $\tau_o = 3.2$ s and $N = 61$; and for 116 nM: $\tau_o = 1.9$ s and $N = 82$. Experiment 831011: 0.05 M Na$^+$, 0.01 M HEPES, $\Delta V = 62$ mV.

Experiments at other [Na$^+$] ($0.02 \leq$ [Na$^+$] $\leq$ 3.5 M) are summarized in Table II. For both STX and TTX, $k_d(0)$ varies as function of [Na$^+$]. As for $K_D$, the variation is more pronounced for STX than for TTX. In contrast, the $k_o(0)$ estimates for STX and TTX vary little with changes in [Na$^+$]. The Na$^+$-dependent changes in $K_D$ result primarily from changes in $k_c$. The $\delta_c$ estimates decrease slightly with decreasing [Na$^+$], while those for $\delta_o$ vary little with [Na$^+$]. Interestingly, there are consistent differences between the $\delta_c$ and $\delta_o$ estimates for
the toxins, the $\delta_e$ estimates for STX being larger than for TTX, with the opposite pattern for the $\delta_o$ estimates.

The $\text{Na}^+$ dependence of the $k_o$ estimates does not arise from a bias in our data-reduction procedures. Similar behavior is observed when the closed-time distributions are analyzed as a function of $[\text{Na}^+]$ at a constant $\Delta V$ (Fig. 8). For each of the three $\text{Na}^+$ concentrations, the data are well described by a single-exponential decay; there is no evidence for the existence of two distinct closed states among the long-lived channel closures. The same ($\text{Na}^+$-dependent) variation is observed in the $k_o(+60)$ values as in the $k_o(0)$ estimates.

**Is There a Negative Electrostatic Potential at the Toxin-binding Site?**

It has been proposed that there is a negative electrostatic potential (relative to the bulk solution) at the toxin-binding site (Henderson et al., 1974; Hille et al., 1975a). Such a potential difference, arising from negative charges at or close to the toxin-binding site, could explain why $K_D$, or $k_e$ estimates for STX vary as a stronger function of $[\text{Na}^+]$ than the corresponding estimates for TTX (Green
Increases in $[\text{Na}^+]$ could change toxin binding by competitively displacing the toxins as well as by changing the potential at the binding site by screening the fixed negative charges. Such a screening will change the local $[\text{T}]$ (and $[\text{Na}^+]$), and the $[\text{T}]$ changes will be larger for STX, which carry a charge of $+2$, than for TTX, which carry a charge of $+1$.

An extension of the two-state model (Scheme I) to include competitive interactions between $\text{Na}^+$ and toxin and fixed charges in the vicinity of the toxin-
### TABLE II

| [Na⁺] M | Toxin | n* | $k_0(0) ± SD$ M⁻¹ s⁻¹ | $k_1 ± SD$ s⁻¹ | $k_0(0)$ s⁻¹ | $k_1 ± SD$ |
|---------|-------|----|----------------|---------------|--------------|----------|
| 0.02 TTX | 10 | 2.2±0.1 x 10⁷ | 0.15±0.05 | 5.8±0.6 x 10⁻⁴ | 0.32±0.07 |
| 0.05 STX | 16 | 5.3±0.5 x 10⁸ | 0.28±0.06 | 5.1±0.3 x 10⁻⁴ | 0.29±0.08 |
| 0.05 TTX | 19 | 1.5±0.1 x 10⁷ | 0.24±0.04 | 4.7±0.5 x 10⁻⁴ | 0.26±0.05 |
| 0.1 STX | 15 | 3.1±0.5 x 10⁸ | 0.35±0.06 | 6.6±0.4 x 10⁻⁴ | 0.19±0.05 |
| 0.1 TTX | 15 | 6.1±0.5 x 10⁸ | 0.25±0.05 | 8.1±0.7 x 10⁻⁴ | 0.35±0.07 |
| 0.1 STX | 13 | 4.7±0.5 x 10⁷ | 0.37±0.05 | 6.6±0.5 x 10⁻⁴ | 0.26±0.05 |
| 0.5 TTX | 17 | 1.5±0.2 x 10⁹ | 0.24±0.05 | 7.1±0.8 x 10⁻⁴ | 0.44±0.04 |
| 0.5 STX | 17 | 4.4±1.3 x 10⁹ | 0.36±0.14 | 8.6±0.5 x 10⁻⁴ | 0.32±0.04 |
| 1.0 TTX | 10 | 1.2±0.1 x 10⁹ | 0.37±0.06 | 5.8±0.9 x 10⁻⁴ | 0.48±0.14 |
| 1.0 STX | 10 | 2.1±0.2 x 10⁹ | 0.52±0.07 | 8.9±0.8 x 10⁻⁴ | 0.21±0.07 |
| 2.5 TTX | 17 | 4.3±0.4 x 10⁸ | 0.26±0.03 | 1.7±1.0 x 10⁻⁴ | 0.28±0.02 |
| 3.5 TTX | 3 | 3.2±0.5 x 10⁸ | 0.40±0.08 | 1.2±0.8 x 10⁻⁴ | 0.1±0.3 |

* n is the number of determinations for $k_0$ and $k_1$ ($-80 ≤ ΔV ≤ +120$ mV).

**Figure 8.** Closed-time survivor plots for TTX at three different [Na⁺]. The data are displayed as ln[$N(t)/N$] vs. $t$. Only channel closures lasting >0.6 s were included in the distributions. The lines denote fits of single-exponential decays to the data. For 0.05 Na⁺ M (rightmost data set): $τ_0 = 11.4$ s and $N = 411$ ($n = 419$); for 0.5 Na⁺ M (middle data set): $τ_0 = 3.6$ s and $N = 545$ ($n = 478$); for 2.5 Na⁺ M (leftmost data set): $τ_0 = 2.7$ s and $N = 226$ ($n = 214$). Experiment 831011: 0.05 M Na⁺, 0.01 M HEPES, ΔV = 62 mV; experiment 840528: 0.5 M Na⁺, 0.01 M HEPES, ΔV = 60 mV; experiment 831122: 2.5 M Na⁺, 0.01 M phosphate, ΔV = 56 mV.
binding site is developed in the Discussion and the Appendix. According to the extended model, $K_D$ (or $k_5$) for STX and TTX will vary as functions of both $[Na^+]$ and the electrostatic potential at the binding site, $V_s$ (see Eqs. 14 and 15). The (competitive) $Na^+$ dependence, however, will not appear in the ratio $K_{D{STX}}/K_{D{TTX}}$:

$$K_{D{STX}}([Na^+], V_s)/K_{D{TTX}}([Na^+], V_s) = (K_{D{STX}}/K_{D{TTX}}) \cdot \exp(V_s \cdot e/kT),$$

(11)

or the ratio $k_{c{STX}}/k_{c{TTX}}$:

$$k_{c{STX}}([Na^+], V_s)/k_{c{TTX}}([Na^+], V_s) = (k_{c{STX}}/k_{c{TTX}}) \cdot \exp(-V_s \cdot e/kT),$$

(12)

where the $K_D$ values on the right-hand side of Eq. 11 denote the toxin dissociation constants when $V_s$ and $[Na^+]$ are both zero, and the $k_c$ values on the right-hand side of Eq. 12 denote the corresponding association rate constants. The relation between the charge distribution, $[Na^+]$, and $V_s$ is not known. As a first approximation, the Gouy-Chapman equation will be used (e.g., Aveyard and Haydon, 1973):

$$V_s = (2 \cdot kT/e) \cdot \text{arcsinh}[\sigma/(8 \cdot [Na^+] \cdot \varepsilon_o \cdot \varepsilon \cdot kT)^{0.5}],$$

(13)

where $\sigma$ is an apparent charge density at the binding site, $\varepsilon_o$ is the permittivity of free space, and $\varepsilon$ is the relative dielectric constant of water (78.5 at 24°C as estimated from Weast, 1972, p. E-49). (The presence of divalent $HPO_4^2-$ was ignored; this will not affect the estimates for $V_s$.) The $K_D$ and $k_c$ ratios are plotted as functions of $[Na^+]$ in Fig. 9. The solid curves were drawn according to Eqs. 11 or 12 and 13 with $\sigma = -0.33 \cdot e \cdot nm^{-2}$, $K_{D{STX}}/K_{D{TTX}} = 1.0$, and $k_{c{STX}}/k_{c{TTX}} = 0.8$. The estimates for $K_{D{STX}}/K_{D{TTX}}$ and $\sigma$ can vary, because the parameters are correlated. An almost equally good fit was obtained when $K_{D{STX}}/K_{D{TTX}} = 1.5$ and $\sigma = -0.4 \cdot e \cdot nm^{-2}$; acceptable fits are obtained when the parameter set varies between these limits. (In Fig. 9B, we also plotted $k_{c{STX}}/k_{c{TTX}}$ as a function of $[Na^+]$, to emphasize that the differential effect of $[Na^+]$ changes on STX and TTX results from changes in the local $[T]$ and that the $Na^+$ dependence of $k_c$ is the same for both toxins.)

Since Eqs. 12 and 13 can describe the $[Na^+]$ dependence of the $K_D$ ratios, there may be a negative potential (and a net negative charge) in the vicinity of the toxin-binding site. The next two sections report results of experiments designed to explore this point further.

**Effects of Monovalent Cations on Toxin-induced Channel Closures**

If there is a net negative charge near the toxin-binding site, the addition of "inert" electrolytes to the extracellular aqueous phase will produce differential decreases in the apparent affinity for TTX and STX. (Ideally, an inert $Na^+$ substitute should not be permeant through the channel; neither should it block the channel, compete with toxins at their binding site, or bind to the channel, thereby altering the net charge. It is not clear that any truly inert cations exist, and some of the ions used here interact with the sodium channel. This should not, however, affect our main conclusions.) The ionic strength was increased using the Cl$^-$ salts of tetraethylammonium (TEA$^+$) and N-methylglucamine (NMG$^+$). Increases in ionic strength using TEA$^+$ decreased the STX affinity...
more than the TTX affinity, without affecting the voltage dependence of the channel closures. A decrease in the STX affinity was also observed when the ionic strength was increased with NMG⁺. The data for $k_c$, $k_o$, and $K_D$ are summarized in Table III.

Importantly, the decreases in $K_D$ were caused by decreases in $k_c$, whereas the $k_o$ values were unaffected by these maneuvers. The $k_c$ changes were related to ionic strength changes in the extracellular phase. (The small increases in $k_o$ for TTX and $k_c$ for STX with 0.02 M TEA⁺ in the intracellular phase are within the uncertainties in our estimates of the rate constants.) The larger decreases in STX affinity, relative to the TTX affinity changes, are consistent with the existence of a net negative charge near the toxin-binding site (see also Table VI and the related text of the Discussion).

![Figure 9](image-url)

**Figure 9.** Differential effects of [Na⁺] on STX- and TTX-induced channel closures. (A) $K_D^{STX}/K_D^{TTX}$ and $K_D^{TTX}/K_D^{STX}$ vs. [Na⁺]. The curves denote fits of Eqs. 11 and 13 to the data (see text). (B) $k_c^{TTX}/k_c^{STX}$ vs. [Na⁺]. The curved line denotes a fit of Eqs. 12 and 13 to the $k_c$ data (see text). The straight line is drawn to emphasize that there is no systematic trend in the $k_o$ data.

**Effects of Divalent Cations on Toxin-induced Channel Closures**

Divalent cations screen fixed negative charges better than monovalent cations (e.g., McLaughlin, 1977). The effects of two divalent cations, Ba⁺⁺ and Zn⁺⁺, were examined to further test for screening of fixed negative charges and to evaluate the relation between the permeation path and the toxin-binding site.

The addition of 0.005 M Ba⁺⁺ to an extracellular solution containing 0.02 M Na⁺ results in a fourfold increase in $K_D$ for STX (Table IV). The effects of Ba⁺⁺ were similar to the effects of TEA⁺ and NMG⁺, except that Ba⁺⁺ was effective at lower concentrations.

Zn⁺⁺ had a much larger effect than Ba⁺⁺. When 0.0008 M Zn(NO₃)₂ was added to an extracellular solution containing 0.02 M Na⁺, $K_D$ for STX and TTX increased 10-fold and 2-fold, respectively (Table IV). Again, the changes in $K_D$...
### TABLE III
Effects of Monovalent Cations on Toxin-induced Channel Closures

| [Na⁺] | n* | M⁺ | [M⁺] | Toxin   | $K_d(0) \pm SD$ | $\delta \pm SD$ | $k_d(0) \pm SD$ | $\delta_k \pm SD$ | $k_d(0) \pm SD$ | $\delta_k \pm SD$ |
|--------|----|----|------|---------|----------------|----------------|-----------------|-----------------|----------------|----------------|
| M      |    | M  |      |         |                |                |                 |                 |                 |                 |
| 0.02   | 5  | TEA⁺| 0.08 | TTX     | 3.7±0.8 x 10⁻⁶ | 0.56±0.14  | 1.3±0.2 x 10⁻⁷ | 0.11±0.08  | 5.5±0.6 x 10⁻⁴ | 0.35±0.07  |
| 0.02   | 6  | TEA⁺| 0.08 | STX     | 1.0±0.4 x 10⁻⁶ | 0.54±0.24  | 2.2±1.1 x 10⁻⁷ | 0.2±0.3   | 5.2±0.5 x 10⁻⁴ | 0.31±0.11  |
| 0.05   | 4  | TEA⁺| 0.05 | STX     | 6.0±1.2 x 10⁻⁹ | 0.55±0.10  | 1.4±0.2 x 10⁻⁸ | 0.32±0.10  | 6.4±5.6 x 10⁻⁷ | 0.2±0.3   |
| 0.05   | 9  | TEA⁺| 0.02²| TTX     | 4.5±0.8 x 10⁻⁹ | 0.41±0.05  | 5.2±0.3 x 10⁻⁷ | 0.18±0.05  | 6.9±0.7 x 10⁻⁷ | 0.24±0.02  |
| 0.05   | 4  | TEA⁺| 0.02²| STX     | 5.3±0.7 x 10⁻¹⁰| 0.50±0.08  | 1.3±0.3 x 10⁻⁹ | 0.20±0.11  | 7.3±0.8 x 10⁻⁷ | 0.29±0.06  |
| 0.05   | 5  | NMG⁺| 0.15 | STX     | 2.3±0.5 x 10⁻⁹ | 0.56±0.15  | 5.9±0.8 x 10⁻⁸ | 0.28±0.12  | 4.5±0.5 x 10⁻⁷ | 0.29±0.06  |

Except for the values marked with asterisks, the inert electrolytes were added to both aqueous phases.

* n is the number of determinations of $K_d$, $k_d$, and $k_k$ ($-60 \leq \Delta V \leq +60$ mV).

² Added to the intracellular solution only.

### TABLE IV
Effects of Divalent Cations on Toxin-induced Channel Closures

| [Na⁺] | n* | D²⁺ | [D²⁺] | Toxin | $K_d(0) \pm SD$ | $\delta \pm SD$ | $k_d(0) \pm SD$ | $\delta_k \pm SD$ | $k_d(0) \pm SD$ | $\delta_k \pm SD$ |
|--------|----|------|------|-------|----------------|----------------|-----------------|-----------------|----------------|----------------|
| M      |    | M   |      |       |                |                |                 |                 |                 |                 |
| 0.02   | 8  | Ba²⁺| 0.005| STX   | 2.6±0.3 x 10⁻¹⁰| 0.38±0.20  | 1.2±0.1 x 10⁻⁷ | 0.14±0.06  | 3.1±0.5 x 10⁻² | 0.24±0.08  |
| 0.02   | 5  | Zn²⁺| 0.008| TTX   | 3.7±1.3 x 10⁻⁹ | 0.64±0.15  | 1.2±0.3 x 10⁻⁷ | 0.14±0.16  | 5.3±1.2 x 10⁻⁴ | 0.59±0.13  |
| 0.02   | 14 | Zn²⁺| 0.008| STX   | 9.8±0.5 x 10⁻¹⁰| 0.46±0.05  | 5.9±0.2 x 10⁻⁷ | 0.14±0.05  | 3.9±0.4 x 10⁻⁷ | 0.34±0.05  |

Zn²⁺ was added to the extracellular side only. Ba²⁺ was added to both aqueous phases. In one of eight determinations for STX, it was added to the extracellular side only.

* n is the number of determinations of $K_d$, $k_d$, and $k_k$ ($-60 \leq \Delta V \leq +60$ mV).
resulted from changes in $k_c$, while there was little effect on $k_o$ or the voltage dependences.

The finding that Zn$^{++}$ has little effect on the voltage dependence of $K_D$ (or of $k_c$ and $K_o$) is important, because Zn$^{++}$ causes a voltage-dependent (fast) block of

\[
\frac{(i_o - i_b)}{i_o} = a \exp\left(\frac{V - V_0}{m}\right) + b
\]

\[
V = 0 \text{ mV} \text{ denotes } \pm 1 \text{ SD for } k_c(0) \text{ and } k_o(0).
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\]

The current through voltage-dependent sodium channels (Green et al., 1987). The characteristics of this block are consistent with Zn$^{++}$ entering and binding some point down-field from the channel entrance (see Green et al., 1987, for details). If the toxin-binding site is close to the site where Zn$^{++}$ binds, there will be electrostatic (repulsive) interactions between Zn$^{++}$ and toxin. These interac-

\[
\frac{(i_o - i_b)}{i_o} = a \exp\left(\frac{V - V_0}{m}\right) + b
\]

\[
V = 0 \text{ mV} \text{ denotes } \pm 1 \text{ SD for } k_c(0) \text{ and } k_o(0).
\]
tions will be more pronounced as the membrane is hyperpolarized, since the fractional occupancy for Zn$^{2+}$ at its blocking site will increase as $\Delta V$ becomes more negative. Any interaction between Zn$^{2+}$ and toxin should alter the voltage dependence of the toxin-induced channel closures. No pronounced effect on the voltage dependence was observed (Fig. 10). In Fig. 10, A and C, the fractional occupancy of Zn$^{2+}$ is plotted as a function of membrane potential. The $k_c$ and $k_o$ estimates for TTX and STX are plotted in B and D, respectively. For TTX, the addition of Zn$^{2+}$ decreased $k_c$ twofold at all potentials, while $k_o$ was unaffected. Electrostatic interactions between toxin and Zn$^{2+}$, at their respective binding sites, will be larger for STX and Zn$^{2+}$ than for TTX and Zn$^{2+}$. For STX, there was an $\sim 10$-fold decrease in $k_c$, with little effect on $k_o$. $\delta_c$ was decreased, but no more than in the Ba$^{2+}$ experiments, and $\delta_o$ was unaffected. There appears to be no electrostatic interactions between a Zn$^{2+}$ at its blocking site and a toxin molecule at its binding site.

DISCUSSION

One Toxin Molecule Closes One Channel

It is generally assumed that STX and TTX bind to a common site and that channel closure results from the binding of one toxin molecule to one channel (Hille, 1968; Colquhoun et al., 1972; Schwarz et al., 1973). This assumption implies a particular channel structure: a single lumen that connects the extracellular and intracellular aqueous phases. However, many channels exhibit multiple conductance states, and recent structural studies on *Escherichia coli* porin channels suggest that a channel may have three separate entrances at one side of a membrane that merge into one large outlet on the other side (Engel et al., 1985). If each entrance could be closed independently, the relation between membrane conductance and toxin concentration would be described by Eq. 4, which is suggestive of all-or-none closures, but an examination of the single-channel closures would show toxin-induced transitions to intermediate conductance levels. This is not observed in native (Quandt et al., 1985) or BTX-modified sodium channels (e.g., Krueger et al., 1983; Green et al., 1984c; Moczydlowski et al., 1984a, b; see also Fig. 2 in this article). The toxins close the channels in an all-or-none fashion. Further, the shape of the dose-response curves for single-channel closures (Figs. 3 and 6) strongly suggests that a channel is closed by one toxin molecule. The data therefore provide no support for cooperative interactions between two toxin molecules (e.g., Benoit and Dubois, 1985) in closing BTX-modified sodium channels. (Channel closures could depend on the binding of two toxin molecules, if the two toxin molecules bind with different affinities. If the second molecule binds with an affinity that is at least 10-fold less than the first, the dose-response curve would approximate a simple Langmuir binding isotherm. Therefore, if the BTX modification disrupted a positively cooperative toxin binding, our data would be consistent with the proposal of Benoit and Dubois [1985]. This model, however, is difficult to reconcile with the lack of interactions between BTX and STX in equilibrium binding studies [Catterall et al., 1979; Krueger et al., 1979; but see Brown, 1986].)
Evidence for Fixed Negative Charges in the Vicinity of the Binding Site

Monovalent cations inhibit toxin binding by a one-to-one competition between cation and toxin (Henderson et al., 1973; Reed and Raftery, 1976; Weigele and Barchi, 1978; Barchi and Weigele, 1979). If simple competition were the sole mechanism for cation-toxin interactions, changes in the (monovalent or divalent) cation concentration should alter the apparent affinities for STX and TTX to the same extent. Contrary to this prediction, increases in \([Ca^{++}]\) decrease the binding and current inhibition by STX more than the binding and inhibition by TTX (Henderson et al., 1974; Hille et al., 1975a). The magnitude of the differential effect on the monovalent and divalent toxins can be accounted for by screening of a net negative fixed charge (Ritchie and Rogart, 1977). Consistent with this interpretation, the addition of \([La^{+++}]\) produces similar differential effects on the STX- and TTX-induced current inhibition as are produced by much larger concentrations of divalent cations (Grissmer, 1984).

Rhoden and Goldin (1979) found that the \(Na^{+}\) inhibition of STX binding to rat brain sodium channels exhibited a higher-order dependence than would be predicted by a one-to-one competitive interaction between \(Na^{+}\) and toxin. This "cooperative" effect of monovalent cations on the STX affinity could result if there were a net negative charge in the vicinity of the binding site, since the ionic strength was not maintained constant in these experiments. When the ionic strength is maintained constant, \([Na^{+}]\) changes do not alter \(V\), and the interaction between STX and \(Na^{+}\) is well described as a one-to-one competition (Weigele and Barchi, 1978; Barchi and Weigele, 1979).

There are thus compelling reasons to conclude that extracellular electrolytes affect toxin binding and channel closure through both competitive interactions with the toxins and screening of fixed charges. This conclusion is confirmed and extended by our findings. Our data strongly support the existence of a net negative charge in the vicinity of the guanidinium toxin–binding site. Similar to previous studies, various cations, i.e., \(Na^{+}\), \(TEA^{+}\), and \(Zn^{++}\), alter STX-induced closures to a greater extent than TTX-induced closures, from which we conclude that there is a net charge in the vicinity of the toxin-binding site. Moreover, since the experiments were done using bilayers that carry no net charge, the charge (or charges) that influences cation and toxin interactions with the binding site must be on the sodium channel protein itself. (We do not know whether these charges originate in the protein proper or in the associated carbohydrate moiety. Indirect evidence suggests that part of the functionally significant negative charge may reside in the carbohydrate. As \([Na^{+}]\) decreases, the channel-to-channel scatter of the STX \(k_c\) values increases and becomes significantly larger than that of the TTX \(k_c\) values [e.g., Fig. 7]. These variations could result from channel microheterogeneities, similar to those observed on sodium dodecyl sulfate polyacrylamide gels, which presumably arise from channel-to-channel variations in glycosylation [Barchi, 1983; J. A. Miller et al., 1983].)

Independent support for this conclusion is provided by studies on BTX-modified rat muscle sodium channels, where \(k_c\) for STX derivatives that carry net charges between -1 and +2 varies systematically with the net charge, which suggests that in 0.2 M NaCl, the binding site is ~40 mV negative relative to the
aqueous phase (Moczydlowski et al., 1986). Models for the interactions between toxins and cations at the toxin-binding site should therefore incorporate specific competitive binding of cations to the toxin-binding site and screening of a net negative charge. (It may also be necessary to consider nonspecific ion binding, which would alter the charge distribution in the vicinity of the binding site.) A simple model, incorporating one-to-one competition between toxin and monovalent cations (e.g., [Na⁺]) and screening of fixed charges in the vicinity of the toxin-binding site, is developed in the Appendix. The [Na⁺] and Vₐ dependence of Kₑ and kₑ for STX and TTX can be expressed as:

\[ K_D([\text{Na}^+]_0, V_a) = K_D \cdot \exp(V_a \cdot z \cdot e/kT) \cdot [1 + [\text{Na}^+] \cdot \exp(-V_a \cdot e/kT)/K_{Na}] \] (14)

and

\[ k_c([\text{Na}^+]_0, V_a) = k_c \cdot \exp(-V_a \cdot z \cdot e/kT) \cdot [1 + [\text{Na}^+] \cdot \exp(-V_a \cdot e/kT)/K_{Na}], \] (15)

where z is the valence of the toxin (+1 for TTX, +2 for STX), K_{Na} is the dissociation for Na⁺ at the toxin-binding site, and K_D and k_c denote the values of K_D and k_c in the absence of competing ions and without an electrostatic potential at the binding site (V_a = 0 mV).

In Fig. 11A, k_c(0) for STX and TTX (from Table II) is plotted as a function of [Na⁺]. As in Fig. 9, the charge distribution in the vicinity of the toxin-binding site is approximated by a uniformly smeared charge density, σ, and the relation between V_a, σ, and [Na⁺] was approximated by the Gouy-Chapman equation (Eq. 13). This combined competition and screening model (Eqs. 13 and 15) provides a good description of the Na⁺ dependence of both the STX and TTX data. The solid curves in Fig. 11A were calculated using the same value of σ as in Fig. 9, −0.33 e·nm⁻², with \( \varepsilon_{STX}^2 = \varepsilon_{TTX}^2 = 3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \), and K_{Na} = 0.3 M.

The difference in the Na⁺ dependence for the STX and TTX data in Fig. 11A cannot be explained by a simple one-to-one competitive interaction between Na⁺ and toxin, where the Na⁺ dependence of k_c(0) is given by:

\[ k_c(0) = k_c(0)/(1 + [\text{Na}^+]_0/K_{Na}), \] (16)

where \( k_c(0) \) is the toxin's association rate constant in the absence of competing ions (at [Na⁺] = 0). For [Na⁺] ≥ K_{Na}, log k_c(0) should decrease as an approximately linear function of log [Na⁺], with a slope whose magnitude is <1. For the TTX data, the slope is about −0.85, while the STX data scatter around a line with a slope of −1.6 (Green and Andersen, 1986). The STX data therefore cannot be fitted by the simple competitive model (Eq. 16). The \( k_c(0) \) and K_{Na} estimates obtained by fitting Eq. 16 to the TTX data are displayed in Table V. These estimates differ by one order of magnitude from those derived from a fit of the combined competition and screening model (Eqs. 13 and 15) to the data.

For a simple (Gouy-Chapman) screening of fixed charges, the Na⁺ dependence of k_c(0) is given by:

\[ k_c(0) = k_c(0) \cdot \exp(-V_a \cdot z \cdot e/kT), \] (17)

where V_a is a function of [Na⁺] (e.g., Eq. 13). In Fig. 11B, k_c(0) is plotted as a function of V_a (calculated from Eq. 13 with \( \sigma = -0.33 \cdot e\cdot \text{nm}^{-2} \)). The data for
FIGURE 11. [Na\(^+\)] dependence of \( k \) for STX and TTX. (A) \( k_0 \) for STX (△) and TTX (○) as a function of [Na\(^+\)]. The curves denote fits of Eqs. 13 and 15 to the data (see text). (B) \( k_0 \) for STX (△) and TTX (○) as functions of \( V_a \). The plot is a transform of A where \( V_a \) was determined from Eq. 13 (with \( \alpha = -1/300 \epsilon \cdot \AA^{-2} \)). The lines denote linear regressions to the data for [Na\(^+\)] ≤ 1.0 M. The slopes were \(-0.018 \text{ mV}^{-1}\) for STX and \(-0.034 \text{ mV}^{-1}\) for TTX (which should be compared with the theoretical expectations: \(-0.017\) and \(-0.034 \text{ mV}^{-1}\), respectively); \( k_0 \) was \( 4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}\) for STX and \( 5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}\) for TTX.

both STX and TTX are well described by the simple screening model (Eqs. 13 and 17) for \( V_a < -20 \text{ mV} \) (or [Na\(^+\)] ≤ 1.0 M); \( k_0 \) estimates obtained from a fit of Eqs. 13 and 17 to the data are listed in Table V. They are one order of magnitude smaller than the estimates obtained when including Na\(^+\)-toxin competition in the model. At high [Na\(^+\)] (≥2.5 M), there are deviations from predictions based on the simple screening model, as would be expected from Na\(^+\)-toxin competitive interactions. The deviations could also result because the approximations and assumptions underlying our use of the Gouy-Chapman theory break down at high ionic strengths.

TABLE V
Comparison of Parameters for Toxin-induced Channel Closures

| Model                              | \( k_0 \)          | \( K_{NN} \) | \( \sigma \) |
|------------------------------------|--------------------|--------------|-------------|
| Simple competition (Eq. 16)*       | \( 3 \times 10^5 \) | \( 3 \times 10^{-2} \) | —           |
| Simple screening (Eqs. 15 and 17)  | \( 5 \times 10^5 \) | —            | \(-0.33\footnote{2} \) |
| Competition and screening (Eqs. 13 and 15) | \( 3 \times 10^5 \) | \( 5 \times 10^{-1} \) | \(-0.33\footnote{2} \) |

* Based on a fit to the TTX data only (Green and Andersen, 1986).

\footnote{2} Based on the fit in Fig. 9.
At a constant [Na⁺], the addition of TEA⁺ or Zn²⁺ decreased the efficacy of the toxin to induce channel closures. The decreases were larger for the STX-induced than for the TTX-induced channel closures. If the only action of the inert cations were to affect $V_0$ through screening of fixed negative charges as described by Gouy-Chapman theory, the changes in $k_c(0)$ should be predicted from Eqs. 13 and 15. These predictions, and predictions based on the simple screening model (Eqs. 13 and 17), are summarized in Table VI and compared with the measured values. There is good qualitative agreement between the predicted and measured rate constants, but neither the simple screening model nor the combined competition and screening model is clearly superior over the other. The argument for competitive interactions between Na⁺ and toxin dep-
calculate \( V \), assuming a constant \( \sigma (-0.33 \text{ e} \cdot \text{nm}^{-2}) \), neither the simple screening (Eqs. 13 and 17) nor the combined competition and screening model (Eqs. 13 and Eq. 15) accurately predict the changes in \( k(0) \). The deviations were larger for STX than for TTX. It appears that TEA\(^+\) and Zn\(^{2+}\) reduce \( V \) more than can be accounted for by their screening action, which suggests that TEA\(^+\) and Zn\(^{2+}\) reduce the net charge by binding in the vicinity of the toxin-binding site. Specific binding of Zn\(^{2+}\) to sodium channels has been postulated to explain the effects of extracellular Zn\(^{2+}\) on the channels' voltage activation (Hille et al., 1975b) and gating charge movement (Gilly and Armstrong, 1982).

Discrepancies between predicted and observed effects may also arise because the charge distribution in the vicinity of the toxin-binding site was approximated by a uniformly smeared charge density. This approximation works well for the lipid bilayer/electrolyte interface (McLaughlin, 1977; Winiski et al., 1986), but may fail when used to describe ligand binding to proteins where only a few (relatively immobile) charged residues are likely to be important. (The cross-sectional areas of STX and TTX are \( \sim 0.5 \text{ nm}^2 \). If the cross-sectional area of the toxin-binding site is similar and involves a carboxyl group with a pK of \( \sim 5.5 \), as suggested by titration studies [Colquhoun et al., 1972; Ulbricht and Wagner, 1975a, b] and covalent modification experiments [Sigworth and Spalding, 1980; Spalding, 1980], this single charge could be the major contributor to the apparent charge density of \( -0.33 \text{ e} \cdot \text{nm}^{-2} \).)

Additionally, STX is not a point charge; it has two guanidinium groups separated by \( \sim 0.4 \text{ nm} \) (as estimated from CPK models). This complication should not be serious, however, because dimethonium \( [(\text{CH}_3)_3\text{N}^+(\text{CH}_2)_2\text{N}^+(\text{CH}_3)_3] \), which has a separation between the charged groups similar to that for STX, behaves as a classic (point charge) divalent cation at the lipid bilayer/electrolyte interface (McLaughlin et al., 1983). Finally, \( K_D \) varies as a function of the electrostatic field in the protein. At a constant \( \Delta V \), changes in the electrostatic potential at the extracellular surface of the protein will alter the field in the protein. This field variation could affect the equilibrium and kinetic aspects of toxin binding over and above what would be expected from the isolated change in \( V \) at the toxin-binding site. It would not, however, affect estimates based on the ratios of STX and TTX equilibrium or rate constants (Fig. 9).

The Location of the Toxin-binding Site

STX and TTX could close voltage-dependent sodium channels by several mechanisms: they could bind and physically occlude the channel (entrance); they could bind at a distant site and exert their effect on ion movement through electrostatic interactions with the permeating ions; or they could close the channel through a conformational change. On the basis of circumstantial evidence, it is widely assumed that the toxins physically occlude the channel by binding at its extracellular entrance (e.g., Catterall, 1980; Hille, 1984), but the other alternatives remain viable (e.g., Mullins, 1973; Hille, 1984, p. 302).

At a first glance, the voltage dependence of the toxin-induced channel closures supports a model in which the toxins enter and block the channel. In this case, the voltage dependence of closing would reflect the electrical distance between the extracellular aqueous phase and the binding site times the toxin valence
(Woodhull, 1973). However, the results of previous studies (Krueger et al., 1983; Green et al., 1984c; Moczydlowski et al., 1984a, b) and of this study are at odds with this interpretation. The similarity of the δ estimates for STX and TTX over a wide range of [Na+] provides additional evidence that only a small fraction (<0.1) of an applied potential difference falls between the bulk extracellular solution and the toxin-binding site (Green et al., 1984c; Moczydlowski et al., 1984b). The binding site must be quite superficial at the extracellular surface of the channel. The voltage dependence therefore results from voltage-dependent changes in the channel (Green et al., 1984c; Moczydlowski et al., 1984b): transitions between high- and low-affinity states (Moczydlowski et al., 1984b) or conformational changes leading to channel closure subsequent to toxin binding (see the next section).

The question remains whether the toxin-binding site is “close to” (e.g., partially covering) or “far from” (spatially separate) the permeation path. We addressed this question indirectly, by studying the effect of the intracellular and extracellular impermeant ions TEA+ and Zn2+ on the toxin-induced channel closures. TEA+ exerts a voltage-dependent block at the intracellular channel entrance (Green et al., 1987); ~0.5 of the applied potential appears to fall between the intracellular solution and the TEA+-blocking site. There were no measurable interactions between a STX or TTX molecule at their binding site and a TEA+ cation that blocks from the intracellular solution (Table III). The lack of a TEA+ effect argues that the TEA+-blocking site and the toxin-binding site are too far apart to have significant electrostatic interactions between cations at these sites.

A similar argument could be made with respect to the position of the toxin-binding site relative to the Zn2+-blocking site. Zn2+ exerts a voltage-dependent block at the extracellular channel entrance (Green et al., 1987); ~0.2 of the applied potential appears to fall between the extracellular solution and the Zn2+-blocking site. The addition of Zn2+ to the extracellular solution decreased k für STX, while k für TTX was hardly affected. In neither case were there apparent effects on k (Table IV).

It is possible to estimate the interaction energy, ΔGint, between Zn2+ at its blocking site and STX at its binding site, from the changes in STX binding in the absence and presence of Zn2+ and the changes in Zn2+ occupancy at its blocking site, ΔfZn. In the absence of Zn2+, the standard free energy for STX binding at an applied potential, ΔV, is

$$\Delta G^\circ(\Delta V) = kT \cdot \ln[K_D(\Delta V)] = \Delta G^\circ(0) + \delta \cdot e \cdot \Delta V,$$

(18)

where $\Delta G^\circ(0)$ is the standard free energy at $\Delta V = 0$. The difference in binding energy at ±ΔV is

$$\Delta \Delta G^\circ(\Delta V) = \Delta G^\circ(+\Delta V) - \Delta G^\circ(-\Delta V) = kT \cdot \ln[K(+\Delta V)/K(-\Delta V)],$$

(19)

Experiments on the node of Ranvier show no evidence for a voltage-dependent TTX inhibition of current flow through native, chloramine T-, or veratridine-modified sodium channels (Rando and Strichartz, 1985, 1986). The voltage dependence of the toxin-induced channel closures is a result of the BTX modification. Note, however, that the voltage dependence of the current inhibition, irrespective of its origin, provides an additional tool for studying voltage-dependent sodium channels.
which can be simplified as
\[ \Delta \Delta G^\circ (\Delta V) = 2 \cdot \delta \cdot e \cdot \Delta V. \] (20)

In the presence of Zn\(^{++}\), one similarly finds that
\[ \Delta \Delta G (\Delta V)_{zn} = 2 \cdot \delta_{zn} \cdot e \cdot \Delta V + \Delta f_{zn} (\Delta V) \cdot \Delta G_{\text{int}}, \] (21)
where \( \delta_{zn} \) is the voltage dependence of \( K_D (\Delta V) \) in the presence of Zn\(^{++}\). If there are no interactions between Zn\(^{++}\) and STX in the aqueous phase,
\[ \Delta G_{\text{int}} = 2 \cdot (\delta - \delta_{zn}) \cdot e \cdot \Delta V / \Delta f_{zn} (\Delta V). \] (22)

For \( \Delta V = 60 \text{ mV} \), \( \Delta f_{zn} (60) \) is \( \sim 0.6 \), while \( (\delta - \delta_{zn}) = 0.11 \pm 0.15 \) (cf. Tables II and IV). We thus estimate \( \Delta G_{\text{int}} \) to be \( 22 \pm 30 \text{ meV} \), and a reasonable upper limit on \( \Delta G_{\text{int}} \) is \( 2kT \). If the interactions between the bound Zn\(^{++}\) and STX are predominantly electrostatic (through the protein):
\[ \Delta G_{\text{int}} = (2 \cdot e)^2 / (4 \cdot \pi \cdot \epsilon_\circ \cdot \epsilon \cdot d_{1,2}), \] (23)
where \( d_{1,2} \) is the distance between the sites, and \( \epsilon_\circ \) is an average dielectric constant. For \( \Delta G_{\text{int}} \) to be \( \leq 2kT \), the \( \epsilon \cdot d_{1,2} \) product must be \( \geq 1.1 \times 10^2 \text{ nm} \). \( \epsilon_\circ \) is between 3 and 80 (Pethig, 1979; Honig et al., 1986), and \( d_{1,2} \) is estimated to be \( >1.5 \text{ nm} \). (A slightly larger minimum separation is obtained if the interactions predominantly result from changes in \( V_n \) owing to changes in Zn\(^{++}\) binding, in which case the minimum separation should be comparable to the Debye length in 0.02 M monovalent salt, \( \sim 2 \text{ nm} \).)

The large separation between the Zn\(^{++}\)-blocking site and the toxin-binding site could occur if the permeation path is long (\( \sim 10 \text{ nm} \)) and fairly narrow, such that it can be occupied simultaneously by Zn\(^{++}\) (or TEA\(^{+}\)) and a toxin molecule with no electrostatic interaction between the two blocking ions. Alternatively, the toxin-binding site is spatially distinct, and distant from the permeation path. The available information supports a structure where the permeation path and the toxin-binding site are distinct. In covalent modification experiments, the single-channel conductance can be modified with no apparent effect on toxin binding (Chabala et al., 1986).\(^2\) Similar experiments on sodium channels in the frog node of Ranvier show that carboxyl groups at the extracellular channel entrance seem to be distinct from those in the vicinity of the toxin-binding site (Gülden and Vogel, 1985). Similarly, it appears that the apparent charge density at the binding site can differ from that at the extracellular channel entrance (Neumcke and Stämpfli, 1986). The lack of electrostatic interactions between toxins at their binding site and ions in the permeation path further implies that the toxins cannot close the channel by physically occluding the permeation path or through electrostatic interactions with the permeating ions. The most likely

\(^2\) Worley et al. (1986) concluded that the toxin-binding site is at the extracellular channel entrance. This conclusion was based on the complete identity between trimethylxoxonium-induced modifications of toxin binding and of single-channel conductance under conditions expected to lead to an almost complete modification of carboxylate groups. An alternative interpretation is that modification of a carboxylate group in the binding site leads to a conformational change in the channel, thereby decreasing the conductance.
mechanism for the toxin-induced closures of voltage-dependent sodium channels is a conformational change that closes the channel.

**A Model for Toxin-induced Channel Closure**

On the basis of the preceding discussion, models for guanidinium toxin-induced closure of BTX-modified sodium channels should incorporate two features: the voltage dependence of the toxin-induced closures should arise from voltage-dependent changes in the channel protein, and the channel closure should result through conformational changes that occur subsequent to toxin binding. A minimal model incorporating these features is obtained by extending Scheme I to include an intermediary (third) state, where the toxins are bound to a sodium channel in the conducting state. A channel in this state can then undergo a voltage-dependent conformational change to close the channel:

\[
O + T \xrightarrow{k_1} OT \xrightarrow{\frac{\alpha(\Delta V)}{\beta(\Delta V)}} CT.
\]  

(Scheme II)

In this scheme, \(O\) is a conducting state with no toxin at its binding site, \(OT\) is a conducting state with a toxin at its binding site, and \(CT\) is the nonconducting state with a toxin at its binding site. For simplicity, the voltage dependence of the inhibition is assigned to the rate constants for transitions between \(OT\) and \(CT\), \(\alpha(\Delta V)\), and \(\beta(\Delta V)\). This model differs from that proposed by Moczydlowski et al. (1984b) in that channel closure is not associated with toxin binding per se, but to conformational changes that occur subsequent to toxin binding. Scheme II can be extended to incorporate a voltage-dependent binding reaction, \(O + T \rightleftharpoons OT\), by introducing additional voltage-dependent equilibria between two \(O\) and between two \(OT\) states, as was done by Moczydlowski et al. (In any case, the voltage dependence of the toxin-induced channel closures appears to be an incidental finding, a consequence of the BTX modification [Rando and Strichartz, 1985, 1986]. The important point for native channels is that the channel closures in Scheme II occur subsequent to toxin binding.)

Solving the kinetic equations corresponding to Scheme II, the dose-response curve is

\[
f_c = \frac{\gamma \cdot [T]}{[K_1 + (\gamma + 1) \cdot [T]]},
\]  

(24)

where \(K_1 = k_- / k_1\), and \(\gamma(\Delta V) = \alpha(\Delta V) / \beta(\Delta V)\). Comparing Eq. 4 with Eq. 24,

\[
f_{c_{\text{max}}} = \gamma / (\gamma + 1),
\]  

(25)

and

\[
K_D = K_1 / (\gamma + 1).
\]  

(26)

Experimentally, \(f_c\) approaches 1 as \([T] \to \infty\) (Fig. 3). Therefore, \(\gamma / (\gamma + 1) \approx 1\), \(\gamma \gg 1\), and \(K_D \approx K_1 / \gamma\) in the potential range investigated here. In this model, the high toxin affinity results from the conformational change(s) that occurs subsequent to the initial binding, not from the binding reaction(s) per se.

According to Scheme II, survivor plots of toxin-induced closed times should be described by single-exponential decays, as was observed (Figs. 2 and 8). This
implies that
\[ k_0 = \beta. \quad (27) \]

[If \( \alpha \) is not much less than \( k_{-1} \), there may be several OT \( \cong CT \) transitions during the time a toxin molecule is bound to the channel. If these transitions cannot be resolved, \( \tau_c^{-1} \approx \beta \cdot k_{-1}/(\alpha + k_{-1}). \)]

According to Scheme II, survivor plots of the intervals between toxin-induced channel closures should generally be described as the sum of two-exponential decays. But only a single-exponential decay was observed (Figs. 2 and 5), which implies that

\[ k_c = \alpha/K_1. \quad (28) \]

[Generally, \( 1/(\tau_o \cdot [T]) \approx \alpha \cdot k_1/(\alpha + k_{-1} + k_1 \cdot [T]) \). For \( k_c \) to be independent of \([T], k_1 \cdot [T] \ll \alpha + k_{-1}; \) further, if \( \alpha \ll k_{-1}, \) Eq. 28 is obtained.]

Toxin action depends on a protonated guanidinium group (C7-C8-C9 in STX [Hille, 1968], and C1-C2-C3 in TTX) and several hydroxyl groups (the C12 gem diol in STX and the C9 and C10 hydroxyls in TTX [Kao and Walker, 1982; Strichartz, 1984; Kao and Yasumoto, 1985]). The existence of two classes of functional groups on the toxin molecules suggests in itself a two-step reaction for toxin-induced channel closures, similar to Scheme II. The hydroxyl groups on the toxin may be involved in the initial binding reaction, O \( \rightarrow \) OT, since the removal of functionally important hydroxyl groups decreases binding affinity without abolishing toxin action. The conformational change that closes the channel may involve interactions between the functionally important guanidinium groups and carboxyl groups at the toxin-binding site, since toxin action is completely abolished by modifying sodium channels with carboxyl group-specific reagents (Shrager and Profera, 1973; Baker and Rubinson, 1975; Reed and Raftery, 1976; Spalding, 1980).

The conformational change(s) could cause a positive entity (e.g., an arginyl or a lysyl residue) to move into the channel lumen to abolish ion movement. Some support for this hypothesis is provided by the observation that the toxin-Na\(^+\) interactions are not classically competitive, as \( k_o \) increased with increasing [Na\(^+\)] (Fig. 8). There thus seem to be (weak) interactions between a toxin at its binding site and Na\(^+\) interacting with the channel at some other site. A similar observation was made by Ulbricht and Wagner (1975b) in studies on toxin-H\(^+\) interactions, where the rate constant for relief of current inhibition increased when the extracellular [H\(^+\)] was increased.

Our conclusion that the toxin-binding site is distant from the channel entrance raises the question, why is the toxin-binding site so highly conserved through evolution? One would expect parts of the channel not essential for function to be more susceptible to evolutionary pressures, and less likely to be conserved, than functionally significant parts. Since TTX-sensitive sodium channels are found in phyla ranging from Annelida to Chordata (e.g., Hille, 1984, p. 374), it appears that the toxin-binding site is essential for channel function, but is not involved in ion movement through the open permeation path. Possible functional roles are that the toxin-binding site is involved in channel gating, e.g., inactivation, or slow inactivation (the voltage dependence of \( K_D \) appears when slow
inactivation is inhibited (Rando and Strichartz, 1985); that the toxin may bind
at a site that is critical for subunit or domain interactions in the channel; or that
the toxins mimic endogenous ligands that bind to the receptor.

**APPENDIX**

**Competitive Toxin-Na\(^+\) Interactions at a Charged Binding Site**

Let, O, CT, and ONa\(^+\) denote channel states where the toxin-binding site is unoccupied
(by toxin or Na\(^+\)), occupied by toxin (T), and occupied by Na\(^+\). The competitive interaction
between T and Na\(^+\) is described by:

\[
O + T \rightleftharpoons \frac{k_c}{k_d} CT, \quad \text{(Scheme A1)}
\]

and

\[
O + \frac{k_{h_{Na}}}{k_{Na}} \rightleftharpoons ONa^+, \quad \text{(Scheme AII)}
\]

subject to the constraint that \(W(O) + W(CT) + W(ONa^+) = 1\), where \(W\) denotes the
probability of finding the channel in the three states.

Solving the kinetic equations corresponding to Schemes A I and A II,

\[
K_D = K_D^0 \cdot (1 + [Na^+] / K_{Na}), \quad \text{(A1)}
\]

where \(K_D = k_d / k_c\) and \(K_{Na} = k_{h_{Na}} / k_{Na}\), and the concentrations (and rate constants and
dissociation constants) refer to the local concentrations at the toxin-binding site.

The local \([T]\) and \([Na^+]\) will differ from their bulk concentrations, \([T]_b\) and \([Na^+]_b\),
when the electrostatic potential at the binding site differs from that of the bulk solution.
If the average time the binding site is unoccupied is long compared with the electric and
diffusive relaxation times of the diffuse part of the double layer adjacent to the binding
site, the local concentrations can be related to the bulk concentrations by the Boltzmann
equation:

\[
[T] = [T]_b \cdot \exp(-V_s \cdot e / kT), \quad \text{(A2)}
\]

and

\[
[Na^+] = [Na^+]_b \cdot \exp(-V_s \cdot e / kT), \quad \text{(A3)}
\]

where \(V_s\) denotes the potential difference between the binding site and the bulk solution
(when the binding site is vacant), and \(e\) is the toxin valence.

When related to the bulk aqueous concentration, the rate constant for toxin association
with the binding site, \(k_c\), becomes:

\[
k_c = k_c \cdot \exp(-V_s \cdot e / kT) / [1 + [Na^+]_b \cdot \exp(-V_s \cdot e / kT) / K_{Na}]. \quad \text{(A4)}
\]

For a simple competitive interaction, \(k_c\) should not be affected by changes in \([Na^+]\), and
the desired expression for \(K_D\), as related to the bulk aqueous concentrations is

\[
K_D = K_D^0 \cdot \exp(V_s \cdot e / kT) / [1 + [Na^+]_b \cdot \exp(-V_s \cdot e / kT) / K_{Na}]. \quad \text{(A5)}
\]

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