Structural Determinants of the Affinity of Saxitoxin for Neuronal Sodium Channels

Electrophysiological Studies on Frog Peripheral Nerve

GARY STRICHARTZ

From the Anesthesia Research Laboratories and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The potencies of saxitoxin (STX) and of five structurally related toxins were determined by their ability to block impulses at equilibrium in frog sciatic nerve. The order of potency, with values relative to STX potency in parentheses, was: neo-STX (4.5) > gonyautoxin (GTX) III (1.4) > STX (1.0) > GTXII (0.22) > 12α-dihydroSTX (0.050) > 12β-dihydroSTX (0.0014). When equipotent solutions of STX and neo-STX were exchanged, impulses in the treated nerve were transiently overblocked or underblocked, thus kinetically distinguishing neo-STX from STX. Similar phenomena occurred with exchanges of STX and GTXIII. No consistent evidence was found for any blocking activity of STX molecules that were not protonated at the C8 guanidinium, but the pH dependence of STX potency cannot be described simply by the titration of this guanidinium group. The effects of pH and of various substituents on STX potency are accounted for by changes in the molecular forms of STX and by alterations in specific electrical charges on STX and at the receptor. The results support a model in which toxin molecules bind in two steps; initial binding of the C8 guanidinium to an anionic group induces the loss of water from the normally hydrated ketone (at carbon 12), which then forms a weak covalent bond with a nucleophilic group on the receptor.

INTRODUCTION

Tetrodotoxin and saxitoxin are used widely to study sodium channels in many excitable membranes (Ritchie and Rogart, 1977). Although it is known that these toxins reversibly block sodium channels by binding with high affinity to a receptor on the external surface of the membrane, the structural details of this receptor have not been characterized definitively. Since these toxins currently

Address reprint requests to Dr. G. R. Strichartz, Anesthesia Research Laboratories, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

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are being used to purify and reconstitute sodium channels (Hartshorne and Catterall, 1981; Barchi et al., 1980), investigations that may ultimately lead to a molecular description of the channel's structure, and, in addition, as their binding properties are being used to classify naturally occurring variants of channels (Rogart et al., 1983; Jaimovich et al., 1983), it is important to define what portions of these toxins are essential for their actions on native membranes. Information about essential groups on the toxin molecules also implies the existence of physicochemical properties of the toxin binding site on the sodium channel (cf. Hille, 1975*a*; Kao, 1983).

Previous work provides the background for the present experiments. Results both from electrophysiological experiments (Ulbricht and Wagner, 1975*a*, *b*) and from binding studies using radiolabeled toxins (Colquhoun et al., 1972; Henderson et al., 1973, 1974; Weigele and Barchi, 1978; Hansen Bay and Strichartz, 1976; Reed and Trzos, 1979) have shown that these ligands bind, with mutual exclusion, to an acidic site that also binds a variety of metal and organic cations. Elimination of binding or of toxin sensitivity by chemical reactions with selective reagents that modify the membranes strongly implicates at least one carboxylic acid group at the toxin receptor (Baker and Rubinson, 1975; Shrager and Profera, 1973; Spalding, 1980).

Previously published hypotheses of tetrodotoxin (TTX) and saxitoxin (STX) action proposed that these toxins blocked channels by partially entering the pore and literally "plugging" it (Kao and Nishiyama, 1965). In one highly explicit model (Hille, 1975*a*), much of the binding site corresponded to a putative ion selectivity filter. The plugging models were based largely on analogous chemical groupings in the two toxins and, in particular, on the guanidinium moieties that are present in both STX and TTX, and which, as free guanidinium ions, can permeate the sodium channels (Tasaki and Spyropoulos, 1961; Kao and Nishiyama, 1965; Hille, 1971). Strict analogies between these two toxins must be drawn cautiously, however, because the parameters that determine the affinity for STX are not identical to those that determine the affinity for TTX. For example, elevating the concentration of calcium ions reduces the affinity of STX more than that of TTX (Henderson et al., 1974; Hille et al., 1975), and replacing solute water by D_2O increases the STX potency, whereas TTX potency is unchanged (Hahin and Strichartz, 1981), and raising the temperature lowers the affinity of TTX by a much greater factor than it lowers that of STX (Hansen Bay and Strichartz, 1980). Thus, the bonds formed by the receptor and STX do not contribute the same energies as those formed by TTX.

In this paper, I present a comparison of the potency of TTX, STX, and a variety of STX derivatives on sodium channels in amphibian nerve membranes. By applying different toxins under different chemical conditions, I have identified some of the chemical groups on STX that are essential for the channel blocking reaction. Recent work by Kao and colleagues (Kao and Walker, 1982; Kao, 1983; Kao et al., 1983) uses an approach similar to the one reported here, although in several cases their findings differ from mine and so do the conclusions. The results of my studies are consistent with an attachment of STX to its receptor via one ionic bond, involving the C8 guanidinium, one weak covalent

bond, and perhaps two to three hydrogen bonds. The guanidinium group at C2 does not bond to the receptor. Fitting of the guanidinium group at C8 into a narrow cleft is neither ruled out nor required by this model. Some of the experimental results have been presented previously (Strichartz, 1981; Strichartz et al., 1984).

MATERIALS AND METHODS

Electrophysiology

The ability of the toxins to reduce the amplitude of compound action potentials from frog (*Rana pipiens*) sciatic nerve was assayed using the sucrose gap method (Stämpfli, 1954). Sciatic nerves were removed from live frogs and used within 2 d. Before mounting in the chamber, each nerve was desheathed and then split longitudinally, the split section being used for the assay without further division. The nerve chamber was like one described previously (Hahin and Strichartz, 1981) with a 300-μl-vol test pool and utilizing Pt-blacked platinum electrodes for stimulation as well as for potential measurements. The entire test pool volume could be replaced twice in <20 s. The nerve was stimulated

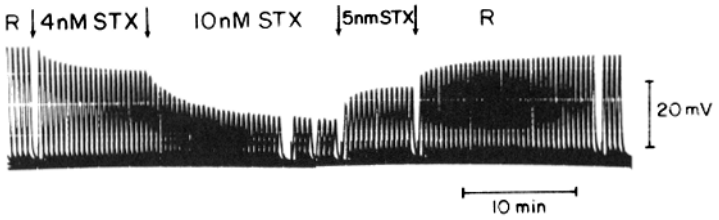


FIGURE 1. Compound action potentials were reversibly depressed by STX. Solutions were completely replaced in the sucrose gap test pool at the time shown by the arrow to the left of each condition. The time axis of the figure refers to the real time of the experiment.

supramaximally by electrodes isolated from the test pool, applying a 50-μs-duration cathodal pulse from a square-wave stimulator (model SD9B; Grass Instrument Co., Braintree, MA), and the compound action potential was recorded directly on a storage oscilloscope (model 5113; Tektronix, Inc., Beaverton, OR). Both stimulator and oscilloscope were gated by a digital clock, which accumulated an incremental delay in triggering the stimulus relative to the horizontal axis of the oscilloscope screen and thus produced a final oscilloscope tracing in which sequentially stimulated action potentials were displaced continuously from left to right on the screen (see Fig. 1).

In almost all of the experiments, the blocking potency of one drug is compared with that of another, usually STX at a standard concentration. By comparing the equipotent toxin concentrations, I assess only the relative potencies of the various toxins, and make no claim to determine the absolute affinities from action potential recordings. A typical nerve "calibration" is shown in Fig. 1. The experimental protocol required that the action potential in Ringer be constant in amplitude for at least 10 min before addition of toxins, and that the test toxin be applied for a time sufficiently long to permit the reduced impulse amplitude to reach a steady state. This took 10–20 min, depending on the particular toxin and its concentration (cf. Fig. 1). No slower components of inhibition of the action potential were evident with any of these toxins. Usually a concentration of standard STX was found that reduced the compound action potential (AP) amplitude by

between 40 and 60%, and the concentration of the toxin being tested that was required to produce the same reduction was determined by switching from solutions of standard STX to solutions of test toxin and back again. Often the relative potencies were compared at multiples of these concentrations and, therefore, at different degrees of block, but never less than 20% or beyond 80% block. The extents of block by standard STX before and after application of the test toxin had to agree within 10% of each other for the data to be included in the potency determinations. All electrophysiological experiments were conducted at room temperatures ranging from 19 to 23°C.

In most of these experiments, a standard frog Ringer with tetraethylammonium (TEA) was used, which contained (in mmol/liter): 115 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 5 or 10 HEPES or MOPS buffer, pH 7.2, adjusted with NaOH, and 12 TEA-Cl. In those experiments where the pH was varied from 6.5 to 10, a Ringer solution was used containing the same inorganic ions, but buffered by MES (5 mM) and glycylglycine (5 mM) as well as 5 mM HEPES. A solution of 0.18 M sucrose was flowed through the chamber "gap" at a velocity of 2–3 $ml \cdot min^{-1}$.

Preparation of Neurotoxins

STX (standard paralytic shellfish poison) was obtained from the Food and Drug Administration, Cincinnati, OH. Gonyautoxin II (GTXII), gonyautoxin III (GTXIII), and neosaxitoxin (neo-STX) were isolated from the cultured cells of *Gonyaulax tamarensis* (Ipswich strain) (Shimizu et al., 1976) according to the previously described procedure (Shimizu et al., 1975, Oshima et al., 1977), and quantitated by the procedure described earlier (Shimizu et al., 1981). 12α-DihydroSTX was prepared by the catalytic hydrogenation of STX in an acidic medium, and 12β-dihydroSTX by the $NaB(CN)H_3$ reduction of STX (Shimizu et al., 1981). Both compounds were exhaustively purified by chromatography to free them of the starting material and the stereoisomers, and the purity and stereochemistry were checked by high-resolution nuclear magnetic resonance (NMR) (Shimizu et al., 1981). A sample of neo-STX, whose purity (>99%) had been established by NMR within hours before delivery and refrigeration (4°C) (cf. Wichmann et al., 1981), was generously provided by Dr. Sherwood Hall, Woods Hole Oceanographic Institute, Woods Hole, MA.

RESULTS

The aim of this research was to identify the properties of the STX molecule that determine its potency in blocking the sodium channel. Toward this end, two factors were manipulated: (*a*) the chemical structure of STX was altered, either by modifying STX itself or by purifying naturally occurring derivatives, and (*b*) the pH of the incubating solution was systematically varied in order to modify the electrical charge and the structure of STX and its derivatives.

Essential Cationic Guanidine Groups

The charge on the two guanidinium groups may be a key factor in determining STX potency. This charge is due to protonation of these basic groups (see Fig. 2) and can be removed by raising the pH. Both NMR and direct titration data (Schantz et al., 1966; Shimizu et al., 1981; Rogers and Rapoport, 1980) show that the guanidinium at C8 has a pK_a of ~8.2, whereas the one at C2 has a pK_a of 11.6 (see Fig. 2). For STX and all the derivatives studied here, except neo-STX, toxin structures and charges are essentially constant below pH 6.5. I

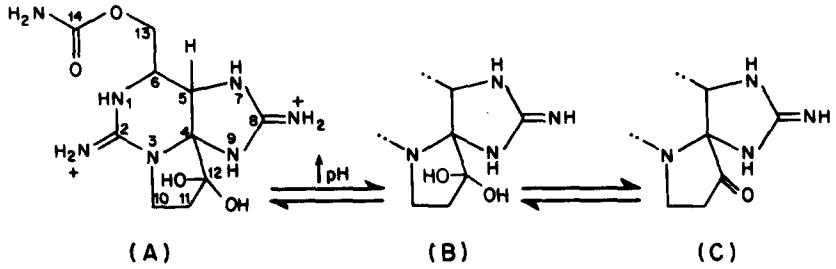


FIGURE 2. The structures of STX. At pH 7.0, >99% of the toxin exists as the divalent hydrate, STX^{2+} (*A*). At the pH is raised, the guanidinium at C8 becomes de-protonated (pK = 8.2), giving the monovalent hydrate (*B*), which exists in equilibrium with the unhydrated ketone (*C*). The region of the molecule beyond the broken lines is unchanged.

assume initially that the toxin binding site is affected little by changes in pH from 6.5 to 10.5 and that the apparent potency differences arise from structural changes of the toxin molecules themselves. This assumption is reviewed in the Discussion.

The potency of STX and of several derivatives in blocking impulses was measured, as in Fig. 1. Toxin concentrations required to reduce the AP by half at different pHs were determined from data like those graphed for neo-STX in Fig. 3. Potency (C_{50}) was defined by the intercept of the diagonal line (fit by

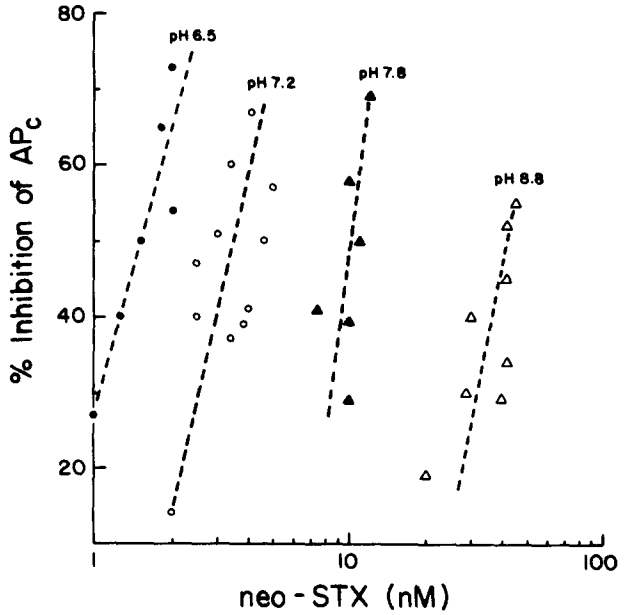


FIGURE 3. A family of dose-response curves for neo-STX measured at different pH values. The lines are fit by linear regression analysis. All data are from the same nerve.

linear regression) with the horizontal axis at 50% AP. The pH dependences of STX and neo-STX potency are graphed in Fig. 4. The potency of STX was relatively constant between pH 6.5 and 8.2, but then decreased rapidly as the pH was raised further, which is consistent with a major involvement of the charged C8 guanidinium in the channel blocking reaction. At pH values above 10, the toxin's potency fell by >98%, preventing a direct test of the importance of the charge at C2. These results support the general finding of Kao et al. (1983) that the C8 guanidinium is essential for the blocking action of STX. (The loss of STX and neo-STX potency in alkaline solutions is not caused by irrevers-

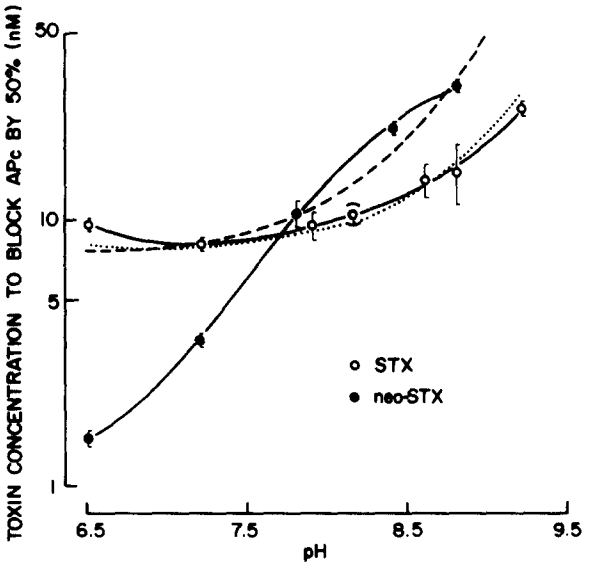


FIGURE 4. The pH dependence of the potency of STX (○) and of neo-STX (●). The toxin concentration for 50% block was determined from three to four separate experiments like that shown in Fig. 3, except for one single determination, in parentheses. The horizontal bars indicate the SEM. The broken line plots the concentration of total STX in solution that would be required to maintain STX^{2+} at 7.4 nM, its concentration producing 50% block at the standard pH of 7.2. The dotted line shows the modification of this curve caused by a titratable basic group fixed near the toxin receptor (see Discussion and Appendix).

ible degradation of the toxin molecules. Solutions of toxins, tested at pH 7.2, lost no detectable AP blocking activity after being incubated at pH 8.8 for 1 h at 22°C.)

Before analyzing the pH dependence of the blocking reactions, I should substantiate my assumption that the impulse-supporting properties of the axons are not compromised at elevated pH. Were this the case here, then any treatment that reduced the net inward current during an impulse would be potentiated at alkaline pH. However, alkaline pH values per se did not increase the sensitivity of impulses in this preparation to treatments that reduce the net inward current

density. This condition was evaluated by comparing the inhibition of the AP by frog Ringer containing one-third the normal Na^+ (substituted by tetramethylammonium ions) at pH 7.2 and 8.9; the degree of inhibition was essentially identical, being 82 and 81%, respectively. Thus, the pH sensitivity of the toxin blocking reactions is not due to changes in the nerve's response to reduced sodium permeability alone and most probably follows from changes in the toxin-channel reaction itself.

The absolute requirement of the channel blocking reaction for the charged C8 guanidinium can be evaluated by comparing the empirical findings with the theoretically predicted pH dependence. The fraction of STX molecules charged at C8 (STX^{2+}) was calculated from the Henderson-Hasselbalch equation assuming a pK_a of 8.24 for this group (Rogers and Rapoport, 1980); the total STX concentration necessary to maintain STX^{2+} at 7.4 nM (the 50% blocking concentration of STX^{2+} at pH 7.2) was determined as a function of pH and is plotted as the dashed line in Fig. 4. The discrepancy between this predicted behavior and the observed potency loss shows that STX potency does not decrease as much as the change in STX^{2+} would predict and thus that the channel blocking reaction does not depend simply and exclusively on the divalent STX^{2+} cation in the bulk solution.

There are several explanations that might account for this discrepancy. For one, the net charge on a toxin molecule appears to influence both its local concentration near the membrane surface and its ability to form specific bonds (Henderson et al., 1974; Hille et al., 1975). Such effects are consequences of the electrostatic interactions between charged drugs and charged membranes. For example, if a positively charged group were fixed to the membrane near, but not at, the toxin binding site, then making the toxin less positive on average, by reducing the degree of protonation of the C8 guanidinium, would tend to increase the local concentration of STX near the receptor and thus increase its apparent affinity. However, such a phenomenon could only account for the discrepancy illustrated in Fig. 4 if the gain in local concentration exceeded the loss in STX charged at C8.

A second, related complication arises from the possible titration by protons not of the toxin, but of charged groups fixed to the membrane surface at or near the toxin binding site. Although there is evidence for acidic groups contributing to negative electrostatic potentials at the toxin binding site (Henderson et al., 1974; Hille et al., 1975), no previous results suggest changes in positively charged groups at alkaline pH values. Direct studies of specific binding of radiolabeled toxins are not possible at high pH, because any effects on the binding site would be obscured by the large effects of alkaline pH on the specific radioactivity of exchange-labeled radioactive toxin molecules (Strichartz, 1982). Still, it is possible to model such effects of titratable basic groups, and this has been done in the Appendix and is described in the Discussion. An arbitrary choice of parameters for this modeling produced the theoretical pH dependence shown by the dotted line in Fig. 4. The fit to the data points is good, although probably fortuitous, and other sources for the pH dependence of STX should not be neglected.

A third explanation for the pH dependence concerns the affinity attributable to monovalent STX^+, unprotonated at the C8 guanidinium, and the secondary changes in toxin structure that accompany the titration of this specific group. In particular, as the pH of toxin solutions is raised and the C8 guanidinium becomes de-protonated ($STX^{2+} \rightarrow STX^+$), the equilibrium between the gem-diol at C12 and its unhydrated ketone shifts toward the latter (Fig. 2, *B* and *C*; Shimizu et al., 1981). Only the monovalent cationic STX molecule (STX^+), deprotonated at C8, displays any ketone character, but, a priori, either of the forms of monovalent STX, the gem-diol (Fig. 2*B*), or the unhydrated ketone (Fig. 2*C*) might have some affinity for the sodium channel. The binding of these molecular species could in theory account for the discrepancy between the pH dependence of the observed potency and the calculated concentration of STX^{2+}. This last hypothesis can be tested by calculating relative potencies for the hydrated monovalent and divalent species and for the ketone form of STX^{1+}. If these calculated potencies are constant over a range of pH values, then the monovalent forms could also be blocking sodium channels. Such calculations are described and summarized in Table I. From these calculations, I conclude that it is not possible to account for the anomalous pH dependence of STX potency by simply assigning constant blocking activity to forms of the toxin other than STX^{2+}. Some alternative explanations are presented in the Discussion. Thus, although it appears that a charged C8 guanidinium moiety is essential for the blocking action of STX, the total reaction probably involves more than the simple formation of an ionic bond.

Blocking Activity of Neo-STX

Neo-STX is a derivative of STX having a weak acidic hydroxyl group at N1 (pK_a = 6.75; Shimizu et al., 1978; cf. Table II). The absolute potency of this toxin at different pH values was also directly measured (Fig. 3) and is distinctly different from that of STX (Fig. 4). At pH 7.2, where 76% of the N1 hydroxyl groups in neo-STX are not protonated (and the net charge at the C2 guanidinium thus differs markedly from that at the C2 in STX), neo-STX is about four times more potent than STX (Table II). This potency difference increases at more acid pH, but decreases under more alkaline conditions (Fig. 4). The potentiation of neo-STX action at acid pH is due to a reversible alteration of the toxin's structure, for when the pH of an acidic solution of neo-STX is changed to pH 7.2 (by the addition of 1 M Tris base), the potency of the solution becomes equal to that of an equimolar toxin solution that was at pH 7.2 originally. The two toxins are equipotent at about pH 7.7. At this pH, the N1 hydroxyl of neo-STX is unprotonated 92% of the time and the average net charge on a neo-STX molecule is +1.0, whereas that on STX is +1.73. Clearly, altering the protonation of the N1 hydroxyl does change the potency of neo-STX, although the net charge on a gonyautoxin molecule is not the only determinant of its potency. If the guanidinium group at C2 were important for ionic bonding, then the presence of a negatively charged hydroxyl group at N1, as occurs in neo-STX, would create major steric and electrostatic interference, strongly compromising toxin potency. Since this does not occur in our experiments, it appears that the

C2 guanidinium group of STX does not bind ionically to the receptor, in agreement with the findings of Kao and Walker (1982).

The pharmacological difference between STX and neo-STX is further manifested by changes in the AP amplitude upon switching between equipotent concentrations of the two toxins. In the experiment shown in Fig. 5, neo-STX is 4.5-fold as potent as STX at pH 7.2. When an STX-containing Ringer solution bathing the nerve is exchanged for one containing approximately equipotent neo-STX, the AP amplitude transiently increases; when the opposite exchange

TABLE I

Calculation of Theoretical Potencies of Monovalent STX

(A) pH	C_{50} – STX^{2+}	STX^{1+} ketone*	Calculated required relative potency
	(α)	(β)	(α/β)
7.7	0.8 nM	0.01 × 9 nM = 0.09 nM	8.89
8.4	6.0 nM	0.24 × 12 nM = 2.88 nM	2.08
9.0	30 nM	0.36 × 20 nM = 7.2 nM	4.20

(B) pH	C_{50} – STX^{2+}	STX^{1+} hydrate*	Calculated required relative potency
	(α)	(β)	(α/β)
7.7	0.8 nM	0.22 × 9 nM = 1.98 nM	0.40
8.4	6.0 nM	0.37 × 12 nM = 4.44 nM	1.35
9.0	30 nM	0.54 × 20 nM = 10.8 nM	2.77

* Values for percent STX^{1+} ketone and STX^{1+} hydrate are from NMR studies of STX in solution (Shimizu et al., 1981).

We first question whether the monovalent ketone alone can provide all of the potency difference between the calculated STX^{2+} and the measured blocking potency. At pH 7.7, the half-blocking concentration (C_{50}) of STX is 9 nM (Fig. 4); and 1% of all the STX molecules, or 0.09 nM, are in the keto form in solution at this pH (term β). The difference (α) between the measured C_{50} and the theoretically predicted concentration of STX^{2+} (the dotted line in Fig. 4) is 0.8 nM at pH 7.7. In order to account for this difference, we must assign the hypothetically active ketone form a potency equal to α/β, about nine times that of STX^{2+} (above, right-hand column). When this procedure is repeated at pH 8.4, however, the calculated potency ratio is equal to ~2, and at pH 9.0 the ratio is ~4. Thus, it is not possible to assign a constant relative potency to the STX ketone form. The same conclusion follows for the monovalent hydrate form of STX (section B), nor can the sum of the two account for the difference in observed and calculated STX potency.

occurs, the amplitude transiently decreases, even though the steady state amplitudes are almost equal in all three solutions. The kinetic transients seen in Fig. 5 can arise from two factors: (*a*) the different concentrations of the toxins diffusing to and from the nerve membrane receptors, and (*b*) the intrinsic differences between the dissociation rate constants of the STX– and neo-STX–receptor complexes (for example, see Wagner and Ulbricht, 1975), differences that are detectable in sucrose gap experiments such as these (Hahin and Strichartz, 1981). But regardless of their origin, the kinetic transients in Fig. 5

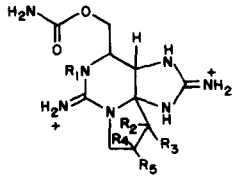
further demonstrate that the active blocking molecules of STX and neo-STX are distinguishably different by their physiological action.

Importance of the Hydroxyl Groups at Carbon 12

Since the gem-diol form of STX^{2+} (Fig. 2*A*) seems to express most if not all of the toxin's potency, can we determine the relative contribution to the toxin's action of the two different hydroxyl groups at C12? Comparison of the blocking potencies of reduced isomers of STX, produced by stereoselective reduction of the α- and β-hydroxyls of the gem-diol, revealed a significant difference between the two (Table II). When the α-hydroxyl group remained, the molecule retained 5–8% of its native activity, but when the β-hydroxyl remained and the α-hydroxyl

TABLE II

Structures and Relative Potencies of STX Derivatives



Compound	Potency*		Substituents				
		n	R_1	R_2	R_3	R_4	R_5
STX	1.0‡		H	OH	OH	H	H
Neo-STX	4.48±0.23	(6)	OH§	OH‖	OH‖	H	H
12α-DihydroSTX	0.052±0.008	(3)	H	H	OH	H	H
12β-DihydroSTX	0.0014±0.0005	(4)	H	OH	H	H	H
GTXII	0.220±0.002	(4)	H	OH	OH	H	OSO_3^-
GTXIII	1.36±0.08	(4)	H	OH	OH	OSO_3^-	H
TTX	0.91¶						

* Potency equals the ratio of STX concentration to equipotent test toxin concentration. This ratio was determined on separate nerves for the number of observations shown in parentheses, n. Conditions were pH 7.2, T = 20–23°C.

‡ Relative STX potency is unity, by definition.

§ Acidic hydroxyl; pK_a = 6.75.

‖ Unhydrated ketone detectable at pH 7.

¶ Data from Hahin and Strichartz (1981).

was reduced to a hydrogen, only ~$1–2 \times 10^{-3}$ of the STX potency was retained, and this could easily be due to undetected residual STX. (Unreacted STX could be present at 1–2% of the total toxin concentration, too little to account for the potency of the 12α-OH compound [Shimizu et al., 1981].) These findings are an explanation for the report that reduced STX, probably a mixture of the α- and β-epimers, had a potency 0.01 that of STX (Kao and Walker, 1982), a value about midway between those we measured for the separate stereoisomers. Apparently, the presence of both hydroxyl groups at C12 is important for full potency, but the α-OH confers more of the potency than does the β-OH group.

The Effect of Sulfate Groups on Carbon 11

The potency of STX is modified when negatively charged sulfate is esterified to a hydroxyl group at carbon 11. This occurs in two natural STX derivatives, GTXII (11α-SO_4-STX) and GTXIII (11β-SO_4-STX), shown in Table II. There is a remarkable stereospecific difference between the potencies of GTXIII and GTXII; solutions of the former are significantly more potent than STX, whereas the latter is only about one-fourth as potent. (However, transitions between the α- and β-epimers do occur in solution at pH >6.0, and at room temperature the equilibrium ratio of GTXII to GTXIII is 3:1 [Shimizu et al., 1976]; therefore, in any solution of GTXIII, there is almost certainly some small concentration of GTXII at physiological pH, and vice versa. Consequently, the stereospecificity of potency reported here should be taken as a lower limit.) As in the experiments with neo-STX, transient overblocks or underblocks were observed when solutions of GTXIII or GTXII, respectively, were replaced by equipotent solutions of STX. Since the direction of the transient depends on whether GTXIII or GTXII is being exchanged, it is highly improbable that these kinetic differences arise from diffusion processes. Instead, these results are consistent with a differ-

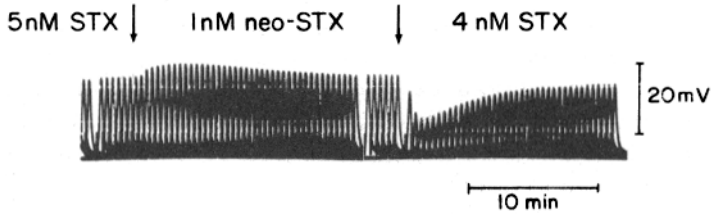


FIGURE 5. Action potentials display transient changes in amplitude when solutions of STX and neo-STX are exchanged in the test pool. Conditions are the same as described for Fig. 1.

ence in the rates of dissociation of the toxin-receptor complexes; GTXIII dissociates more slowly than STX, which dissociates more slowly than GTXII.

Although these sulfate substituents are negatively charged groups of relatively large van der Waals dimension (diam ~5 Å), their presence on the molecules reduces the potency, at most, by less than an order of magnitude compared with STX^{2+}. Therefore, it seems unlikely that the region of the bound STX molecule around C11 makes intimate contact with the receptor. The presence of the sulfate ester at C11 can, in principle, modulate the toxin's activity by several means. First, the additional negative charge could interact with charged groups located at the receptor site or nearby on the membrane. Second, sulfate substitution could influence the structure of the toxin, for example, by shifting the gem-diol $\rightleftharpoons$ ketone equilibrium at carbon 12, or by altering the pK_a of the guanidinium groups. The last of these possible modes of influence was tested by measuring the pH dependence of the potency of GTXII and GTXIII.

The blocking activities of GTXII and GTXIII were changed by different extents at alkaline pH. Compared with the decrease in STX potency resulting

from an elevation of pH to 8.8, which was a fall to 0.53 ± 0.14 (4) of the potency at pH 7.2, the potency of GTXII was reduced to 0.55 ± 0.01 (3) and that of GTXIII to 0.34 ± 0.03 (3) by the same pH change. On the basis of simple electrostatic interactions, the presence of a nearby anionic substituent should elevate the pK_a for protonation of a dissociable group (Edsall and Wyman, 1958), producing less of a pH dependence in the extent of charge in the C8 guanidinium. But in the STX derivatives, this simple behavior is not apparent; rather than being less sensitive to rises of pH, the 11-OH-SO_4 derivatives are as (GTXII) or more (GTXIII) sensitive than STX. As noted above, GTXIII isomerizes spontaneously to an equilibrium mixture of the epimers GTXII and GTXIII, and it is possible that this epimerization reaction is alkaline catalyzed. However, the absence of an obvious difference in pH sensitivity between GTXII and STX, which differ structurally by addition of one $-SO_4$ moiety at 6–7 Å distance from the center of the C8 guanidinium, again demonstrates that simple ionic bonding does not account totally for the action of these toxins.

DISCUSSION

In this study, the amplitudes of compound action potentials have been measured to assay the relative potency of neurotoxins in blocking sodium channels. The advantage of this assay is the stability of the preparation; AP amplitudes of frog sciatic nerves remain constant in the sucrose gap for up to 6 h, and several different toxins and conditions can be tested on the same preparation. The disadvantage is that the amplitudes of compound APs are both less sensitive to sodium channel blockade than are direct measures, such as voltage clamp or toxin binding studies, and can change in response to alterations of other parameters, such as increases in potassium currents or changes in channel gating. To avoid interference from changes in potassium channels, I conducted all experiments in Ringer containing TEA ions. Although I cannot be absolutely certain that the toxins do not alter channel gating, none of the previous voltage clamp studies have detected such an effect (Hille, 1968; Hille et al., 1975; Wagner and Ulbricht, 1975; Ulbricht and Wagner, 1975*a*, *b*; Hahin and Strichartz, 1981; Kao, 1983). Furthermore, the ratio of relative potencies of TTX to STX measured in sucrose gap, 0.91 (Table II), is essentially identical to that measured directly under voltage clamp, 0.89 (Hille et al., 1975). The relative toxin potencies from sucrose gap also agree closely with the affinities for the toxins measured by equilibrium binding experiments on brain membranes (Strichartz, 1981; Strichartz et al., 1984) and from analyses of their actions on single channel currents in membranes reconstituted in lipid bilayers (Moczydlowski et al., 1984). Indeed, measurements of the blockade of single channels by these toxins independently provide both the association and dissociation rate constants for the blocking reaction. These data confirm the relative potencies reported here, demonstrate that there are no impurities giving irreversible inhibitions, and substantiate the observation of the slow rate of reversal of bound neo-STX (E. Moczydlowski, S. Hall, G. Strichartz, and C. Miller, unpublished observations). For these reasons, I believe that compound action potentials provide valid assays of relative toxin potencies.

The results of this study provide strong clues about the parts of the STX molecule that are important for its action in blocking sodium channels. Groups on STX and its derivatives affect blocking activity in three ways: (*a*) by interacting directly with the receptor or with the nearby membrane to form bonds, or (*b*) by influencing local toxin concentrations, or (*c*) by modifying the chemistry of other parts of the toxin molecule. I will evaluate the potency contributions produced by specific regions of the toxins in light of such considerations.

STX: Charge

The electrical charge on specific parts of STX is important for potency. Binding of STX to its receptor on the sodium channel appears to involve ionic bonding because it can be inhibited competitively by cations and protons (Henderson et al., 1973, 1974; Weigele and Barchi, 1978; Hansen Bay and Strichartz, 1978; Reed and Trzos, 1979). From the pH dependence of potency, it appears that the positively charged guanidinium group at C8 is an essential element for the toxin's blocking action, and since the presence of an additional −OH group at the C2 guanidinium (as in neo-STX) does not eliminate potency, but instead elevates it at pH values below 7.7, we conclude that ionic bonding probably involves the guanidinium at C8 but not that at C2, which confirms the results of Kao and Walker (1982) and Kao et al. (1983).

Adding negatively charged groups to STX has different and, at first glance, unpredictable effects. At C11 (GTXII, GTXIII), at N1 (hydroxy-acid form of neo-STX), and at the carbamyl nitrogen (an additional sulfate in compounds GTXVIII and B1; Strichartz et al., 1984; and unpublished observations), negative charges appear either to increase (GTXIII) or reduce toxin potency but do not abolish it. There is no direct correlation between the net electrical charge of a toxin molecule and its potency.

My analysis also shows that the pH dependence of STX potency is not interpretable only in terms of simple ionic binding of the cationic C8 guanidino moiety to an anionic group on the receptor; i.e., with regard to STX, the potency loss with increasing pH did not behave as a simple titration of the C8 guanidinium (Fig. 4). STX maintained its relative potency to a greater extent than the C8 guanidinium would remain protonated in solution, and this difference could not be accounted for by attributing some constant blocking activity to the monovalent STX^{1+} or to the ketone molecules in solution. The sulfate ester derivatives GTXII and GTXIII also demonstrated a pH dependence that is not explainable simply on the basis of intra- or intermolecular charge interactions. The formation of an ionic bond between the STX guanidiunium group and one anionic receptor moiety is insufficient to describe the blocking action of STX, although I believe it is a necessary step in the overall mechanism.

Any process that increases the free energy of toxin binding in a pH-dependent manner could account for the difference between the titration behavior of toxin in solution and the pH dependence of its blocking action. However, a change in the toxin's pK_a caused by a lower pH near the charged axonal surface is not a valid explanation. These changes do not occur, because the equilibrium constant for protonation, K_a, is equal to $[H^+]\ [STX^{1+}]/[STX^{2+}]$, and the electrostatic

effects on $[H^+]$ are exactly canceled by those on $[STX^{1+}]/[STX^{2+}]$. Therefore, in the absence of binding, the pH dependence of toxin groups at the membrane surface is like that in the bulk solution. One hypothesis to explain the observed pH dependence of STX action is presented later in this Discussion.

STX: Hydration

STX can exist in two chemical forms, which differ by the addition of H_2O at C12, the gem-diol (or hydrated ketone; Fig. 2, *A* and *B*) and the unhydrated ketone (Fig. 2*C*). At pH 7.0, no STX ketone is detectable in solution, but as the pH is raised, this form increases so that at pH 8.4, 24% of the toxin molecules are in the ketone form (Shimizu et al., 1981). In STX, the gem-diol–ketone equilibrium shifts with changes in pH because of titration of the charge on the C8 guanidinium. Loss of protons from this group (pK ~8.2) results in an electron shift from the guanidine nitrogens to C4, which consequently destabilizes the gem-diol (Fig. 2*B*) relative to the unhydrated ketone (Fig. 2*C*), resulting in more of the latter at equilibrium. Other modifications of the STX molecule that increase the electron density of C4 will also increase the ketone form and decrease the gem-diol form. Since the keto form is required for exchange of the methylene hydrogens at C11 on STX, a reaction that occurs via a keto-enol tautomerism, this exchange can be used to detect the presence of the unhydrated ketone. Tritium exchange from C11 is greatly accelerated when STX is bound to anionic groups on resins or bound to isolated membranes at "nonspecific" sites, other than high-affinity receptors (Strichartz, 1982). Like deprotonation, these ionic bonding reactions increase the electron-donating power of C4 and thereby stabilize the ketone form of STX molecules.

Exchange of C11 protons, detected by NMR, is also much faster in neo-STX than in STX in solutions at neutral pH (Shimizu et al., 1978), which indicates the presence of the unhydrated ketone. These facts taken together provide the basis for a binding scheme for STX and its derivatives that accounts for almost all of my experimental observations.

A Two-Stage Binding Reaction: Ionic Bonding and Ketone Induction

Because neo-STX is markedly more potent than STX and exists in the ketone form at C12 to a greater extent than STX, and because the ketone form occurs when STX bonds to anionic groups, I propose the following mechanism for toxin binding. STX binds to the receptor in a two-stage process (Fig. 6). First, the cationic C8 guanidinium group binds to a receptor anionic group. This initial interaction induces the dehydration of the gem-diol to the unhydrated ketone at C12, which then proceeds, in the second stage, to form a covalent bond with a nucleophilic group of the receptor. This covalent bond is relatively weak, which accounts for the rapid reversibility of the binding reaction; examples would be the formation of a hemiketal or a Schiff base with a hydroxy or an amino group, respectively.

Toxins must be protonated at the C8 guanidinium in order for binding to occur. Thus, although raising the pH of an STX solution results in the formation of the ketone, it does so because of deprotonation of this guanidinium; the

monovalent gem-diol would not be reactive. We would also expect relatively little binding of the monovalent STX ketone, based on the analysis of Table I. In contrast, neo-STX molecules in solution have both a charged C8 guanidinium (pK = 8.65; Shimizu et al., 1978) and significant unhydrated ketone character at C12. The net greater potency of neo-STX results from the opposing effects of having more charged molecules with potentially reactive C12 ketones but also having a negatively charged group at N1, which reduces the apparent affinity, probably by electrostatic repulsion from anionic groups at or near the receptor (Henderson et al., 1974; Strichartz et al., 1984). At slight acidic pH values, the

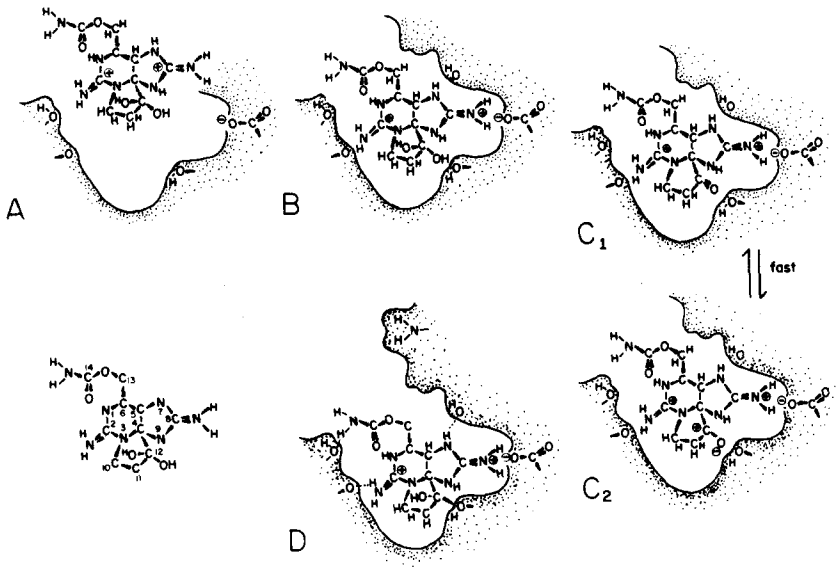


FIGURE 6. Two-stage binding reaction of STX with receptor. The charged guanidinium at C8 binds at an acidic group on the receptor in the initial step ($A \rightarrow B$). This binding induces the dehydration of the gem-diol with concomitant formation of the ketone, which exists as a resonance hybrid of carbonyl (C_1) and carbo-cation (C_2) species. The ketone carbon at C12 is then susceptible to attack, for example, by an alcohol on the receptor with the eventual formation of a hemilactal (D). The amine group on the membrane above the binding site represents the fixed basic charge that modifies the pH dependence of STX potency.

potency of neo-STX increases because the acidic N1 hydroxy group becomes neutralized (solution $pK_a = 6.75$) and, with it, the electrostatic repulsion caused by this group disappears.

The potencies of the other gonyautoxins can also be explained within this scheme. GTXIII potency exceeds that of STX because the β-OH sulfate group on C11 is negatively charged and by its nearness exerts an electrostatic effect that stabilizes the carbo-cation form of the ketone (see Fig. 6 *C*). This direct electrostatic effect is greater than the rather weak electron-withdrawing activity of $-SO_4^-$, which occurs by induction through carbon-carbon bonds. In addition,

the carbo-cationic form of the ketone is the major reactive intermediate for nucleophilic attack in the formation of a hemiketal bond. An identical role is provided by the 11α-OH sulfate in GTXII, but because of its steric position, it strongly compromises the initial ionic bonding of the guanidinium. Nevertheless, covalent bonding can occur and some GTXII molecules do bind to the receptor.

Reversible covalent bonds can account for a wide range of free energy values and their presence is not inconsistent with the observed toxin equilibrium dissociation constants. For example, a variety of substituted aldehydes and ketones can inhibit serine proteases, such as papain, with K_i values ranging from >0.5 to 5.2×10^{-9} M (Poulos et al., 1976; Lewis and Wolfenden, 1977*b*). Measurements of the positive deuterium isotope effects on several of these K_i values testify to the likely formation of a reversible thiohemiacetal between inhibitor and protein. The deuterium isotope effect may arise from the equilibrium hydration reaction of the aldehyde or ketone (Lewis and Wolfenden, 1977*a*) and that hydration has a large energetic component that is catalyzed by general acids and general bases (Pocker and Dickerson, 1973; Lavery et al., 1979). The chemistry of these compounds as enzyme inhibitors parallels much of the behavior of STX as an inhibitor of sodium channels and produces circumstantial evidence in support of the involvement of a weak reversible covalent bond.

The stereoselective reduction of STX to 12α-dihydroSTX or 12β-dihydroSTX produces a 50-fold difference in potency loss. I interpret this to show that weak bonding, such as hydrogen bonding, can still happen to the 12α-OH group but, because of steric differences, not to the 12β-OH group. The energy of a typical (-O-H---N-) hydrogen bond of ~2 kcal/mole could easily account for a 100-fold difference in the toxin dissociation constant and thus in the measured relative affinities and potencies. In the gem-diol form of STX, the 12α-OH group also can form a hydrogen bond, but it will be markedly weaker than that formed with 12α-dihydroSTX because the electron-withdrawing power of the 12β-OH group lowers the ability of the 12α-OH group to share its hydrogen. Nevertheless, we cannot rule out completely the receptor binding of the gem-diol form of STX.

A previous study (Strichartz, 1982) showed that the exchange rate of the C11 methylene hydrogens from STX that was specifically bound to sodium channels was no different from the slow exchange from toxin molecules in solution. In the current binding scheme, the induced ketone reacts rapidly and covalently with the receptor and cannot participate in the keto-enol tautomerism that accounts for this hydrogen exchange in solution. Accordingly, although the ketone does occur, exchange is limited to the brief periods before or after it has reacted with the receptor; at these times, the C11 methylene may be accessible to the solvent and the measurable hydrogen exchange occurs. For this reason, and because the 11-OH sulfate derivatives (GTXII and GTXIII) bind successfully, we propose that intimate contact between the region of the toxins at C11 and the receptor is not required for tight binding.

Alternative Models

An alternative explanation to account for the greater potency of neo-STX hypothesizes the formation of a hydrogen bond between the N1 hydroxy group

and a donor group on the receptor. Depending on the ability of the N1 hydrogen in STX also to contribute to hydrogen bonding, the additional energy from this bond could account for as much as 3 kcal/mol, corresponding to an increase in affinity and toxin potency of 10^3. Since the observed potency increase is about fourfold at pH 7.2, but increases as the N1 oxygen is protonated (Fig. 4), the formation of such a hydrogen bond is tenable. However, the net charge on a toxin molecule modulates its apparent potency, with more positively charged toxins apparently more strongly attracted to the membrane surface (Henderson et al., 1974; Hille et al., 1975; Strichartz et al., 1984), and protonation of neo-STX's N1 hydroxy group would increase its apparent potency by this mechanism also.

The increase of STX affinity in D_2O solvent over H_2O was interpreted as evidence for some role of hydrogen bonding (Hahin and Strichartz, 1981). This positive deuterium isotope effect was also observed with neo-STX, but not with TTX. The increased STX affinity at equilibrium was resolved kinetically as a halving of the dissociation rate, which indicates the formation of a stronger toxin-receptor complex. While hydrogen bonding described these results quantitatively, there are other explanations. For one, the reversal of a covalent bond, as postulated in this Discussion, could be acid catalyzed and its rate could be dependent on vibrational energy levels directly coupled to proton mass (Lavery et al., 1979). Another explanation concerns entropic changes in STX molecules, where the carbamyl "tail" moiety may be able to assume more positions in solution than when the molecule is bound. Removal of this group reduces the toxin's potency (Kao and Walker, 1982), but the energetics of its contribution to binding are still unknown. Deuterium isotope effects on that group's population states could account for the selective presence of a D_2O effect on STX when none is observed on the more compact TTX molecule which has fewer degrees of freedom. Until we know the thermodynamics of the microscopic rate constants for the toxin-receptor interactions and the energetic contributions of nearby electrostatic charges, we will have an incomplete and equivocal description of the toxin binding reactions.

Hypothesized Membrane Amino Group

The two-stage binding model requires the initial reaction of divalent STX (STX^{2+}) and therefore predicts the same dependence of binding on pH as is plotted in Fig. 4 (broken line). The discrepancy between this predicted behavior and the experimental results, which could not be accounted for by other forms of STX in solution or by changes in the pK_a of STX, must still be addressed. One explanation for the observed pH dependence posits the existence of a positively charged basic group(s) fixed to the membrane near the STX binding site. According to this hypothesis, the free energy of the toxin bound at the receptor is affected by the electric potential from this charge, and so the apparent affinity is reduced by the repulsion of the divalent STX cation by the cationic group fixed to the membrane. As the pH is raised, this hypothetical group loses its proton (and charge) and the apparent affinity of the reactive STX^{2+} is increased. Exact modeling of this phenomenon requires the specification of several parameters, including the pK of the membrane group and the relative

distance and angular position of the bound STX^{2+}. In an initial approximation of this situation, I have assumed arbitrarily the presence of a typical 2° amine, having a pK of 9 (e.g., the side chains of arginine or lysine in a protein), located 10 Å from the charge center of bound STX (see Appendix). The electrical potential from this amino group at the toxin binding site is ~15 mV at pH 7.0, which lowers the apparent affinity for STX^{2+} by a factor of 3.25. The effect of this titratable potential on the potency of STX at pH values from 6.6 to 9.2 is graphed as the dotted line in Fig. 4. Its close agreement with the measured change in STX potency must be somewhat fortuitous, since the several essential parameters were arbitrarily selected, but it still encourages belief in the postulated 2° amine near the STX binding site.

Selective chemical reaction already has indicated the presence of specific amino groups that contribute potentials of ~20 mV at the inactivation voltage sensor of sodium channels in frog excitable membrane (Cahalan and Pappone, 1981). The spatial relation of this site to the toxin binding site is not known; however, in preliminary STX binding experiments to rabbit brain membranes, I have found that reaction with the same large amino group-selective reagent, trinitrobenzenesulfonic acid, had two effects on specific STX binding parameters. The number of binding sites was reduced, and the remaining sites bound STX with a much higher K_D. The second effect is indicative of a reduction in the free energy of binding, possibly because of conversions of groups near the binding site but also because of more general modifications of the receptor, of an allosteric nature, for example.

Comparison with Previously Reported Results

The structure-potency study presented here supports several aspects of STX action that others have previously proposed. The involvement of the C8 guanidinium confirms the finding of Kao et al. (1983), who showed that toxin potency fell rapidly in the pH range of 7–8.25. These authors, however, reported no discrepancy between the pH dependence of STX potency and the fraction of toxin charged at the C8 guanidinium (Kao, 1983). Since their studies were done on squid in artificial seawater (ASW) containing much higher concentrations of monovalent (432 mM: $Na^+ + K^+$) and divalent (59 mM: $Ca^{2+} + Mg^{2+}$) cations than those in frog Ringer, the electrostatic effects that I posit here may have been effectively screened in their measurements. They also used $\dot{V}_{max}$ as a measure of blockade, although this parameter is a nonlinear measure of relative sodium conductance (Cohen et al., 1981); and at pH 8.25, the highest pH that they studied, this parameter is only reduced by 30% (Kao et al., 1983). The absence of measurements beyond this pH, coupled with the assumption that the pK_a of STX is unchanged in ASW, confuses any comparison between Kao's (1983) conclusions and my own.

My finding of a greater potency of neo-STX over STX differs from the results of Kao and Walker (1982) and Kao et al. (1983), who found that neo-STX was equipotent with STX in skeletal muscle and in squid at pH values of 6.5 and 7.25. It is highly improbable that the differences that I observed between these two toxins was due to an error in concentration, for the neo-STX sample was

purified and analyzed within days of its testing and the kinetics are clearly different (Fig. 5). At present, I see no explanation for the discrepancy between my results and those of Kao and co-workers.

A Hypothetical Receptor

A graphic interpretation of this structural investigation is summarized in Fig. 6. The receptor forms one ionic bond with the C8 guanidinium group, a weak covalent bond with the C12 carbonyl moiety and one or several hydrogen bonds, perhaps with the amino groups of the C8 and C2 guanidinium groups and of the carbamyl "tail" (cf. Kao and Walker, 1982). A hydrogen bond might also be formed with the protonated acid hydroxy moiety on N1 in neo-STX. The region around the C11 methylene does not contact the receptor. In addition to the one anionic group on the receptor that bonds ionically to the C8 guanidinium, other negatively charged groups nearby modulate toxin binding through electrostatic interactions (Henderson et al., 1974; Hille et al., 1975; Strichartz, 1981; Strichartz et al., 1984). At least one basic group with pK = 8–9 also is fixed near the STX binding site and influences binding through electrostatic effects.

In several respects, this model resembles one published by Hille (1975*a*). A hemiketal bond between the C12 ketone and receptor was proposed, as well as the formation of ionic bonds with the C8 guanidinium and numerous hydrogen bonds. (Camougis et al. [1967] had previously suggested the presence of a hemilactal bond linking TTX to its receptor.) The new aspects of the model presented here are the two-stage binding with induction of the ketone form and the presence of the local basic group. Unlike Hille's speculated structure, this one does not require binding to a "selectivity filter" at the inner opening of the sodium channel and the consequent blockade by toxin acting as a plug.

The model is intended as a hypothetical structure for the STX binding site, based solely on the potency data from this and other studies. No information about the mechanism of blockage of sodium channels by STX or TTX is available directly from these data, and the relationship of the toxin binding site to other aspects of the sodium channel is not specified.

Nevertheless, some comments on existing models for toxin action are pertinent to this discussion. Two classes of models for toxin action have been proposed in the literature. One specifies that STX or TTX molecules enter the outer opening of the channel pore and "plug" it at a cation binding site that is part of the so-called "selectivity filter" of the channel (Kao and Nishiyama, 1965; Henderson et al., 1974; Hille, 1975*a*; Weigele and Barchi, 1978). The other model suggests that the toxin binding site is located more superficially, near the outer opening of the channel, and that block occurs by steric or electrostatic interference with sodium ions rather than by a literal "plug" (cf. Spalding, 1980; Kao and Walker, 1982; Kao, 1983). A third possibility, infrequently entertained, is that toxins interfere with a channel's conductance via some allosteric mechanism rather than by direct occlusion at a cation binding site.

Although the "plugging" models originally were accepted widely and are consistent with some experimental results from competition experiments between

toxins and metal cations (Henderson et al., 1973, 1974; Weigele and Barchi, 1978), recent experiments provide evidence that cannot be reconciled with a plugging action for the toxins within the pore of the channel at a site for ion selectivity. These experimental results include (*a*) changes of a channel's toxin sensitivity with no change in cation selectivity properties following modifications produced either biologically (Huang et al., 1979; Pappone, 1980) or chemically (Spalding, 1980), and (*b*) a discrepancy between the strong ability of certain cations (e.g., NH_4^+) to block toxin binding and their weak interference with transport of sodium ions through the channel (Hansen Bay and Strichartz, 1978; Hille, 1975*b*). Furthermore, if the C8 guanidinium group plugged the channel at the putative selectivity filter, the site of voltage-dependent proton and Ca^{2+} binding (cf. Woodhull, 1973), then the binding of STX also should be voltage dependent. Although a voltage-dependent action of TTX has been reported for the block of batrachotoxin-activated sodium channels incorporated into lipid bilayers (Kreuger et al., 1983), the voltage dependence is the same for TTX, STX, and neo-STX, as well as GTXII and GTXIII, which have sulfate substituents within 4–6 Å of the charged C8 guanidinium (Green et al., 1984; Moczydlowski et al., 1984). These compounds would be expected to bind with less voltage dependence than STX if the C8 guanidinium were plugging at the H^+ and Ca^{2+} binding site of Woodhull's (1973) model, 0.25 of the equivalent electrical distance through the membrane (but also cf. Campbell, 1982). Their equal voltage dependence, together with the fact that equilibrium STX binding to brain synaptosomes (Kreuger et al., 1979) and TTX binding to intact frog skeletal muscle (Almers and Levinson, 1975) are conspicuously voltage independent, means that the impact of membrane potential on TTX and STX binding is not a direct influence of voltage on the charged toxin molecules but probably requires a change in the receptor itself.

Although none of these results unequivocally disproves the toxin plug at the selectivity filter model, because the alterations of toxin affinity caused by biological, chemical, and membrane potential perturbations could all be explained through an allosteric mechanism, this complicated response seems improbable and the more parsimonious model in which toxins obscure the ion pathway of an otherwise conducting channel is preferable to me.

STX binding is competitively inhibited by metal cations, and in the same order in which they block sodium currents at frog nodes (Henderson et al., 1974; Weigele and Barchi, 1978; Gitschier, 1981; Hille, 1975*b*). The competitive inhibition by organic cations, however, occurs with a different sequence (Hansen Bay and Strichartz, 1978; Gitschier, 1981) and certain organic cations displace STX more effectively than they block sodium current. This could not occur if there were one and the same cation binding site for toxin competition and for the blockade of channel permeability, and I propose that at least part of the toxin receptor is located at the exterior surface of the sodium channel rather than within the pore. The properties of this hypothetical receptor correspond to a cation binding site at the opening of the channel, which participates in the dehydration of ions, rather than to a plugging site within the channel pore.

APPENDIX

Calculation of the Effects on STX Affinity from a Titratable Charge Near the Binding Site

Assume that a basic 2° amino group is fixed on the membrane at distance r from the charge center of STX^{2+} when it is bound to the receptor. If the amino group, having an assumed pK of 9, is charged +1 at pH 7.0, then it will produce a potential, Ψ_N, in a spherically symmetrical solution described by the Debye-Huckel theorem:

$$\Psi_N = \frac{eZ_N}{4\pi\epsilon_0\epsilon} \frac{\exp(a\kappa)}{(1 + a)} \frac{\exp(-r\kappa)}{r}, \tag{1}$$

where e is one positive electron charge, Z_N is the valence of the amine group, ϵ is the dielectric of the medium (H_2O: we take $\epsilon = 70$ here), ϵ_0 is the permittivity of free space, a is the radius of the amino group (2 Å used here), and κ^{-1} is the Debye length, which depends on the electrolyte concentration of the solution and is calculated to be ~10 Å for frog Ringer (see Robinson and Stokes, 1959). Using the parameter values just noted, from Eq. 1, the Ψ_N at pH 7.0 (where $Z_N = 1.0$) and $r = 10$ Å is 7.7 mV. Since we position the group at the surface of a planar membrane of relatively low dielectric constant (ϵ = 3–10), the condition of spherical symmetry is removed. The electric field, E, from the charge has a much shallower gradient through the membrane than through the aqueous solution and, since $E = -\nabla\Psi$, the potential at the membrane surface is greater than it would be if the charge were surrounded by electrolyte solution; for this calculation, we assume that the potential is doubled, and thus Ψ_N ($r = 10$ Å) $\simeq$ 15 mV.

The STX concentration will depend on the potential according to Boltzmann's equation:

$$STX_s = STX_b \exp(-\Psi Z_T e/kT) \tag{2}$$

where STX_s and STX_b are the STX concentrations at the binding site and in the bulk solution, respectively, Z_T is the charge on STX in solution, k is Boltzmann's constant, and T is the temperature in degrees Kelvin. Since we know that STX has basic groups with pK_a values of 8.25 and 11.6, we can calculate Z_T from the Henderson-Hasselbalch equation. The value of Z_N can be computed similarly, and the expected variation of [STX] at the binding site as a function of pH can be calculated from Eq. 2. For the +15-mV potential at pH 7.0, the toxin concentration at the site would be 0.32 of the concentration in the absence of this potential. As the pH is raised, Ψ_N falls, as does Z_T, so that at pH 9.2 they equal 5.8 mV and 1.11, respectively, and the binding site STX concentration is reduced by a factor of only 0.9. The calculated change in STX concentration caused by this electrostatic effect, $[STX]_s/[STX]_b$, can be multiplied by the calculated decrease in STX^{2+} (assumed here to be the reactive molecular species) to get the concentration of reactive STX^{2+} at the toxin binding site at different pH values. The result is shown by the dotted line in Fig. 4. The experimental points fit this line far better than the curve that describes the pH dependence of STX^{2+} concentration in the bulk solution (the broken line).

Several comments on the assumptions included in these calculations are appropriate. We have localized the charge on STX at a single point, although the center carbons on the two charged guanidinium groups are separated by ~4 Å (Schantz et al., 1975), and the distance from the toxin site to the 2° amine group is assumed to be only 10 Å. The electrostatic effects on the nearer guanidinium will exceed those on the farther one, depending on the dielectric of the substance separating the two groups, and since one

group is selectively depronated, the effects of the field will vary at the two STX charges at different pHs. But for a first approximation, the assumption of a point charge provides a good fit to the experimental data.

The valence for STX used in Eq. 2 is the average charge on any STX molecule, even though we assume that only the divalent STX^{2+} binds to the receptor (see Results). The validity of this treatment depends on the fact that the protonation-deprotonation of the guanidinium groups and the diffusion of STX to the receptor are faster processes than the binding and dissociation reactions of the toxin, which have a relaxation time of ~1 min at 20°C in frog nerve (Hahin and Strichartz, 1981). Thus, the repelling force of the amino group rapidly affects all the STX molecules in solution, even though only STX^{2+} molecules actually bind.

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