

Voltage-dependent Blockade of Muscle Na⁺ Channels by Guanidinium Toxins

Effect of Toxin Charge

EDWARD MOCZYDLOWSKI, SHERWOOD HALL, SARAH S. GARBER,
GARY S. STRICHARTZ, and CHRISTOPHER MILLER

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254; the Department of Chemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543; and the Anesthesia Research Laboratories, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Na⁺ channels from rat muscle plasma membrane vesicles were inserted into neutral planar phospholipid bilayers and were activated by batrachotoxin. Single channel blocking events induced by the addition of various guanidinium toxins were analyzed to derive the rates of channel-toxin association and dissociation. Blocking by tetrodotoxin, saxitoxin, and six natural saxitoxin derivatives containing sulfate or hydroxyl groups were studied. Although the binding affinities vary over 2,000-fold, all of the toxins exhibit identical voltage dependence of the blocking reactions, regardless of the toxin's net charge. The results suggest that the voltage dependence of toxin binding is due to a voltage-dependent conformational equilibrium of the toxin receptor, rather than to direct entry of the charged toxin molecule into the applied transmembrane electric field.

INTRODUCTION

Integral proteins of cell membranes normally experience voltages of ~100 mV falling over a distance on the order of 100 Å. Thus, some portion of a protein spanning a lipid bilayer dwells in an electric field of ~10 million V/m created by ionic diffusion potentials. While it is recognized that voltage is a primary regulatory signal for some excitable membrane proteins, the mechanisms by which voltage is coupled to protein function are poorly understood. One straightforward mechanism can be explained by Eyring rate theory. If an ion in solution can bind to a site in the transmembrane electric field, then the rates of ion movement onto and off of the site would be expected to vary with voltage according to a Boltzmann distribution, where the steepness of the voltage dependence is determined by the valence of the ion and the fraction of the field that the ion traverses to reach the site (Woodbury, 1971). This theory has been

Address reprint requests to Dr. Edward Moczydlowski, Dept. of Physiology, University of Cincinnati Medical Center, Cincinnati, OH 45267.

used to explain voltage-dependent blocking of ion channels by many types of small ions (Woodhull, 1973; Adelman and French, 1978; Neher and Steinbach, 1978; Miller, 1982). Alternatively, voltage-dependent motions and rearrangements of charged and dipolar amino acid residues within membrane proteins may underlie many voltage-driven channel phenomena.

In the preceding paper (Moczydlowski et al., 1984), we described a voltage-dependent blocking reaction of individual Na^+ channels induced by tetrodotoxin (TTX). In the present study, we attempt to gather more evidence concerning the molecular details of this voltage-dependent reaction, by exploring the effects of varying toxin structure and charge. For this purpose, we take advantage of organisms of the genus *Protogonyaulax*, which produce saxitoxin (STX) and a variety of natural derivatives that have been purified and chemically characterized (for a review, see Hall, 1982). These derivatives differ from STX by containing one or two $-\text{SO}_3^-$ groups or a dissociable $-\text{OH}$ group at specific positions on the STX molecule. By comparing block by molecules with different net charges ranging from 0 to +2, we can directly test whether the voltage dependence varies according to the charge on the blocking ion, as expected for the simple case of a binding site in an electric field. The results demonstrate that there is no effect of toxin charge on the voltage dependence of toxin binding rates, and that an alternative explanation must be sought. Analysis of a simple four-state kinetic scheme suggests that a voltage-dependent conformational equilibrium of the protein could explain the effects of voltage on toxin binding.

METHODS

The STX derivatives listed in Fig. 1 were extracted from dinoflagellate cultures and purified by chromatography and recrystallization as described (Hall, 1982). Toxin concentrations are based upon dry weight measurements of highly purified samples. Standard solutions were stored at -80°C , and working stock solutions were freshly prepared by dilution into 1 mM citrate buffer, pH 5. Batrachotoxin (BTX) was the generous gift of Dr. John Daly, National Institutes of Health, Bethesda, MD.

Rat skeletal muscle membrane microsomes were prepared and a purified plasma membrane fraction was used for Na^+ channel incorporation into planar bilayers as previously described (Moczydlowski et al., 1984). Lipid bilayers cast from decane, channel incorporation methods, and the recording system for single channel current were also described in the preceding paper. Voltages are referred to normal cell convention: positive is depolarizing. All data were obtained at 24°C from neutral lipid bilayers (80% phosphatidylethanolamine/20% phosphatidylcholine) under symmetrical buffer conditions: 10 mM MOPS-NaOH, pH 7.4, 200 mM NaCl, 0.1 mM EDTA. Single Na^+ channels were studied in the absence of inactivation by adding $0.2 \mu\text{M}$ BTX on either side of the bilayer. Toxin-blocking rate constants were measured in bilayer experiments from the corrected mean blocked or unblocked times as described in detail in the preceding paper. At least 50 blocked or unblocked events were collected at a given voltage and toxin concentration for measurement of mean dwell times. Data from at least five different bilayers were pooled for each toxin. For most toxins, a lower limit of 0.4 s was used as a rejection limit for short blocked states to avoid contamination of blocking kinetics with gating kinetics. Mean dwell times were corrected for this cutoff limit as described previously (Moczydlowski et al., 1984). Because of the short blocked times of toxins B1 and C1, a cutoff limit of 0.2 s was used to keep maximum correction factors within 20%. Blocked and unblocked

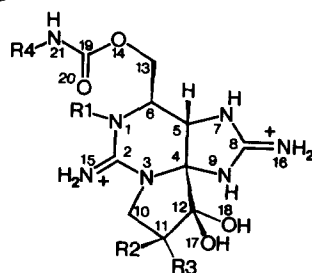
events were usually measured from chart paper records by hand with an accuracy of 0.1 s, except for some experiments using B1, where data were digitized at 100 Hz and analyzed by computer. [³H]STX binding assays on rat muscle membranes were performed as described (Moczydlowski and Latorre, 1983), except that the buffer for the assay was the same as that for the bilayer experiments.

RESULTS

Binding of STX and Its Derivatives to Single Channels

Fig. 1 shows the structure of STX and several of its natural derivatives as previously determined (Schantz et al., 1975; Shimizu et al., 1978; Boyer et al., 1978; Fix-Wichman et al., 1981; Hall, 1982). At a working pH of 7.4, the various molecules have net charges ranging from +2 for STX with its two guanidinium groups, to 0 for the C1 and C2 isomers, which contain two guanidinium and two SO₃⁻ moieties. We investigated the interaction of each of these toxins with single BTX-modified Na⁺ channels in order to study the effect of different structural modifications on the blocking kinetics.

Fig. 2 shows examples of single Na⁺ channel records obtained in the presence of various toxins, with an applied voltage (+50 mV) at which the channels are, in the absence of toxin, open virtually all the time (Moczydlowski et al., 1984). The control record shows occasional brief closing events, which are voltage independent. Addition of guanidinium toxin induces slow fluctuations or "block-



	R1	R2	R3	R4
STX	H	H	H	H
B1	H	H	H	SO ₃ ⁻
GTX 2	H	H	OSO ₃ ⁻	H
C1	H	H	OSO ₃ ⁻	SO ₃ ⁻
GTX 3	H	OSO ₃ ⁻	H	H
C2	H	OSO ₃ ⁻	H	SO ₃ ⁻
NEO	OH	H	H	H

FIGURE 1. Structure of saxitoxin and several natural derivatives. Abbreviations: STX, saxitoxin; B1, 21-sulfoSTX; GTX2, 11 α -hydroxysaxitoxin sulfate; C1, 21-sulfo-11 α -hydroxysaxitoxin sulfate; GTX3, 11 β -hydroxysaxitoxin sulfate; C2, 21-sulfo-11 β -hydroxysaxitoxin sulfate; NEO, N-1-hydroxysaxitoxin. At pH 7.4, the derivatives have the following approximate net charges: STX: +2; B1, GTX2, GTX3, NEO: +1; C1, C2: 0.

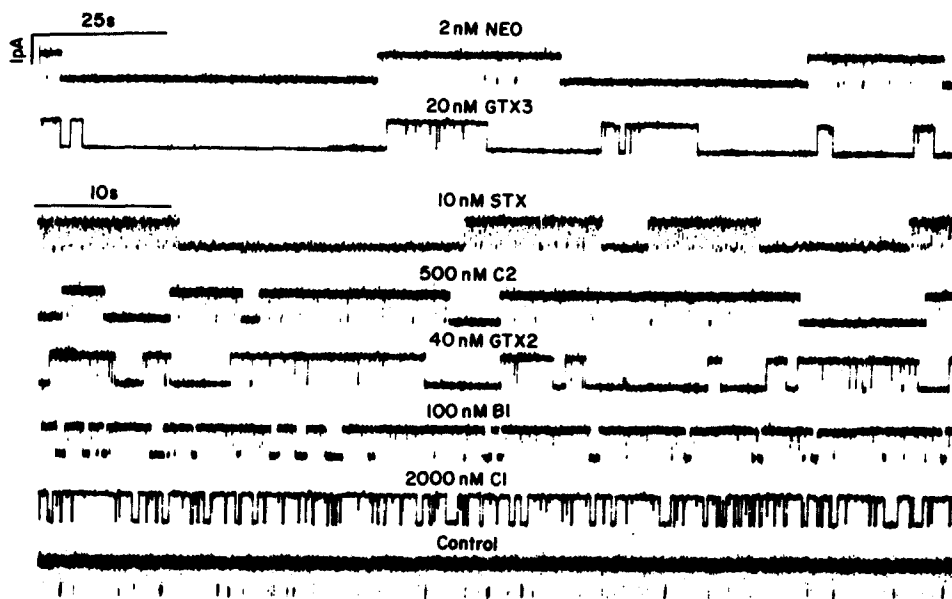


FIGURE 2. Single Na^+ channels in the presence of various STX derivatives. The planar bilayer aqueous phase contained 200 mM NaCl, 0.1 mM EDTA, 10 mM MOPS-NaOH, pH 7.4, 0.2 μM BTX, and the indicated concentration of STX derivative. Each trace is from a separate bilayer. The applied voltage was 50 mV, except for STX, for which it was 45 mV. Records were filtered at 20–100 Hz. In all figures, channel opening is upward. The records are presented in the order of decreasing mean block time; the compressed time scale refers to NEO and GTX3. The control record was obtained in the absence of any guanidinium toxin.

ing events," each of which is due to the binding of a single toxin molecule to the channel protein (Krueger et al., 1983; Moczydlowski et al., 1984). While the single channel conductance is the same (20 pS) in all records, it is evident that the various toxins differ vastly in the affinities and kinetics of their action.

Fig. 3 shows a single channel record at 50 and at -50 mV obtained in the presence of 100 nM B1 (21-sulfosaxitoxin). This record shows that this toxin induces blocking events that are on the average much shorter than those of TTX or STX. The duration of the blocked state increases at negative voltages, while that of the unblocked state decreases. Thus, B1 toxin displays the same type of voltage-dependent binding reaction as seen for TTX and STX (French et al., 1984; Moczydlowski et al., 1984).

We found that blocked and unblocked states are well described by single-exponential probability distributions for each of the toxins. This was verified by constructing cumulative probability histograms, as shown in Fig. 4 for B1, or by checking that the mean and standard deviation are equal, as required by an exponential distribution. Also, as previously documented for TTX (Moczydlowski et al., 1984), the mean blocked time for each of the toxins is independent of toxin concentration, while the mean unblocked time is directly proportional to concentration, as expected for a pseudo-first-order bimolecular process.

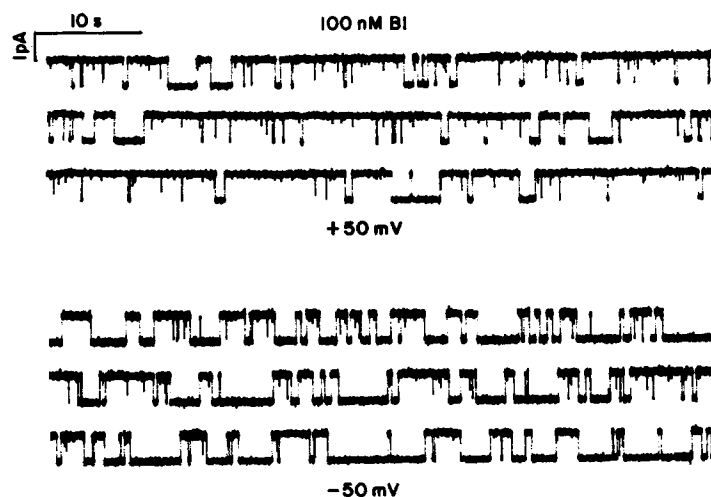


FIGURE 3. Blocking events due to 21-sulfosaxitoxin (B1). Aqueous buffer conditions are as in Fig. 2, with 100 nM B1 present. Applied voltages are indicated on the figure. Each group of three traces is one continuous record from the same bilayer.

For some of the toxins in which the sulfate groups are easily hydrolyzed (Hall et al., 1980), we did find instances of multiexponential blocked state distributions that would be expected for a mixture of toxins with different dissociation rates. However, these artifacts were avoided by using freshly prepared solutions of

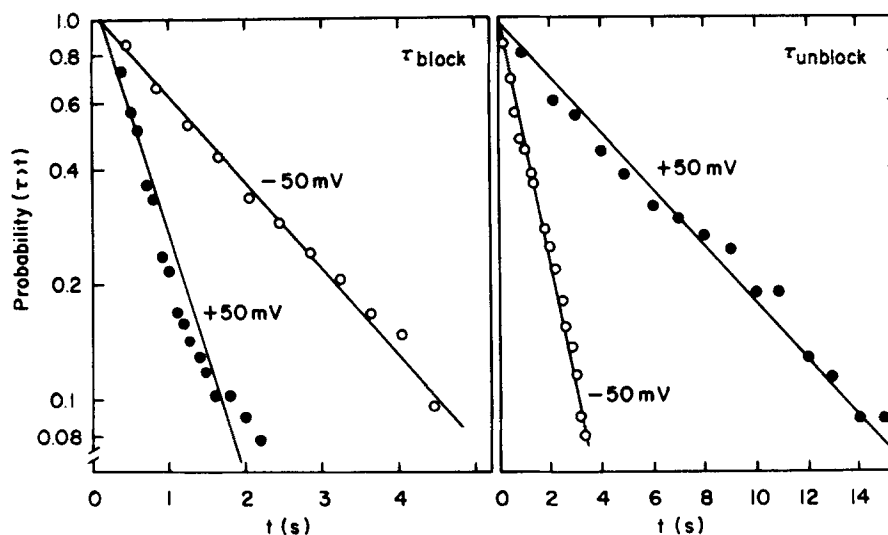


FIGURE 4. Dwell-time distributions for B1 block. Dwell-time histograms were tabulated by computer after digitization at 100 points/s. The cutoff time for short blocking events was 0.2 s. Bilayer conditions were as described in Fig. 2. The total number of events was 78 for 130 s of input at +50 mV and 188 for 170 s of input at -50 mV.

highly purified toxin samples. Because each toxin has a characteristic dissociation rate (or mean blocked time), single channel analysis provides a sensitive method for checking the purity of toxin samples. From experiments as in Fig. 2, for example, it is apparent that the toxins C1 and gonyautoxin 2 (GTX2) block on very different time scales, and that these two compounds are kinetically distinct. Prior to this study, it was not possible to determine whether the low toxicity of C1 was due to minor contamination of the toxin with its hydrolysis product, GTX2 (Hall et al., 1980). This possibility is now directly ruled out.

Voltage Dependence of Binding Rates

All of the toxins listed in Fig. 1 exhibited voltage-dependent binding rates, as shown in Figs. 3 and 4 for the example of toxin B1. The absolute magnitudes of the rate constants varied widely from toxin to toxin. Fig. 5 shows the measured blocking rate constants for several toxins as a function of voltage. The results suggest that the binding rate constants and the equilibrium dissociation constant may be approximated as exponential functions of voltage according to the following equations:

$$k_{-1}(V) = k_{-1}(0)\exp(A_{-1}V); \quad (1)$$

$$k_1(V) = k_1(0)\exp(A_1V); \quad (2)$$

$$K_D = k_{-1}/k_1; \quad A = A_{-1} - A_1; \quad (3)$$

$$K_D(V) = K_D(0)\exp(AV), \quad (4)$$

where k_{-1} is the first dissociation rate constant, k_1 is the bimolecular association rate constant, and the A terms are slope parameters in mV^{-1} . The data for all eight toxins were fitted according to these equations as shown for some examples in Fig. 5. These results are summarized in Table I.

An unambiguous result of our experiments is that the voltage dependence (A parameter) of toxin binding is not related to the net charge of the toxin. Table I shows that the dissociation rate constant increases e -fold per 60–90 mV and the association rate constant decreases e -fold per 70–100 mV of depolarization for all the toxin blockers. These two rate effects result in an e -fold increase in the equilibrium binding K_D per 30–40 mV of depolarization. Given the inherent statistical limitations of our measurements, the small differences in the voltage dependences of the various toxins are not significant. We also found (Table I) the voltage dependence of the association reactions to be slightly less than that for the dissociation reactions in most experiments. Thus, whatever the mechanism for the voltage dependence, in terms of Eyring rate theory the transition state of the binding reaction lies at the top of a nearly symmetric energy barrier with respect to voltage.

Table I also shows that the various toxins can be distinguished from each other

FIGURE 5. (*opposite*) Voltage dependence of binding rate constants for various toxins. Corrected dissociation (*A*) or association (*B*) rate constants were measured as described in Methods. In *B*, bimolecular rate constants were obtained by dividing the pseudo-first-order rate constant by the toxin concentration. Solid lines are semilogarithmic least-squares fits. Zero-voltage rate constants and slope values for these fits are given in Table I.

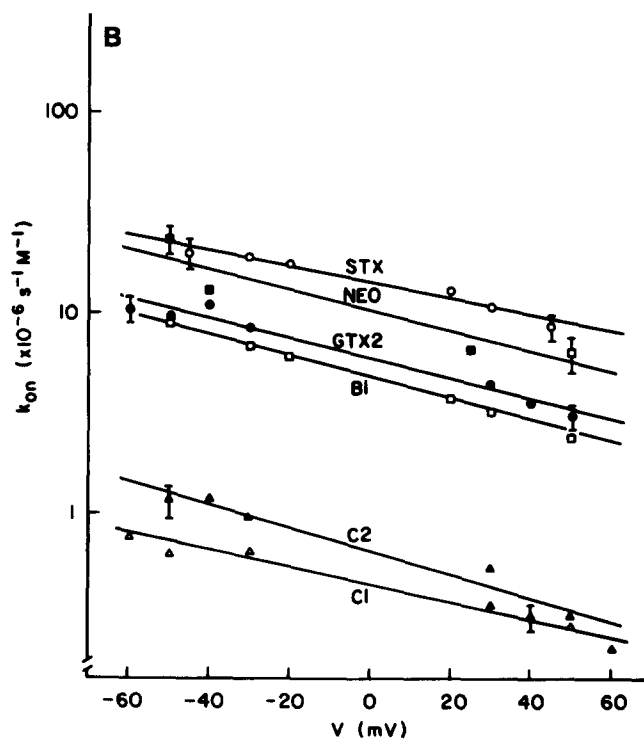
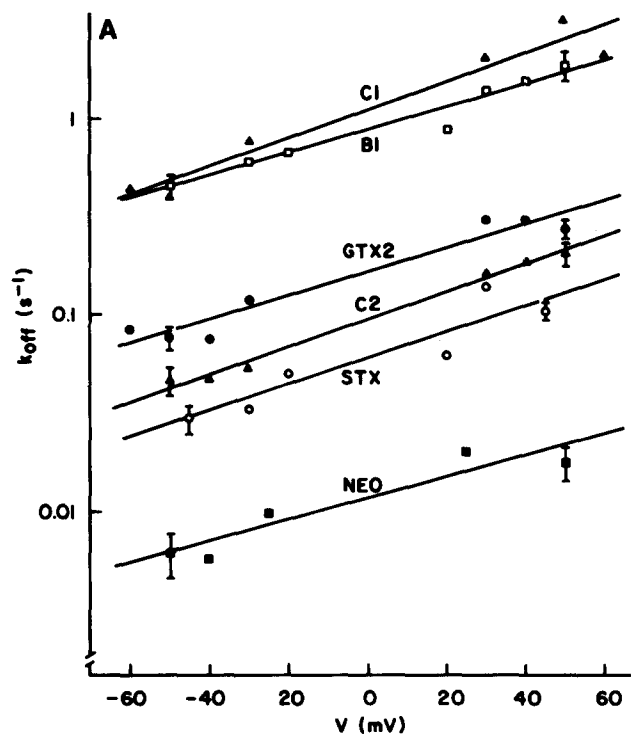


TABLE I
Zero-Voltage Blocking Rate Constants of Various Toxins for Rat Muscle Na⁺
Channels in Planar Bilayers

Toxin	Dissociation rate		Association rate		Equilibrium constant	
	$k_{-1}(0)$	A_{-1}	$k_1(0)$	A_1	$K_D(0)$	A
	s^{-1}	mV^{-1}	$\times 10^{-6}$ $s^{-1}M^{-1}$	mV^{-1}	nM	mV^{-1}
STX	0.061	0.015	14	-0.0092	4.3	0.023
B1	0.88	0.013	4.8	-0.012	180	0.025
GTX2	0.17	0.014	6.0	-0.011	28	0.025
C1	1.1	0.016	0.44	-0.0098	2,500	0.026
GTX3	0.039	0.011	8.7	-0.013	4.5	0.024
C2	0.095	0.016	0.65	-0.014	150	0.030
NEO	0.012	0.013	10	-0.012	1.2	0.025
TTX	0.12	0.013	4.5	-0.010	27	0.023

Dissociation and association rate constants were measured as a function of voltage as shown in Fig. 5. The values are observed rate constants measured at 24°C in the presence of 200 mM NaCl, 10 mM MOPS-NaOH, pH 7.4, 0.1 mM EDTA. Zero-voltage parameters were obtained from semilogarithmic least-square fits to Eqs. 1 and 2.

by their zero-voltage rate constants. Addition of the various sulfate moieties to the STX nucleus reduces the binding affinity by a combination of an increased dissociation rate and a decreased association rate. By contrast, the introduction of a hydroxyl group on the N1 nitrogen to form neosaxitoxin (NEO) actually increases the binding affinity by a fivefold reduction in the dissociation rate.

Comparison of STX Binding in the Bilayer and Native Membrane Fragments

In order to determine if the toxin receptor was altered in the process of incorporation into the planar bilayer, we measured STX binding parameters for the muscle membrane preparation by a direct binding assay using [³H]STX. The rate of [³H]STX dissociation was too fast for us to measure directly at the temperature of the bilayer experiments (24°C); however, we measured it at two lower temperatures, 0 and 18°C, in order to extrapolate to 24°C. Fig. 6 shows the time course of [³H]STX dissociation from its specific receptor site. The dissociation reaction closely follows an exponential time course, as expected for a homogeneous population of sites. The rate constants that we measured were 0.0047 s⁻¹ and at 0°C and 0.026 s⁻¹ at 18°C. These dissociation rates were the same whether or not the membranes were preincubated for 30 min in 4 μM BTX. Since this amount of BTX is saturating for its effects on Na⁺ channels in cells (Huang et al., 1982), this result argues that STX binding rates for the normal and BTX-modified channel are equivalent under these conditions. Since the membranes were washed free of KCl and preincubated with NaCl, these measurements were performed at zero membrane potential. We may thus compare the zero-voltage single channel rate constants with the values obtained using [³H]STX. By extrapolating the dissociation rates measured at 0 and 18°C from the Arrhenius plot, we obtained a dissociation rate constant of 0.046 s⁻¹ at 24°C for [³H]STX. This value agrees with the bilayer measurement of $k_{-1}(0) =$

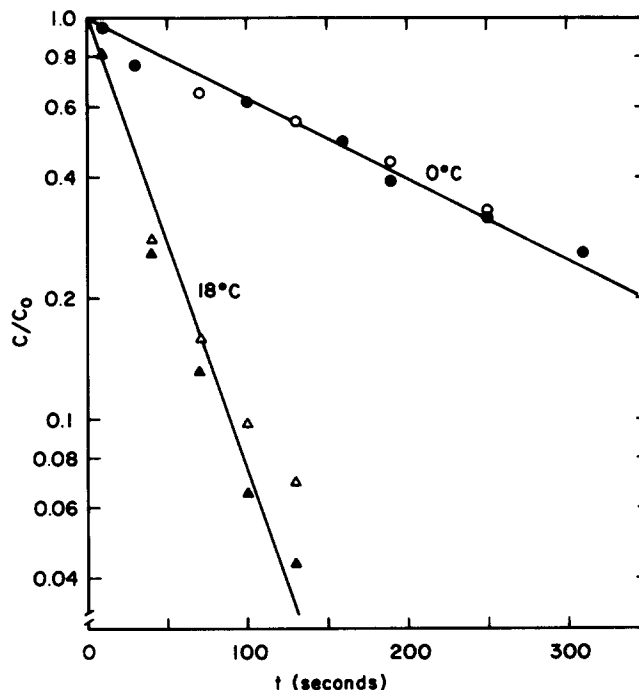


FIGURE 6. Dissociation kinetics of [³H]STX from rat muscle membranes. [³H]-STX bound to toxin receptor sites was measured at various times after displacement with excess TTX. The buffer conditions of the assay were the same as for the bilayer experiments, except that the temperature was maintained at 0°C (circles) or 18°C (triangles), and the experiment was done in the absence (solid symbols) or presence (open symbols) of 4 μM BTX. The data are plotted as the ratio of bound radioligand at the time after TTX addition to that bound at time zero according to $C/C_0 = \exp(-kt)$. The measured rate constants were: 0.0047 s⁻¹, 0°C; 0.026 s⁻¹, 18°C.

0.061 s⁻¹. Similarly, we obtained an extrapolated equilibrium dissociation constant of 8.2 nM at 24°C from binding data at 0 and 18°C. That this value is within a factor of 2 of the bilayer value [$K_D(0) = 4.3$ nM] suggests that the association rate constant for STX is also not drastically altered upon incorporation of the channel into the bilayer. Moreover, these K_D values are similar to those measured by [³H]STX binding to rat sarcolemma vesicles ($K_D = 2.6$ nM) or to cut pieces of rat skeletal muscle ($K_D = 5.9$ nM) (Barchi and Weigele, 1979; Hansen-Bay and Strichartz, 1980).

DISCUSSION

Relationship of Toxin Structure to Binding Rates

The plethora of toxins specific for the voltage-dependent Na⁺ channel (Catterall, 1980) greatly facilitates the direct comparison of parameters measured by biochemical methods with those measured by electrical methods. This is an asset

for planar bilayer studies, where one would like to assess the "normalcy" of the channel inserted in the bilayer. Such a comparison involves the additional assumption that bilayer data measured at the microscopic level on individual channels can be directly compared with biochemical measurements at the macroscopic level, typically involving 10^{12} channels per assay. The comparison shows remarkable agreement between the binding parameters of [^3H]STX measured directly on isolated membranes and those of STX measured in the artificial bilayer (Fig. 6 and Table I). The agreement between the relative affinities and potencies of various toxins as measured by blocking studies of single channels, by binding competition to rabbit brain membranes and by depression of the action potential in frog nerve is also quite good (Table II).

These results suggest two conclusions. The first is that it seems safe to assume that specific binding of the guanidinium toxins is equivalent to blocking of the channel. This allows us to discard the possibility of kinetically significant intermediate states of the channel that have toxin bound, but are still conducting. The second point is that the lipid environment of the protein probably does not greatly affect the toxin binding rates, since we have used neutral phospholipid bilayers, while cell plasma membranes contain significant amounts of negatively charged lipids and cholesterol. This point can be tested directly in the future by modifying the lipid composition of the planar bilayer.

With respect to binding rates of the various STX derivatives, our data support the previous suggestion (G. Strichartz, manuscript submitted for publication) that the net charge is not the sole factor in determining these rates. Rather, tight binding seems to be due to a combination of several molecular interactions in the binding site as discussed below.

Before discussing our results, we review some basic chemical characteristics of toxin binding as described by previous work. Many studies suggest that there is at least one essential carboxyl group in the toxin binding site: the carboxyl

TABLE II
Relative Potencies of Various Toxins Measured by Different Methods

Toxin	Planar bilayer (this work)	[^3H]STX*	Action potential†
	(K_D , STX/ K_D , toxin)	(K_D , STX/ K_D , toxin)	($K_{1/2}$, STX/ $K_{1/2}$, toxin)
STX	1.0	1.0	1.0
B1	0.024	0.029	—
GTX2	0.15	0.29	0.22
C1	0.0017	—	—
GTX3	0.96	1.9	1.4
C2	0.029	0.094	—
NEO	3.6	4.3	4.5
TTX	0.16	0.20	0.33

* From competition binding experiments to rabbit brain membranes using [^3H]STX at 4°C (Strichartz, 1984).

† Relative potencies as measured by depression of action potential in frog sciatic nerve at 20–22°C (Strichartz, 1984).

alkylating reagents trimethyl- and triethylxonium inactivate toxin binding (Reed and Raftery, 1976; Baker and Rubinson, 1975; Spalding, 1980); toxin binding is competitively inhibited by decreasing pH with an apparent pK_a of 5.3; toxin binding is inhibited competitively by monovalent cations with a selectivity sequence indicating a high field strength site (Henderson et al., 1974; Reed and Raftery, 1976; Barchi and Weigele, 1979). Since TTX has one guanidinium group and STX has two such groups, it seems likely that the carboxyl may be required for ionic bonding with a toxin guanidinium. Recent studies have suggested that the positively charged C-8 guanidinium of STX may be required for binding, since this guanidinium has an abnormally low pK_a of 8.2 (Rogers and Rapoport, 1980; Shimizu et al., 1981), which correlates with loss of STX potency at high pH (Kao et al., 1983; G. Strichartz, manuscript submitted for publication).

Effect of the Sulfo Group at N21

The introduction of this substituent has an adverse effect on the binding affinity as seen by comparing B1 with STX, C1 with GTX2, and C2 with GTX3. The OFF rate was increased as much as 14-fold and the ON rate was reduced as much as 13-fold for these three pairs of molecules that differ only by the sulfo-carbamyl group vs. the parent carbamyl. The effect on the OFF rate may imply that an unhindered nitrogen on the carbamyl is necessary for hydrogen bond formation in the site or that the introduction of a negative charge in that region of the site destabilizes the bound toxin because of electronegative groups on the protein. The former explanation receives additional support from the observation that decarbamoyl-STX has about a fivefold lower potency in blocking the action potential in frog sartorius muscle (Kao and Walker, 1982).

In considering effects on the association rate, we note that the measured ON rate constants for the various toxins fall in the range of the highest association rates of small molecules to enzymes (Fersht, 1977). If we correct for the effect of Na⁺ competition, the zero-voltage association rate constants for TTX and STX are 0.3×10^8 and $1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively. Thus, these reactions occur within two orders of magnitude of diffusion limitation. If STX binds to a recessed site with a snug fit, we might expect the addition of bulky groups to reduce the ON rate by decreasing the frequency of productive collisions. The relatively large size of the sulfate groups (5 Å diam) can be appreciated by the crystal structure of the C2 molecule shown in Fig. 7. The addition of two $-\text{SO}_3^-$ groups increases the dimensions of the STX molecule and reduces the ON rate for the C1 and C2 derivatives by a factor of 20–30. The reduction of the ON rate caused by these groups may also be due to a greater time required for proper configurational rearrangement of the toxin or the site, as needed to result in productive binding.

For an alternative interpretation, we may consider that the ON rates are correlated with the net charge of the toxins. STX, with a net charge of +2, exhibits the largest ON rate, while C1 and C2, with zero net charge, show the smallest. This correspondence may imply that a negative surface charge on the protein is a major factor in determining the ON rate, as suggested by Strichartz

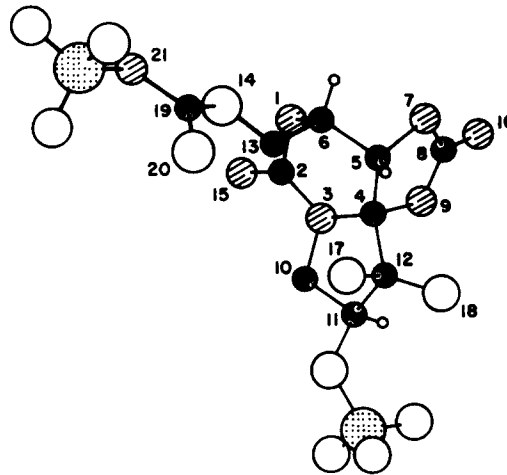


FIGURE 7. X-ray crystal structure of toxin C2. This structure, determined by S. Darling, was redrawn from Hall (1982). The positions of protons on carbons 5, 6, and 11 were calculated and are included for stereochemical clarity. Atoms are designated as follows: carbon, black; nitrogen, striped; oxygen, unshaded; sulfur, stippled.

et al. (1984). Further experiments are required to distinguish between the relative contributions of toxin charge and size to the association rate constant.

Effect of the Hydroxysulfate Group at C11

Replacement of a hydrogen atom on STX by a single hydroxysulfate group at either isomeric position at C11 does not have a severe effect on binding. This can be seen by comparing the binding rates of GTX2 and GTX3 with those of STX, which are within a factor of 2–3 of the parent molecule. Substitution at the β position vs. the α position at C11 results in stronger binding, however, as noted by the higher affinity of GTX3 compared with GTX2 and that of C2 compared with C1. We can conclude that the C11 position is not a critical determinant of the toxin binding energy. It does appear that the addition of the hydroxysulfate in the C2 molecule reduces the increase in the OFF rate caused by the sulfo-carbamyl of the B1 molecule. Specific ligand site interactions and steric effects would have to be invoked to explain the discrimination between the α and β isomers at C11.

Effect of the Hydroxyl Group at the N1 Nitrogen

The existence of NEO is a particularly fortunate instance of a subtle molecular alteration revealing functional detail. The extra hydroxyl group on NEO gives a pK_a of 6.75 (Shimizu et al., 1978) and thus, at our pH of 7.4, NEO in solution exists as a mixture of the deprotonated (82%) and protonated (18%) forms. If the C2 guanidinium of STX were required to be in the cationic form for ion-pair formation in the binding site, we would expect that deprotonated NEO might be incapable of binding. This is not the case, since NEO has a three- to fourfold-enhanced affinity relative to STX at this pH. The ON rate constant of

NEO, which should be sensitive to the concentration of the potent species, is nearly the same as that of STX, which suggests that the deprotonated species does bind. The dissociation rate constant of NEO is about fivefold slower than that of STX, which reflects a greater stability of the receptor occupied by NEO. Thus, the cationic guanidinium at C2 is not essential for ion-pair formation in the site. This result implicates the C8 guanidinium as the active group, in harmony with previous conclusions reached from pH studies of Kao et al. (1983) and G. Strichartz (manuscript submitted for publication). The reason for the reduced OFF rate of NEO is unclear from our work alone. However, this observation may support the hypothesis of a weak covalent bond between toxin and protein via the keto form of STX at the C12 position (Hille, 1975; Strichartz et al., 1984), since NEO in neutral solution exhibits a faster exchange between the keto and hydrate forms than does STX (Shimizu et al., 1978, 1981).

Is the Voltage Dependence Related to Toxin Charge?

Our results show that the voltage dependence of toxin binding is independent of toxin charge. Can we rationalize this result in terms of the usual picture of voltage-dependent blocking: that voltage dependence is conferred upon the binding reaction by the movement of the charged blocking molecule into an applied electric field within the channel pore (Woodhull, 1973)? It is possible to retain this simple mechanism, but only by requiring that the C8 guanidinium of the STX nucleus enter the field and that all of the other charges be completely excluded. How reasonable is such a possibility?

If we assume that TTX does block by this mechanism, the substantial voltage dependence demands that the TTX guanidinium sense a large fraction, 0.65, of the voltage drop. The structural evidence discussed above implicates the C8 guanidinium of STX as the essential cationic residue. Then, assuming a homologous blocking mechanism for TTX and STX, we would have to propose that the charge on the C2 guanidinium lies completely outside the field, while the C8 guanidinium enters it as deeply as TTX, to obtain the same voltage dependence. Such a hypothesis is not unprecedented, but it is difficult to justify with only a 4-Å charge separation between the C8 and C2 guanidiniums. Thus, if we assume that 60% of the applied voltage drops across a 4-Å distance, then only the C8 guanidinium, but none of the other charges on the molecule, would experience the applied field, and all the toxin derivatives would display identical voltage dependence of binding.

Besides being awkward in the extreme, this picture has one major difficulty. It requires the C8 guanidinium to behave as a point charge in the binding site and to be impervious to the induction effects of the other charged substituents. However, the fact that the C2 and C8 guanidinium groups of STX have different pK_a 's of 11.2 and 8.2, respectively, refutes this assumption. The effect of the sulfate groups on the pK_a 's and the charge distribution at the two guanidinium groups is unknown, but they would also be expected to have an electron-withdrawing influence. In view of such tenuous assumptions regarding the structure of the binding site and the nature of charge effects, it appears that a

Woodhull-type mechanism for the voltage dependence of toxin block is untenable.

Hypothesis of Last Resort: a Voltage-dependent Conformational Change

Entry of a charged species into an electric field is the most direct and economical explanation for voltage-dependent blocking rates. Because there is no effect of toxin charge on the voltage dependence of blocking, we are forced to abandon this simple interpretation. In the preceding paper (Moczydlowski et al., 1984), we also ruled out another possible mechanism—a voltage-dependent competitive Na^+ binding. Therefore, in the absence of direct evidence, we take refuge in the a priori postulate that a conformational change may explain the phenomenon.

Fig. 8 shows the scheme we use to consider the hypothetical effect of a voltage-dependent conformational equilibrium of the protein on the apparent rates of toxin binding. In this scheme, E1 and E2 represent interconvertible states of the toxin receptor, where depolarizing voltage shifts the equilibrium toward the E2 states. Since depolarization also reduces the affinity of toxin binding, this requires that E2 be a low-affinity state for toxin and E1 be a high-affinity state. In this scheme, we also assume that the actual toxin binding rates k_1 , k_2 , k_{-1} , and k_{-2} are voltage independent, while the K_3 and K_4 equilibria are voltage dependent and very fast compared with the binding rates. In this situation, the probability distribution functions of the blocked and unblocked states will behave as single exponentials, as we actually observe. This scheme is analogous to one previously considered by Hanke and Miller (1983) to explain the pH dependence of the *Torpedo* Cl^- channel.

According to the definitions of equilibrium constants given in the legend of

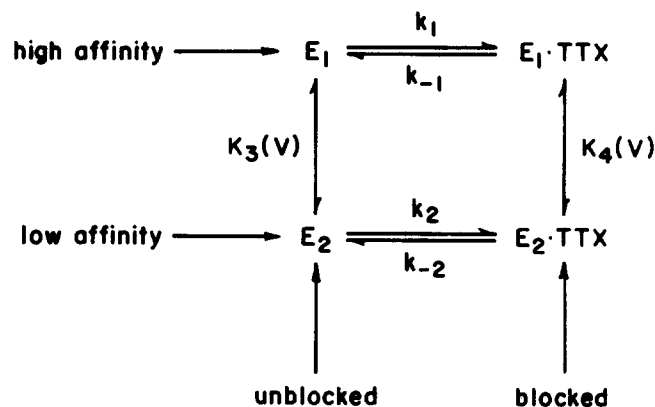


FIGURE 8. Hypothetical scheme to explain voltage-dependent toxin binding. E represents a toxin binding site that can exist in either a high-affinity (E1) form, or a low-affinity (E2) form. Only the K_3 and K_4 equilibria are voltage dependent and are defined as the ratio of the E2 form to the E1 form. K_1 and K_2 are dissociation constants. TTX represents tetrodotoxin or any of the other guanidinium toxins.

Fig. 8, the following relationships can be derived:

$$K_1K_3 = K_2K_4; \quad (5)$$

$$k_{\text{on}} = [k_1 + k_2K_3(V)]/[1 + K_3(V)]; \quad (6)$$

$$k_{\text{off}} = [k_{-1} + k_{-2}K_4(V)]/[1 + K_4(V)]; \quad (7)$$

$$K_D = K_1\{[1 + K_3(V)]/[1 + K_4(V)]\}. \quad (8)$$

Eq. 5 requires that if only K_3 and K_4 are voltage dependent, then these two reactions must have the same voltage dependence. We shall consider that these reactions follow a simple Boltzmann distribution with respect to the voltage, V :

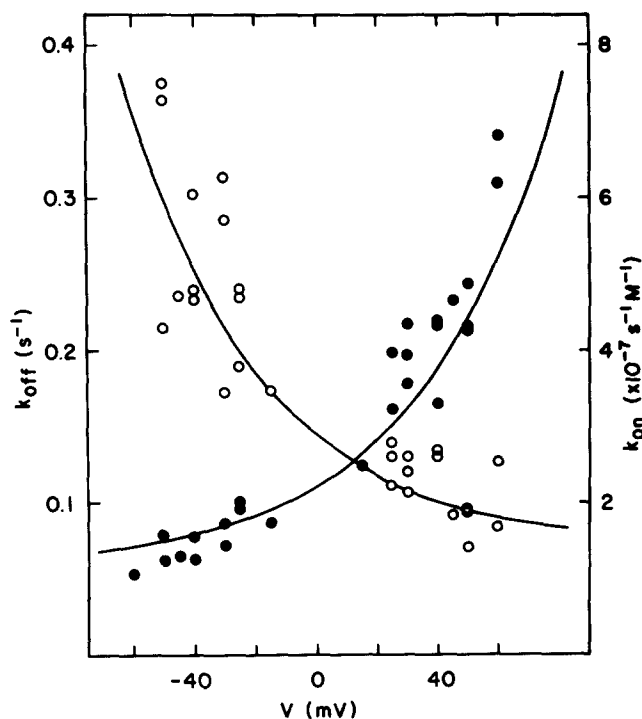


FIGURE 9. Fit of TTX binding rate constants via scheme of Fig. 9. Data for TTX blocking were taken from Moczydlowski et al. (1984). The different values plotted at a given voltage were from different experiments at various Na⁺ concentrations, ranging from 40 to 600 mM NaCl. The association rate constants (O) were corrected to zero Na⁺ concentration by multiplying the measured value by $(1 + [\text{Na}^+]/32 \text{ mM})$, as expected for a simple competition model, discussed in the above reference. The data were fit to the model of Fig. 8 and Eqs. 5–9 using an iterative method as discussed in the text. The solid lines were drawn according to Eq. 6 (k_{ON} , O) and Eq. 7 (k_{OFF} , ●) using the following values for the constants: $K_1 = 3.8 \times 10^{-11} \text{ M}$, $K_2 = 3.8 \times 10^{-7} \text{ M}$, $K_3 = 100 \exp(0.023V)$, $K_4 = 0.01 \exp(0.023V)$, $k_1 = 1.5 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, $k_2 = 1.4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 0.057 \text{ s}^{-1}$, and $k_{-2} = 5.4 \text{ s}^{-1}$.

$$K_{1,3}(V) = K_{1,3}(0)\exp(AV). \quad (9)$$

The scheme shown in Fig. 8 can be distinguished from a single-step voltage-dependent binding reaction in that, at the limits of infinitely high positive and negative voltage, the apparent binding rate constants given by Eqs. 6 and 7 approach limiting values k_1 , k_2 , k_{-1} , and k_{-2} . Since we do not clearly observe limiting values of the apparent rate constants at high voltage (Fig. 5), we cannot obtain a unique fit to our data of the model in Fig. 8. However, this model is sufficient to fit the data, using one set of plausible values for the kinetic constants. Such fits for the case of TTX binding are shown in Fig. 9.

While the model of Fig. 8 and the satisfactory fits of Fig. 9 were contrived to solve our dilemma, we do not consider the model only as a mental exercise. One clear prediction of the model is that the binding rates should saturate at high positive and negative voltages. This prediction should provide the impetus to use other methods of bilayer formation to measure binding rates at voltages up to 250 mV, where such saturation might be observed. We also note that the model of Fig. 8 is not without precedent in the Na^+ channel literature. A similar model was used by Catterall (1977) to account for the strongly voltage-dependent binding of scorpion toxins to Na^+ channels of neuroblastoma cells.

A conformational mechanism for the voltage dependence of toxin binding thus becomes, if not positively attractive, then at least inoffensive. It seems reasonable to conclude that the Na^+ channel protein may be a dynamic structure that is able to sense the transmembrane electric field and alter its conformation to affect the binding energies of specific toxin receptors on its surface. A future challenge will be to obtain structural correlates of this conformational flexibility.

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