

Rapid Voltage-dependent Dissociation of Scorpion α -Toxins Coupled to Na Channel Inactivation in Amphibian Myelinated Nerves

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ABSTRACT The voltage-dependent action of several scorpion α -toxins on Na channels was studied in toad myelinated nerve under voltage clamp. These toxins slow the declining phase of macroscopic Na current, apparently by inhibiting an irreversible channel inactivation step and thus permitting channels to reopen from a closed state in depolarized membranes. In this article, we describe the rapid reversal of α -toxin action by membrane depolarizations more positive than +20 mV, an effect not achieved by extensive washing. Depolarizations that were increasingly positive and of longer duration caused the toxin to dissociate faster and more completely, but only up to a limiting extent. Repetitive pulses had a cumulative effect equal to that of a single pulse lasting as long as their combined duration. When the membrane of a nonperfused fiber was repolarized, the effects of the toxin returned completely, but if the fiber was perfused during the conditioning procedure, recovery was incomplete and occurred more slowly, as it did at lower applied toxin concentrations. Other α -type toxins, from the scorpion *Centruroides sculpturatus* (IVa) and the sea anemone *Anemonia sulcata* (ATXII), exhibited similar voltage-dependent binding, though each had its own voltage range and dissociation rate. We suggest that the dissociation of the toxin molecule from the Na channel is coupled to the inactivation process. An equivalent valence for inactivation gating, of <1 e per channel, is calculated from the voltage-dependent change in toxin affinity.

INTRODUCTION

Several scorpion α -toxins and sea anemone toxins specifically modify the inactivation of Na channels in excitable membranes. In myelinated nerve fibers, they produce a steady state Na current during prolonged depolarization, but have no effects on channel activation (Koppenhofer and Schmidt, 1968*a, b*; Conti et al., 1976; Bergman et al., 1976; Romey et al., 1976; Neumcke et al., 1980; Mozhayeva et al., 1980; Ulbricht and Schmidtmayer, 1981; Wang and Strichartz, 1983, 1985). Since these toxins act only from the external solution, the toxin binding

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site is believed to be located on the outer surface of the Na channel (Narahashi et al., 1972).

Several reports have already suggested that these toxins do not bind tightly to depolarized membranes. Catterall (1977) first reported that radiolabeled *Leiurus* toxin bound with high affinity to Na channels in resting membranes but with low affinity when the membrane was depolarized by external KCl. This voltage-dependent equilibrium binding of *Leiurus* toxin coincides with the voltage dependence of the Na channel activation process. On the basis of this relationship, Catterall (1979) proposed that *Leiurus* toxin binds to a site that "senses" the activation process. More recently, Mozhayeva et al. (1980) and Warashina et al. (1981) also found, independently, that both *Buthus* scorpion toxin (M₇ and 2001) and sea anemone toxins (ATXII and PaTX) had less effect on Na channel inactivation when the membrane was depolarized for minutes under voltage-clamp conditions. Furthermore, Mozhayeva et al. (1979) found that the fast inactivation process was partially restored by repetitive pulses in the *Buthus* toxin-treated axon. Their results suggest that a relatively rapid binding and unbinding of scorpion α -toxin may occur. They attributed the decrease of the toxin binding mainly to changes in the conformation of Na channels that occur during fast inactivation, or more specifically, to "the difference in electrical energy between inactivated states of normal and poisoned channels" (Mozhayeva et al., 1980).

In this report, we examine the role of fast inactivation on the voltage-dependent binding of a purified *Leiurus* scorpion toxin and two other α -type toxins, *Centruroides* IVa and ATXII. Our results indicate that the conformational changes associated with fast inactivation processes dramatically affect the binding of all three α -toxins. Finally, our analyses also appear to be in qualitative agreement with the recent report from Meves et al. (1984) using *Centruroides* toxin V and ATXII, but with different protocols. We will compare their results with ours in the Discussion. A brief preliminary publication of these results has appeared (Wang and Strichartz, 1984).

METHODS

Toxin Preparation

Leiurus α -toxin IIa (α LQIIa) and *Centruroides* α -toxin IVa (α CSIVa) were each purified from the supernatant of the crude venoms *Leiurus quinquestratus* and *Centruroides sculpturatus*, respectively, by sequential chromatography on two cation exchangers (BioRex-70 column followed by CM-52 column). The details of the purification methods have been described previously (Wang and Strichartz, 1983). Sea anemone toxin II of *Anemonia sulcata* (ATXII) was purchased from Calbiochem-Behring Corp., San Diego, CA.

Voltage Clamp of Single Myelinated Nerve Fibers

Single myelinated nerve fibers were isolated from sciatic nerves of the toad *Bufo marinus* and voltage-clamped using the method of Dodge and Frankenhaeuser (1958). Leak and capacitance currents were subtracted by an analog circuit. Current traces were photographed from an analog storage oscilloscope and the amplitudes were digitized using a Digiplot (Houston Instrument Co., Austin, TX) in conjunction with an eight-bit micro-computer (Horizon 2, NorthStar Computers, San Leandro, CA). The currents were

calibrated by dividing the internodal ohmic potential by an assumed internodal resistance of 20 M Ω . The solution pool that bathed the node had a volume of 0.1 ml, and 2.0 ml of solution was passed through this pool to change the solution. In some cases, this pool was continuously perfused with precooled solution at a flow rate of 1 ml/min. To remove K current, the internodes were cut in 0.12 M CsCl, or in 60 mM CsCl and 60 mM NaCl. Purified toxins were dissolved in 2 ml of standard TEA Ringer containing 12 mM TEA-Cl, 110 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 5 mM HEPES buffer, pH 7.2, and 1 mg bovine serum albumin (BSA) per milliliter of solution, which was also used for control conditions. Unless indicated otherwise, most of the nodes in this report were treated with 200 nM *Leiurus* toxin, which gave maximal physiological effects (Wang and Strichartz, 1985). The temperature was maintained at $10 \pm 0.5^\circ\text{C}$.

RESULTS

General Description of the Action of Leiurus Toxin on Na Currents

We recently described the effects of *Leiurus* toxin on the kinetics of Na channels in single myelinated nerve fibers (Wang and Strichartz, 1985). Briefly summarized, the report showed that within minutes after its addition to the bathing Ringer, *Leiurus* toxin produces a slowing of the declining phase of Na currents (I_{Na}), as shown in Fig. 1. The fall in I_{Na} during depolarization is usually taken to represent the closing of Na channels by fast inactivation processes (Hodgkin and Huxley, 1952); the slowing and reversibility of fast inactivation by *Leiurus* toxin are saturable, toxin concentration-dependent, specific effects that have an apparent dissociation constant ($K_{\text{D}}^{\text{app}}$) of ~ 15 nM (Wang and Strichartz, 1985). The toxin is without effect on channel activation and ion selectivity (Wang and Strichartz, 1983, 1985). In addition, *Leiurus* toxin appears to have no effects on K channels, measured when the Na currents are blocked by tetrodotoxin (TTX). This factor is noteworthy, since in toad nodes, even with Cs⁺ inside and TEA⁺ outside, K currents may not be completely eliminated, particularly at the most positive potentials. Small residual K currents may sometimes be present at the end of depolarizing pulses larger than +40 mV (Fig. 1A); alternatively, there may be a small late Na current (<3% of peak I_{Na}) present after prolonged depolarization of control axons (Dubois and Bergman, 1975). However, the αLQIIa toxin-induced currents, even after voltage-dependent reversal, were at least three to five times greater than these toxin-independent signals.

The inhibitory action of *Leiurus* toxin on Na channel inactivation appears to be voltage dependent. The fraction of Na channels still open at the end of a 150-ms pulse becomes larger when measured at a more depolarized potential (up to +50 mV) than at 0 mV or lower. Normally, in a control nerve fiber, the more positive the potential, the less Na current remaining at the end of a prolonged depolarization, because of steady state Na inactivation (Hodgkin and Huxley, 1952; Dodge and Frankenhaeuser, 1958). The inverse response in toxin-treated nerves indicates that the voltage dependence of the inactivation processes can be altered or even reversed. Similar phenomena occur after application of crude *Leiurus* venom to frog node (Koppenhofer and Schmidt, 1968*a, b*) or squid axon (Gillespie and Meves, 1980), or in crayfish axon after application of a purified sea anemone toxin (Warashina and Fujita, 1983).

To study directly the effects of *Leiurus* toxin on Na channel inactivation at very positive potentials, i.e., $> +50$ mV, it is necessary to raise the internal Na ion concentration, since the outward currents are too small to be measured accurately in the usual 0.12 M CsCl intracellular solution. With an intracellular solution of 60 mM NaCl and 60 mM CsCl, the Na reversal potential is usually around +10 mV, as shown in Fig. 1, A–C (see also Shapiro, 1977). Again, we observed that in more depolarized membranes, up to +30 to +50 mV (Fig. 1, B

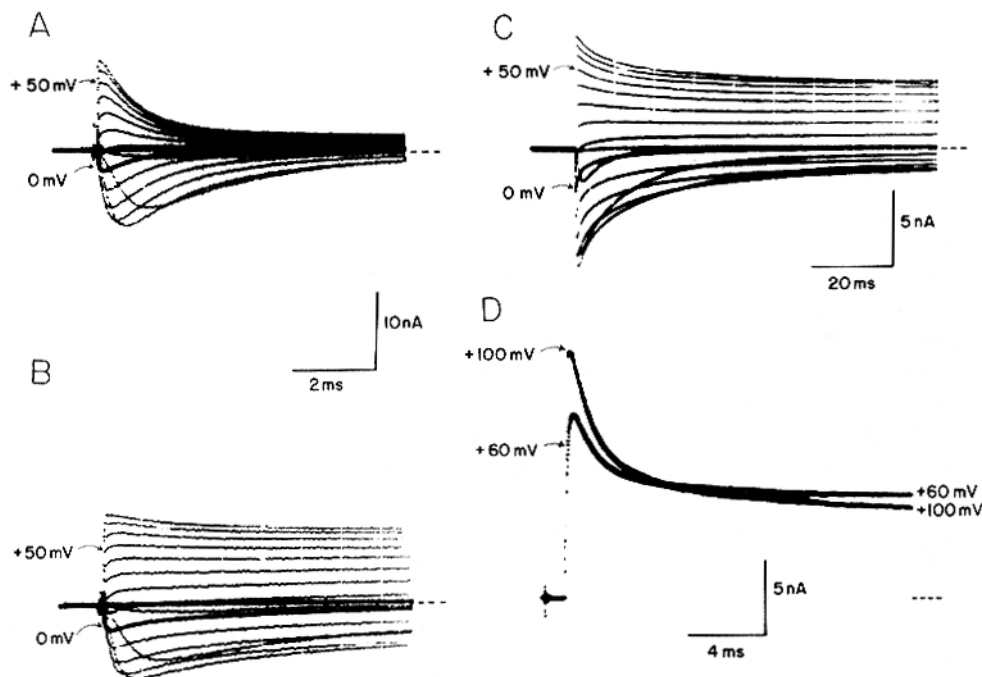


FIGURE 1. General effects of *Leiurus* α -toxin in node. A family of Na currents before (A) and after (B and C) external application of 200 nM *Leiurus* toxin in TEA Ringer. The internal solution contained 60 mM NaCl and 60 mM CsCl to produce large outward currents above +20 mV. The numbers indicate the membrane potentials, each pair separated by 10 mV, corresponding to some current traces. $E_H = -100$ mV; $E_{pp} = -130$ mV for 50 ms. Different time scales are shown in B and C. In D, a separate node was treated under the same conditions as in B. Note that the current trace at $E_m = +100$ mV crosses under the current trace at $E_m = +60$ mV. Current crossover is also found in C but is less apparent.

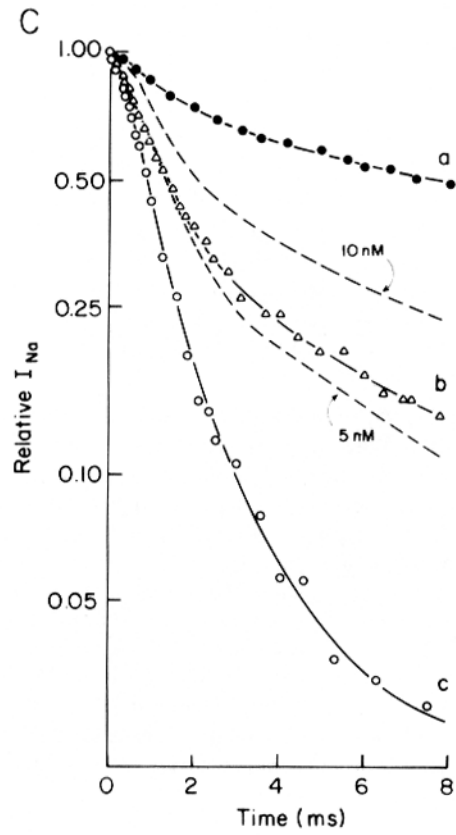
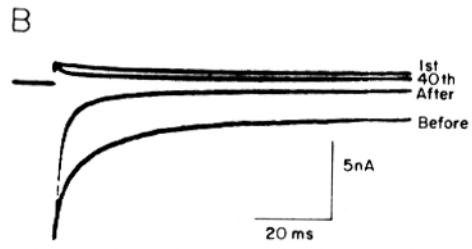
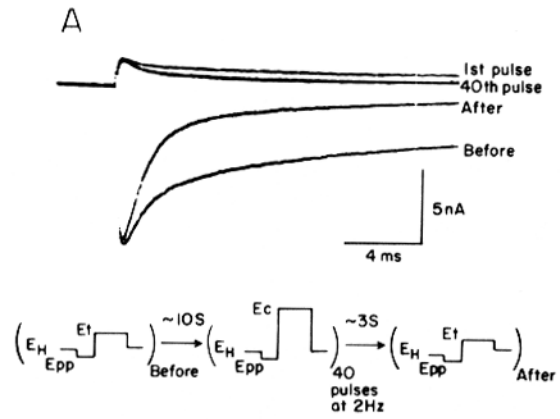
and C), the slowing of inactivation was greater. This result is qualitatively similar to that found with low Na ions internally, although it is difficult to compare them quantitatively since the peak amplitude and the direction of Na currents are very different between these two ionic conditions and the small contamination by residual K currents cannot easily be corrected. However, at potentials beyond +50 mV, the effect of *Leiurus* toxin on Na inactivation appears to be smaller than that at +30 mV; at these large depolarizations, more Na channels become

nonconducting during a prolonged pulse (Fig. 1C). Indeed, at +100 mV, the inactivation process again appears to be relatively fast, so that less current remains at the end of that depolarization than after a +60-mV pulse (Fig. 1D). (The apparently inverted voltage dependence of inactivation is not found in the control node, and the inactivation time course remains fairly constant over the high voltage range [Stampfli and Hille, 1976].) In the remaining sections, we will describe the voltage-dependent effect of *Leiurus* toxin in detail.

Reversible Voltage-dependent Action of Leiurus Toxin

The reduced effect of *Leiurus* toxin at very high voltages may arise from two different reactions: the effectiveness of bound toxin may be altered as a result of changes in the channel conformation, or toxin may actually dissociate from its receptor site during the large pulse, exemplifying a change in potency. To examine directly the toxin-channel interactions, we have applied a train of repetitive depolarizing pulses. The advantage of using repetitive pulses is to eliminate the various slow inactivation reactions that occur during one prolonged pulse (Fox, 1976), which might reduce the current in channels from which toxin has dissociated. Slow inactivation usually occurs at depolarized membrane potentials with a time constant longer than 300 ms, much slower than the normal fast inactivation processes in node (Dodge and Frankenhaeuser, 1958). Fig. 2 shows the kinetics of I_{Na} during repetitive depolarization of a node with internal 0.12 M CsCl. The pulse program is shown in the middle panel. After 40 conditioning pulses, each to +100 mV with a duration of 100 ms, the inactivation current kinetics became much faster. In general, the peak current amplitude was not changed. We found that in control nerve fibers not previously exposed to *Leiurus* toxin, such repetitive pulses had little effect on either the peak current or the kinetics, which proves that slow inactivation does not occur significantly during this "conditioning."

Since the outward current could be contaminated with residual K currents and is relatively small in nodes having an internal solution of 0.12 M CsCl, we also examined the inward current at $E_m = 0$ mV immediately before and after the +100-mV conditioning pulses (Fig. 2A). Again, the inactivation kinetics were clearly faster and nearly complete after conditioning, but the peak current amplitude and its rising kinetics were little changed. A view of this effect during a longer pulse is shown in Fig. 2B. The kinetics of the declining phase of I_{Na} at $E_m = -20$ mV are plotted semilogarithmically in Fig. 2C. The toxin's effect of slowing Na channel inactivation was not completely reversed by repetitive depolarizations (trace *b*, Fig. 2C); the Na current kinetics after reversal by conditioning are very similar to those resulting from exposure to 5 nM toxin with no conditioning (shown by the dotted line added to Fig. 2C). Before conditioning, the kinetics correspond to a toxin concentration of 200 nM, by definition. Assuming that binding of *Leiurus* toxin causes the slowing of inactivation and that unbinding restores the faster inactivation, the results imply a weaker but finite toxin binding (40-fold less) at this very positive potential. The reversal of *Leiurus* toxin action is strongly dependent on the duration and the voltage of



the conditioning pulses, and the "precondition" action of the toxin is restored after a recovery period.

These aspects of the toxin-channel interaction, defined in this article as "reversible voltage-dependent actions" of *Leiurus* toxin, contrast with the effects of toxin on the Na current kinetics observed under infrequent pulses to $E_m \leq 20$ mV when no reversal of the effects is detectable (Wang and Strichartz, 1985).

The Effects of Conditioning Pulse Duration on Voltage-dependent Reversal

Conditioning pulses of longer duration produce a more complete reversal of the toxin effects (Fig. 3). A significant reversal is readily observed at $E_m = +80$ mV for durations of >30 ms, using a pulse program like that shown in Fig. 2. If a single long pulse lasting several seconds is applied, the result is similar to that from repetitive pulses of shorter duration (Fig. 4, *A* and *B*). The single, 1-s pulse has a duration equal to the sum of the durations of the short, repetitive depolarizing pulses. However, a 500-ms repolarization to the holding potential was inserted between conditioning depolarizations to allow slow and fast inactivation to recover. The effect of *Leiurus* toxin was diminished after the single long pulse, and a slight reduction of the peak current was also observed, which was probably caused by slow inactivation (Fig. 4*B*). In comparison, during repetitive depolarization of the same nerve fiber, the inactivation kinetics became faster during each early conditioning pulse and reached a steady state after about the first six pulses (Fig. 4*A*). It appears that the primary mechanism of the voltage-dependent action is not directly related to slow inactivation, which, by definition, occurs only during a long pulse with a duration of more than several hundred milliseconds.

Parenthetically, the time course of Na channel inactivation in control fibers was little affected under our pulse protocol (Fig. 3, solid circles). Previously, both Frankenhaeuser (1963) and Meves et al. (1984) reported that the time course of the falling phase of the Na current was accelerated by a preceding depolarizing conditioning pulse. The absence of this phenomenon in our experiments may be due to the long interpulse (850 ms at $E_H = -100$ mV) and the hyperpolarizing conditioning pulse ($E_{pp} = -130$ mV for 50 ms). In contrast, Meves et al. (1984)

FIGURE 2. (*opposite*) Na currents in the *Leiurus* toxin-treated node before and after repetitive pulses. The internal solution contained 0.12 M CsCl. The pulse program is shown in the middle panel. (*A*) Four separate current traces were recorded. "Before" corresponds to the current measured at $E_t = -20$ mV before the repetitive conditioning pulses. The "1st" and "40th" traces correspond to the first and the last current traces measured at conditioning pulses ($E_c = +100$ mV for 100 ms) applied at 2 Hz for a total of 40 pulses. "After" corresponds to the current at $E_t = -20$ mV measured immediately after conditioning. In *B*, current traces are shown on a time scale different from that in *A*. The inactivation kinetics of Na currents before (*a*) and after (*b*) repetitive conditioning pulses are shown in panel *C* under conditions similar to those in *A*. The control kinetics (*c*) before 200 nM toxin treatment are also included, as well as the current kinetics in another node exposed to 5 nM *Leiurus* toxin. The currents at various times were normalized by the peak current amplitude, set at time zero.

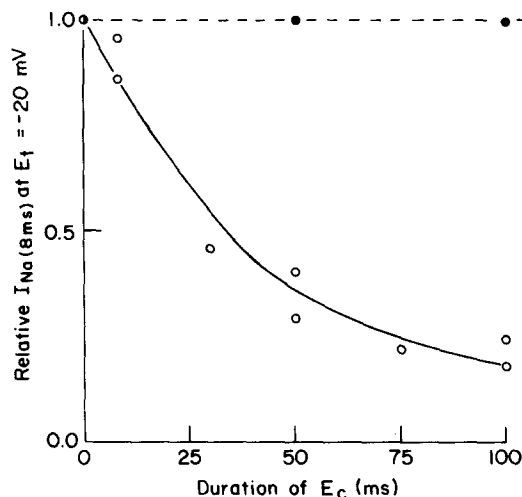


FIGURE 3. Dependence of reversal on the conditioning pulse duration. The length of the repetitive conditioning pulse (+80 mV), applied as in the pulse program in Fig. 2, is plotted against its relative effect on I_{Na} at the end of an 8-ms test pulse to -20 mV. The results from one control (●) and two separate *Leivirus* toxin-treated nodes (○) are shown. The absolute amplitude of the current at 8 ms in the control is normally $<5\%$ of the current in *Leivirus* toxin-treated nerve measured under this condition.

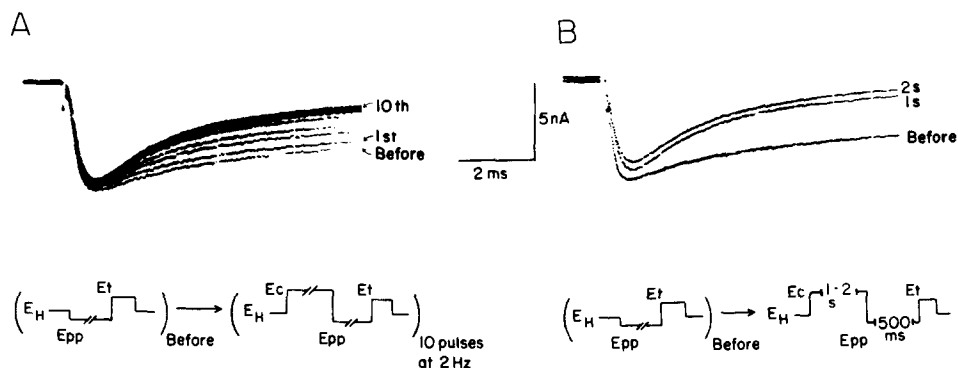


FIGURE 4. Effects of brief repetitive conditioning pulses vs. a single pulse. The pulse programs are diagrammed below the current traces. (A) An 8-ms test pulse to -20 mV was applied after each 100-ms conditioning pulse to $E_c = +100$ mV, with a 50-ms hyperpolarizing interpulse to $E_{pp} = -130$ used to allow the recovery of both normal activation and inactivation processes. The current traces during the test pulses are labeled "Before" (conditioning pulse), and "1st" and "10th" (after conditioning pulses at 1 Hz). (B) Current traces are shown for $E_t = -20$ mV before and after a 1- or 4-s conditioning pulse to $E_c = +100$ mV. A 500-ms interpulse was used to allow the recovery of the normal activation, inactivation, and slow inactivation processes. Both nodes contained an internal solution of 60 mM NaCl and 60 mM CsCl.

used a much briefer (20 ms) interpulse at $E_H = -92$ mV between the conditioning and test pulses. They also reported that the recovery from the effect of the conditioning pulse is nearly complete after an interpulse of 500 ms. Taken together, our measurements of the inactivation kinetics do not include any of the accelerated inactivation produced by a preceding conditioning pulse, as reported for toxin-free nodes using a different procedure.

Effects of Conditioning Pulse Potential on the Voltage-dependent Reversal

The reversible voltage-dependent action of *Leiurus* toxin is sensitive to the conditioning pulse potential; both the extent and the rate of reversal are affected. At potentials less than +20 mV, there was insignificant reversal after 40 pulses of 100 ms duration. A single 4-s pulse over the same voltage range also provided

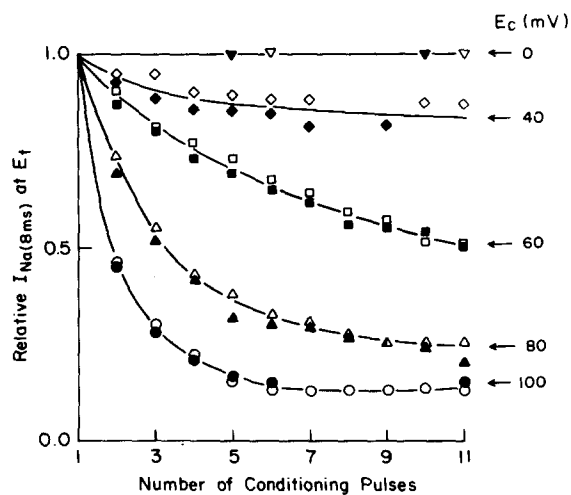


FIGURE 5. The rates of reversal of *Leiurus* toxin action at various conditioning potentials, assayed according to the pulse program in Fig. 4A. Each current value, measured at 8 ms, was normalized to the current amplitude without the conditioning pulse. Open symbols: 120 M CsCl internal solution; solid symbols: 60 mM NaCl and 60 mM CsCl internal solution.

no detectable reversal of toxin action (data not shown). However, when the potential was larger than +20 mV, the voltage-dependent reversal began to appear, and both the rate and the extent increased with increasingly positive potentials (Fig. 5) and saturated at about +120 mV. Still, the saturated reversal was incomplete and some slowing of Na current remained for conditioning potentials up to +150 mV. It appears that at very positive potentials, *Leiurus* toxin still affects the channel but with a much lower apparent affinity, i.e., only a small fraction of channels is modified by the toxin under these conditions.

Time constants for the reversal (τ_{rev}) are also voltage dependent. Reversal appears to be a first-order process at all potentials, fitted by a single exponential when plotted semilogarithmically against the time of depolarization (not shown).

Apparent rate constants for this reversible first-order process are calculated from measured values of τ_{rev} , and the steady state levels of modification shown in Figs. 5 and 6 are graphed in Fig. 7. The absolute value of the equilibrium constant for reversal cannot be determined (see Discussion), but the rate constants, expressed on an arbitrary scale, saturate at the positive potentials where toxin occupancy reaches its minimum. Thus, the kinetic basis for the limited dissociation is also voltage dependent.

The voltage dependence of the toxin-channel interaction is not affected by the direction of Na currents, since reversals measured in both low and high internal Na ion concentrations have the same voltage dependence (Figs. 5 and 6). We believe that the local accumulation or depletion of Na ions has little influence on the voltage-dependent reversal.

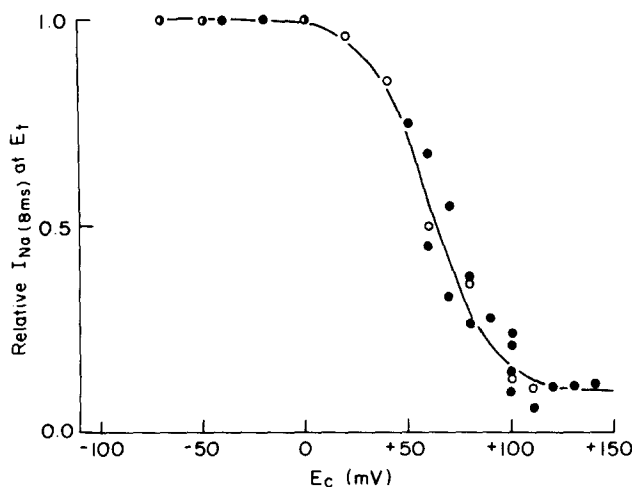


FIGURE 6. The steady state of voltage-dependent action at various conditioning potentials. The pulse program was the same as in Fig. 4A, except that the final current amplitudes were measured after 40–80 pulses at a frequency of 1–2 Hz. (○) 120 mM CsCl internal solution; (●) 60 mM NaCl and 60 mM CsCl internal solution. The curve can be fit by the equation $Y = 1 - B / \{1 + \exp[(E_c - \bar{E})/s]\} + B$, where $\bar{E} = 67$ mV (the potential at the inflection point of the curve), $s = 15$ mV (maximum slope of the curve), and $B = 0.1$ (extent of maximal effect).

Two different processes can account for voltage-dependent reversal of α -toxin action: (a) the bound toxin could become less effective in slowing inactivation, or (b) the toxin could dissociate from the channel. To test these possibilities and to determine whether dissociation of the toxin truly occurs during the reversal of action, we analyzed the voltage-dependent reversal and recovery at two concentrations of *Leiurus* toxin, 20 and 200 nM. We found that at both concentrations repetitive conditioning pulses reduced the toxin effects similarly. The \bar{E} (the potential at the inflection point of the curve; see Fig. 6) was 58 ± 5 mV in two separate experiments at 20 nM, a value insignificantly different from the 67 mV measured at 200 nM. Also, the rate of toxin dissociation at 20 nM became

measurable only at $E_c = +20$ mV and saturated at $E_c = 100$ – 120 mV. Overall, the only significant difference was in the recovery phase, which was seven times slower in 20 nM toxin than in 200 nM toxin. These results show that the rapid voltage-dependent reversal of the toxin effect is independent of toxin concentration, whereas its subsequent recovery is strongly dependent on toxin concentration and cannot follow uniquely from the conformational change of a stable toxin-channel complex.

The Recovery from Voltage-dependent Reversal

Upon returning to the -100 mV holding potential after the voltage-conditioned reversal, the toxin's effect of slowing inactivation is restored completely, as shown

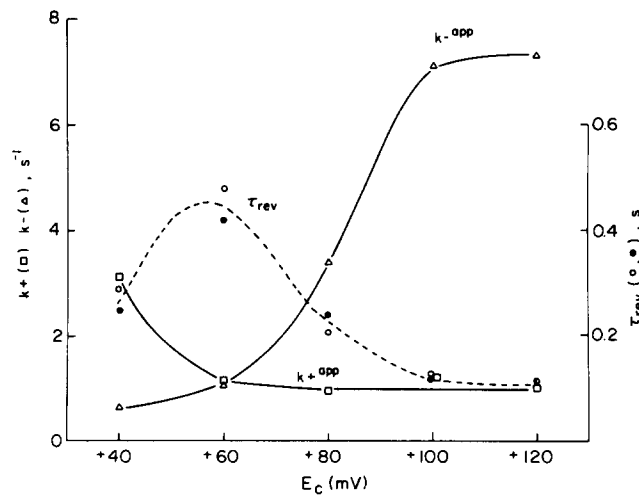


FIGURE 7. Measured time constants (τ_{rev}) and calculated rate constants (k_+^{app} , k_-^{app}) for the reversal of αLQIIa toxin action at different membrane potentials. Time constants were determined by linear regression fits of semilogarithmic graphs (not shown) of the data of Fig. 5. The rate constants were calculated, as described in the text, from the average τ_{rev} values and the corresponding apparent K_D 's, derived from the fraction of modified channels at steady state reversal, as shown in Fig. 6.

in Fig. 8. The time constant for this exponential recovery at 200 nM toxin is 31 ± 1 s (SD, $n = 3$), determined by fitting each of three semilog plots by eye. At 20 nM toxin, the time constant is 216 s (average of two experiments). The recovery time appears not to be sensitive to the application of a prepulse (-70 to -140 mV, for 50 ms) before the test pulse, nor is it dependent on the internal Na ion concentration. Apparent rate constants for toxin binding and dissociation, k_{+1} and k_{-1} , respectively, can be calculated from the two recovery time constants (τ_{rec}) measured at 20 and 200 nM toxin. The expression relating these terms is:

$$\tau_{\text{rec}} = (k_1[T] + k_{-1})^{-1}. \quad (1)$$

The calculated values are: $k_{+1} = 1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = 1.6 \times 10^{-3} \text{ s}^{-1}$.

During the recovery period (after exposure to 200 nM toxin), with the

membrane potential held at -100 mV, perfusion of the nodal pool (0.1 ml) with a 20-fold excess volume of toxin-free TEA Ringer had no significant effect on the rate or extent of recovery. It thus appears that neither the specifically nor the nonspecifically bound toxin molecules freely diffuse away from the vicinity of the nodal membrane during recovery. However, when the reversal conditioning is performed during continuous perfusion of the nodal chamber, the subse-

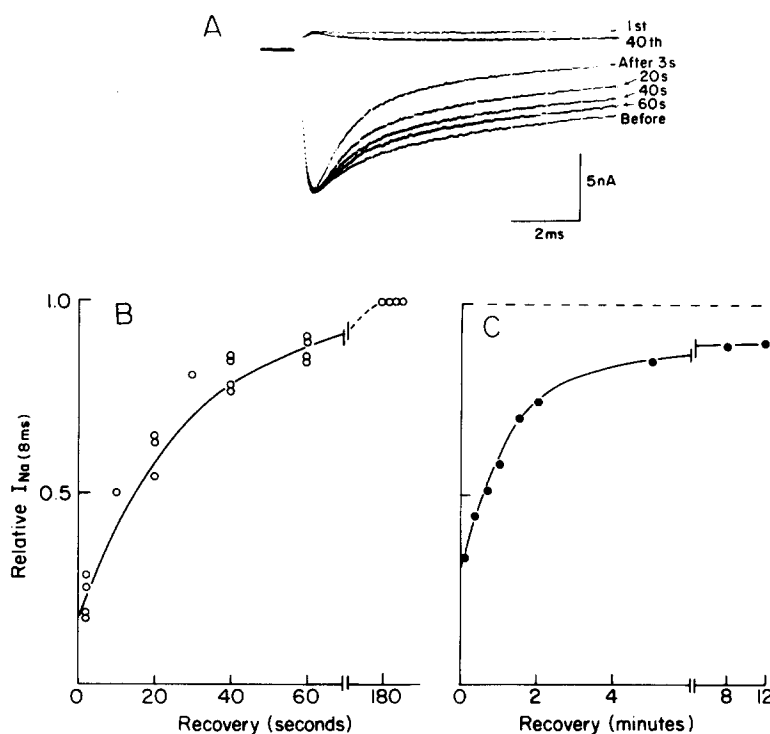


FIGURE 8. Recovery of toxin action after voltage-dependent reversal. (A) Current kinetics during the recovery are labeled at times after cessation of the repetitive conditioning pulses. (B) Relative I_{Na} at 8 ms into the test pulse are normalized to the I_{Na} (8 ms) before repetitive conditioning pulses and plotted against recovery time. Data were taken from A and from three other nodes. (C) Incomplete recovery when a node was continuously perfused with toxin-free TEA Ringer. The recovery time course was measured by the same method as in B (note the different time scale).

quent recovery of modified currents is slowed. The time constant of recovery grows to ~ 120 s and the recovery becomes incomplete, i.e., not all of the maintained current present before the repetitive pulse train is restored during the period of recovery (Fig. 8C). Without large, repetitive depolarizations, the effect of the toxin on inactivation seems to be irreversible, even after extensive washing. But washing during a train of large depolarizations accomplishes reversal of a limited extent, which is not subsequently recovered.

Comparison with Other Neurotoxins

Both *Anemonia sulcata* sea anemone toxin II (ATXII) and *Centruroides sculpturatus* α -toxin (α CSIVa) slow Na channel inactivation. However, the action of ATXII and α CSIVa can be reversed in the node of Ranvier by simple washing alone. These toxins also reduce the peak current amplitude, unlike purified *Leiurus* toxin. Since they have very different apparent affinities for the channel and their molecular weights are also different, 5,000 for ATXII and $\sim 7,000$ for α LQIIa and α CSIVa, we were interested in examining their reversal by voltage.

Both the actions of ATXII and *Centruroides* α -neurotoxins display a voltage-dependent reversal similar to α LQIIa action, but with some differences. First,

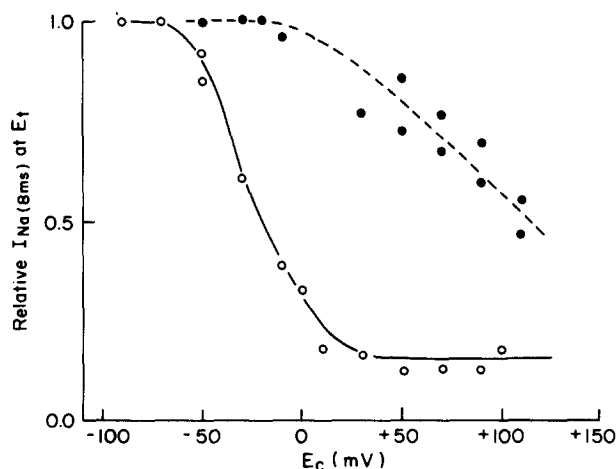


FIGURE 9. The steady state voltage-dependent action of *Centruroides* scorpion α -toxin (IVa) and sea anemone toxin ATXII. The assay was performed as in Figs. 4A and 6, except when the peak current amplitude was changed after conditioning pulses, the normalization of relative I_{Na} (8 ms) included this change. (O) 770 nM α CSIVa; (●) 5 μ M ATXII. Curves were fitted by eye. The data of α CSIVa can also be fitted by the equation $y = 1 - B / \{1 + \exp[(E_c - \bar{E})/s]\} + B$, where $\bar{E} = -27$ mV, $s = 11.5$ mV, and $B = 0.15$.

the voltage range of the reversal is quite different. The *Centruroides* α -toxin appears to be more sensitive than α LQIIa to membrane depolarization; the reversal of action occurs at pulses ~ 80 mV less positive than those required to reverse *Leiurus* toxin, and the voltage dependence is also slightly less steep ($s = 11.5$ vs. 15 mV; Fig. 9). In contrast, only a moderate voltage-dependent reversal can be obtained for sea anemone toxin, even by large conditioning pulses at high frequency (Fig. 9). There are also differences in the rates of reversal. First, whereas voltage-dependent reversal of α CSIVa reached its steady state within a single 100-ms conditioning pulse to +100 mV (Fig. 10A), ATXII reversal never achieved a steady state level, even at repetitive conditioning pulses of 200 ms duration to +110 mV (Fig. 10B). Second, the actions of both toxins recover very

rapidly after conditioning, too fast to be estimated by the method used in Fig. 8; complete recovery can be achieved within 5 s. This is not surprising since high concentrations of these toxins (between 700 nM and 5 μ M) were used in these experiments because of their relatively low binding affinity. The association time course, therefore, is considerably faster than that of 31 s, which corresponds to the lower *Leiurus* concentration (200 nM) that we used.

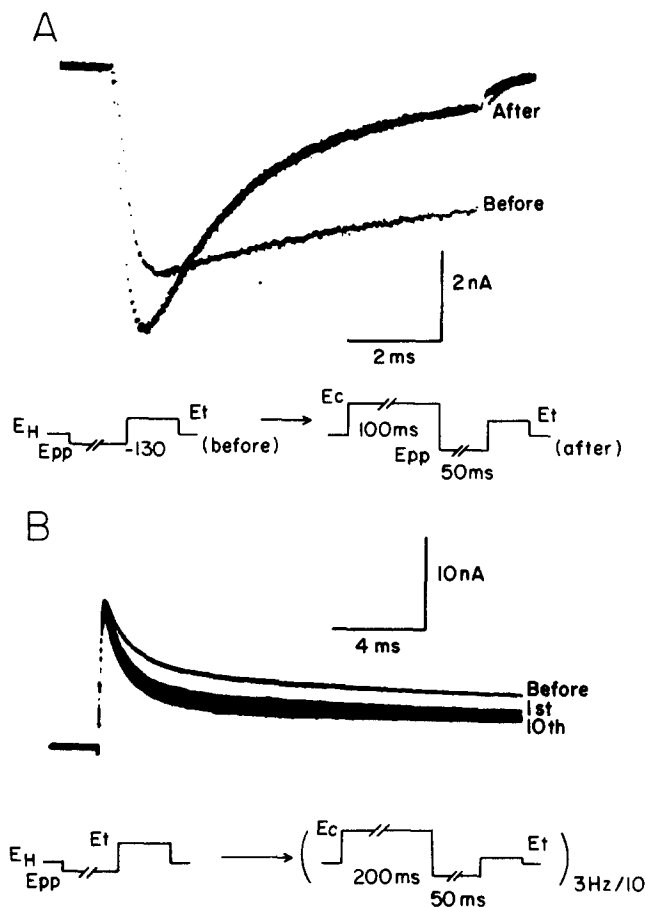


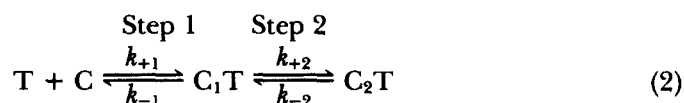
FIGURE 10. The rate of voltage-dependent dissociation of *Centruroides* α -toxin IVa and sea anemone toxin ATXII. (A) α CSIVa (770 nM) requires only a single conditioning pulse to reach its steady state reversal. $E_c = 100$ mV, $E_t = -30$ mV. Node bathed in 120 mM CsCl. In B, sea anemone toxin ATXII (5 μ M) requires many repetitive pulses with longer duration and higher frequency in order to produce some dissociation of the toxin. $E_c = 100$ mV, $E_t = +30$ mV, $E_H = -100$ mV. Node bathed in 60 mM NaCl and 60 mM CsCl.

DISCUSSION

This article describes a rapid, reversible, voltage-dependent reaction of α -type neurotoxins with the neuronal Na channel. Similar voltage-dependent binding reactions were observed for three different toxins: α LQIIa from *Leiurus quin-*

questriatus and α CSIVa from *Centruroides sculpturatus* scorpions, and ATXII from *Anemonia sulcata* sea anemone. The effect was characterized through extensive experiments with α LQIIa. Three kinetic phases can be resolved: at large depolarizations, the conductance falls rapidly ($\tau = 1$ –10 ms; Fig. 1); the fraction of modified channels is decreased during prolonged or repetitive depolarization ($\tau = 100$ –500 ms; Fig. 5); toxin modification recovers, with a concentration-dependent rate, at the resting potential ($\tau = 31$ s at 200 nM toxin). The reversal rate and extent were dependent on the size and duration of the conditioning pulses, but were independent of the toxin concentration. Even the most positive conditioning potentials could only reverse 85% of the toxin-modified currents, when 200 nM α LQIIa toxin was used. Although neither extensive washing in toxin-free Ringer nor repetitive conditioning alone sufficed to lower the extent of toxin-modified I_{Na} at steady state, together they produced a reversal that recovered incompletely and more slowly than normal.

At least two steps seem to be involved in the reversal process and its recovery. The rapid loss of current and the slower disappearance of modified channels is obviously voltage dependent, but the recovery process, which is due at least in part to toxin binding, was only assayed at negative potentials, so its voltage dependence is unknown. A reaction scheme with two sequential steps can be used to model some of these kinetics.



The intermediate toxin-channel complex, C_1T , has a lower affinity for the toxin than does C_2T and, in principle, may be less or equally effective in modifying channel inactivation. Similar schemes have been proposed by Schmidt-mayer et al. (1982) to explain the observed kinetics of onset of the action of ATXII and, independently, by Barhanin et al. (1981) to account for an anemone toxin that bound to the channel without producing any physiological effect.

Since the reversal of the toxin action recovers in a concentration-dependent mode, net dissociation must account for the loss of modified channels. The apparent K_D increases as a continuous function of voltage from +20 to +100 mV, and is related to the fraction of toxin-modified channels, y , by the equation $K_D = T(1 - y)/y$ (see Results). "Effective" equilibrium dissociation constants saturate at 15 nM for $E_m < +20$ mV, and 1.8 μ M for $E_m \geq +100$ mV. If the membrane potential directly affected the toxin binding reaction, rather than the receptor conformation, then the toxin affinity would fall to zero at sufficiently positive potentials. The fact that this does not occur (see Figs. 6 and 9) implies that K_D values in the intermediate range represent a distribution of toxin receptors between at least two forms, one of high affinity ($K_D = 15$ nM) and one of low affinity ($K_D = 1.8 \mu$ M).

We assume here, as Schmidt-mayer et al. (1982) did, that a toxin-bound channel has the same gating behavior regardless of whether it is in the C_1T or C_2T conformation. (By our assay, this means that its ability to conduct current, at 8 ms after a standard test pulse, is modified identically by bound toxin.) If C_1T were less effective than C_2T , then functional reversal could happen unaccom-

panied by dissociation. However, such a process does not describe the voltage-dependent effects we are characterizing.

The reversible conversion of C_1T to C_2T has major consequences for the equilibrium and rate relationships that describe the population of α -toxin-modified channels. The total toxin occupancy of channels at equilibrium is given by:

$$Y = \frac{(C_1T) + (C_2T)}{\bar{R}} = \frac{[T](K_2 + 1)}{[T] + [T]K_2 + K_2K_1}, \quad (3)$$

where \bar{R} is the total channel number and the equilibrium constants for the two steps are defined as $K_2 = k_{-2}/k_{+2}$; $K_1 = k_{-1}/k_{+1}$.

Thus, K_2 is a factor modifying the apparent toxin affinity. Since the apparent K_D varies with potential from 15 nM to 1.8 μ M, K_2 must correspondingly increase some 120-fold to accomplish the voltage-dependent reversal.

Effective rate constants are also modulated by step 2. The conversion of C_1T to C_2T lowers the availability of the rapidly dissociating form of the toxin-channel complex and thus modifies the reversibility of the toxin modification. The true dissociation rate constant, k_{-1} , is thus reduced by a factor of K_2 , the distribution constant between high- and low-affinity forms. The measured time constant for toxin binding will be: $\tau_{app} = (k_1[T] + k_{-1}K_2)^{-1}$, and the apparent equilibrium dissociation constant will be: $K_D = K_1K_2 = (k_{-1}/k_{+1}) K_2$.

By measuring the recovery times for modified currents at two toxin concentrations, we derived apparent rate constants for $k_{+1} = 1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = 1.6 \times 10^{-3} \text{ s}^{-1}$ for the resting node (see Results). The apparent time constant for the reversal of modified currents (τ_{rev}) is strongly voltage dependent, and at $E_m = +120 \text{ mV}$ has a value of 0.11 s (derived from semilog plots of the data in Fig. 5). Using an effective $K_D = 1.8 \mu\text{M}$ for the conditions of reversal, we also calculated the apparent rate constants at very large potentials: $k_{+1} = 4.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = 8.2 \text{ s}^{-1}$. Thus, the apparent dissociation rate constant is increased by 5,000-fold and the association rate constant by 30-fold at +120 mV relative to their values at rest. The net change in their ratio, 170, is about equal to that calculated for the change in K_2 that accounts for the shift in the apparent equilibrium constant, 120.

The apparent rate constants for the recovery from voltage-dependent reversal at rest are close to the values calculated for the initial appearance of the effect when α LQIIa toxin is first added to the nodal bathing solution. The time constant for that effect is $\sim 5 \text{ min}$ when the toxin is at 15 nM, its effective K_D (Wang and Strichartz, 1985), which gives values of $k_{+1} = 1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} (= K_D k_{+1}) = 1.6 \times 10^{-3} \text{ s}^{-1}$. Apparently, the functional rebinding of toxin after voltage reversal is kinetically equivalent to the binding from the nodal bath, with no additional sources, sinks, or barriers to diffusion.

If the recovery of toxin-modified currents represents binding of free toxin, then dissociation times should be calculable from the apparent rate constants. In the absence of free toxin, channel modification should disappear at rest with a time constant of 10 min ($= 1/k_{-1}^{app}$). Were there no high-affinity state coupled to the rapidly dissociative complex, then reversal at high potentials would occur in

0.12 s, as $k_{1}^{pp} \geq 8.2 \text{ s}^{-1}$. Neither of these rates was observed under these respective conditions, however, and the actual reversal of toxin action was far slower. In resting or infrequently stimulated fibers that had repeatedly been perfused, the effect persisted in Ringer for several hours. Furthermore, recovery after voltage-dependent reversal occurred despite the absence of free toxin. In order to explain these results, we postulate that the density of residual, nonspecifically bound *Leiurus* toxin molecules remains high near the nodal membrane, even after extensive washing. This toxin, loosely bound and present in amounts that greatly exceed the number of receptors, is readily available to the channels. Accordingly, the τ_{off} measurement, which usually assumes a sudden decrease in the toxin concentration after washing, is not a valid measure of the true dissociation of toxin from the receptor. Although the kinetics of the toxin-channel interaction in the node deviate significantly from the free toxin-channel binding that would occur in a homogeneous solution, the final K_D is likely to represent the true equilibrium affinity for the receptor, since the limitations on the rates are due to a buffer of ligand near the receptor, which should not affect the thermodynamics of equilibrium binding. The free toxin concentration at the actual binding site may also be altered, but this is impossible to assess in the myelinated nerve from our data alone.

The Voltage Dependence of Leiurus Toxin Binding Corresponds to Modified Fast Inactivation Processes

Several lines of evidence indicate that the rapid voltage-dependent dissociation in *Leiurus* toxin is related to the fast inactivation processes of modified channels. First, the binding of *Leiurus* toxin to the Na channel results in specific modification of Na channel inactivation and has no effects on activation; similarly, its voltage-dependent dissociation restores inactivation, again with no effect on activation. Second, the large depolarizations that most effectively reverse the action of toxin on subsequently measured currents are accompanied by a concomitant rapid decline in their own maintained current, which shows that, at the highest voltages, channels close, probably by inactivating, before toxin dissociates. Other α -toxins, having different voltage-dependent binding, behave similarly; they elicit specific effects on Na channel inactivation, but not activation. Again, the time course of the declining phase of the Na current at large depolarizations ($E_m = +100 \text{ mV}$) is faster than that at less positive potentials ($E_m = +60 \text{ mV}$) in these toxin-treated nodes (data not shown), as if the inactivation process occurred with a voltage dependence similar to that for the reversal of toxin binding.

A mechanism based on previously described kinetic modifications (Wang and Strichartz, 1985) accounts for the correlation between toxin dissociation and channel inactivation, and provides certain quantitative predictions (Fig. 11). Like the scheme suggested originally by Mozhayeva and Naumov (1980) and by Meves et al. (1984), our scheme proposes that depolarizing conditioning pulses accelerate inactivation and cause dissociation of the toxin-channel complex. It is an adaptation of the modulated-receptor hypothesis, originally proposed to account for the effects of local anesthetics on Na currents (Hille, 1977).

Na channels are specified in four states, resting (R), open (O), and two

inactivated states (I_1 and I_2). The inactivated states lie in sequence; I_1 is reversibly convertible to O and irreversibly converted to I_2 . Bound α -toxins inhibit the I_1 -to- I_2 reaction, resulting in slowed and incomplete inactivation and in an absence of the delay in repriming the R state after depolarization (Wang and Strichartz, 1985; see Chiu, 1977). The resting, open, and first inactivated (I_1) states have a relatively high affinity for α -toxin, but I_2 , destabilized itself by the toxins, has a reciprocally lower affinity, reflected in both the higher effective K_D and the relatively large dissociation rate constant. (The O and I_1 states may have a lower affinity for T , but toxin dissociation from them is too slow to occur measurably during the brief depolarizations used in this study. Therefore, the modified activation reaction $R \cdot T \rightarrow O \cdot T$ is kinetically indistinguishable from $R \rightarrow O$ and all open states can be considered as one; activation steps are thus enclosed in a domain of effective high affinity, indicated by the dashed line.) By

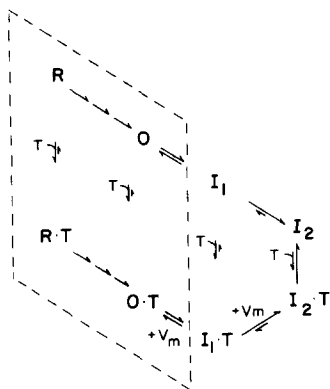


FIGURE 11. A simple kinetic scheme to describe the voltage-dependent reversal of α -toxins by state-dependent affinities. Toxin binding reactions are represented by the vertical transitions, and gating processes by the horizontal and oblique transitions. The arrows, within these separate categories, are scaled to be proportional to the reaction rates reported here and in another article (Wang and Strichartz, 1985) for α LQIIa = 200 nM and $E_m = +100$ mV. See text for details.

analogy with the reaction scheme introduced previously in this Discussion, C_1T corresponds to the second inactivated state, and C_2T to the high-affinity resting and open states. Despite the low probability of I_2 in toxin-modified channels, the $I_1T \rightarrow I_2T$ transition retains its modest voltage dependence such that sufficiently large potentials can force channels into this inactivated state, overcoming the action of the toxin and leading to its dissociation.

Balancing the free-energy relationships around this scheme provides a quantitative accounting of the changes in toxin affinity and the inactivation equilibria:

$$\Delta G_{(R \cdot T \rightarrow I_2 \cdot T)} - \Delta G_{(R \rightarrow I_2)} = \Delta G_{(R \rightarrow R \cdot T)} - \Delta G_{(I_2 \rightarrow I_2 \cdot T)}. \quad (4)$$

The left-hand side of this equation equals the difference between the free energies of the resting-inactivated equilibria for toxin-bound and normal channels; the right-hand side of the equation equals the difference in free energy of

toxin binding between the R and I states. Since $\Delta G' = -RT \ln K_D$, the change in K_D from 15 nM at rest (binding to R) to 1.8 μM at large depolarizations (binding to I_2) corresponds to a difference in the binding energy of 2.68 kcal/mol, or 114 meV/mol.

The free energy for the inactivation reaction is the sum of nonelectrical (W_o) and electrical (W_e) terms and is related to the fraction of channels in the inactivated state through Boltzmann's equation:

$$I/R = \exp(-\Delta G_{R \rightarrow I_2}/RT); \quad (5)$$

$$I/(I + R) = \frac{1}{1 + \exp[(W_o - W_e)/RT]}; \quad (6)$$

$$I/(I + R) = \frac{1}{1 + \exp[(W_o - z_i e E)/RT]}, \quad (7)$$

where z_i is the equivalent valence for inactivation gating and e is the unitary electron charge. Toxin binding causes an increase in the energy for the inactivation transition, expressed as the midpoint potential of inactivation, \bar{E} . Half of the channels are in the inactivated form when the electrical energy equals the nonelectrical energy; ergo, when channels are bound by toxin, a greater electrical energy is required to balance out the additional nonelectrical term that stabilizes the noninactivated state. This additional electrical energy is equal to the product of the difference between midpoint potentials ($\Delta \bar{E}$) times the equivalent charge for channel inactivation, $z_i e$. In our unmodified nodes, \bar{E} is -80 mV (Wang and Strichartz, 1985), whereas the value of \bar{E} corresponding to inactivation (absence of maintained I_{Na}) of half of the αLQIIa -modified channels is 67 mV (Fig. 6). Therefore, assuming that z_i is unchanged by toxin, the electrical free-energy difference per mole equals $\Delta \bar{E} z_i e = +147 z_i$ meV. Returning to Eq. 4 and equating changes in inactivation energy to the difference in energy of toxin binding gives $z_i = 114e \text{ meV}/147 \text{ meV} = 0.78$ electron charge equivalents. A charge of <1 electron thus corresponds to the voltage-dependent component of Na channel inactivation that accompanies αLQIIa dissociation.

The identical calculation can be performed for the rapid reversal of the αCSIVa toxin. For this ligand, the low-affinity K_D equals 4.3 μM (15% modified channels at 0.77 μM toxin; Fig. 9) and the high-affinity K_D equals 200–300 nM (unpublished observation). Thus, the difference in binding energies equals 1.48–1.72 kcal/mol or 63–73 meV/mol. The corresponding shift in inactivation midpoint potential is between -80 and -27 mV ($\Delta \bar{E} = 53$ mV), yielding an equivalent inactivation valence of 0.78–0.90 electron equivalents, a value near that calculated from the actions of *Leiurus* α -toxin.

How does this value for z_i compare to calculations of the equivalent charge for the state change that drives the reversal of toxin binding or to the charge equivalent of normal inactivation gating? The maximum slope of the toxin-dissociation curve against conditioning voltages (Fig. 6), is 15 mV, giving an equivalent valence of 1.6. The slope of normal inactivation against membrane potential (h_∞) has a somewhat higher value in frog node, giving an apparent

equivalent charge of 3–4; however, much of this voltage dependence originates in the activation process, which, in other excitable membranes, is kinetically coupled to the inactivation reaction (Goldman and Schauf, 1972; Bezanilla and Armstrong, 1977; Patlak and Horn, 1982). A closer agreement is found in recent experiments measuring the true activation gating charge in squid axons, which used $0.6e$, the maximum equivalent inactivation charge, to model the true voltage dependence of gating charge (Stimers et al., 1985).

Biophysical Mechanisms for Toxin Reversal

The interaction between an α -toxin molecule and its binding site is reciprocal: the toxin restricts the conformational changes of inactivation through binding at an external site, but voltage can force these conformational changes and thus modulate the binding affinity of the toxin. A model in which voltage acts directly on the binding reaction, for example by electrostatically raising the positively charged toxin's energy at the binding site, is incompatible with the saturation of both equilibrium and kinetic parameters of voltage-dependent reversal. The overall binding reactions of different toxins reverse at different potentials and have different dissociation rates because the individual α -toxins bind differently to the site, but all show this reciprocal action to some degree.

Meves et al. (1984) proposed a similar interpretation in their study on the *Centruroides* α -toxin (their toxin V). They suggested that a depolarizing conditioning pulse accelerates inactivation and causes dissociation of the toxin-receptor complex. On the other hand, in their view a depolarizing conditioning pulse does not cause dissociation of ATXII from the Na channel. Our results, obtained with a different procedure, indicate that ATXII does dissociate from the channel, but only under the influence of strong, prolonged, repetitive depolarizations applied at high frequency. This is probably because of the high concentration of ATXII required in the experiments, which effected a very rapid reassociation of toxin-receptor complex during the interpulse and prevented a true equilibrium of toxin dissociation from being achieved.

*Relationship to Biochemical Studies of *Leiurus* α -Toxin Binding*

Catterall and his colleagues have previously demonstrated a strong voltage dependence of the binding of radiolabeled α -toxin from *Leiurus*, measured directly by biochemical methods (Catterall, 1977, 1979; Ray et al., 1978). This dependence on membrane potential corresponds closely to that of the Na activation parameter, in both frog muscle and neuroblastoma cells (Catterall, 1979). The large discrepancy between this voltage-dependent binding and the one we report here may be due to several factors. Different tissues were used for the biochemical studies and their responses to scorpion α -toxin may differ from that of amphibian node. In frog muscle, for example, the inactivation process is slowed somewhat by toxin but still reaches completion after 10 ms at -24 mV (Catterall, 1979); this contrasts with the prolonged steady state currents seen in α -toxin-treated nodes and is consistent with the open (activated) state of the channel having a low affinity for the toxin. A second difference is apparent in the time courses of the dissociation reaction. Even in the most depolarized

neuroblastoma cells ($E_m = 0$ to -5 mV in 135 mM KCl; Catterall et al., 1976), the toxin binding reversed by half in no less than 4–5 min, whereas the reversal times we measured at $+20$ to $+40$ mV were no longer than 300 ms (cf. Figs. 6 and 7). However, we never depolarized nodes to potentials of <0 mV for periods longer than several seconds (in order to avoid slow inactivation) and we would not have seen such slow reversal reactions. Dissociation of bound toxin from open channels in node may well occur, albeit much more slowly than from inactivated channels. Under conditions of prolonged depolarization, the open state probability would be altered by the toxin, and the scheme we have proposed (Fig. 11) would have to be modified to show this change. Then the strongly voltage-dependent activation transitions would dominate the modulation of toxin affinity, and the true equilibrium toxin binding would mirror the voltage dependence of that transition, but with an offset appropriate to the energy of toxin binding as described above. Curiously, no such offset appears in the voltage-dependent binding of toxin to frog muscle, synaptosomes, and neuroblastoma cells.

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