

# Kinetic Analysis of the Action of *Leiurus* Scorpion $\alpha$ -Toxin on Ionic Currents in Myelinated Nerve

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**ABSTRACT** The effects of a neurotoxin, purified from the venom of the scorpion *Leiurus quinquestriatus*, on the ionic currents of toad single myelinated fibers were studied under voltage-clamp conditions. Unlike previous investigations using crude scorpion venom, purified *Leiurus* toxin II $\alpha$  at high concentrations (200–400 nM) did not affect the K currents, nor did it reduce the peak Na current in the early stages of treatment. The activation of the Na channel was unaffected by the toxin, the activation time course remained unchanged, and the peak Na current vs. voltage relationship was not altered. In contrast, Na channel inactivation was considerably slowed and became incomplete. As a result, a steady state Na current was maintained during prolonged depolarizations of several seconds. These steady state Na currents had a different voltage dependence from peak Na currents and appeared to result from the opening of previously inactivated Na channels. The opening kinetics of the steady state current were exponential and had rates  $\sim$ 100-fold slower than the normal activation processes described for transitions from the resting state to the open state. In addition, the dependence of the peak Na current on the potential of preceding conditioning pulses was also dramatically altered by toxin treatment; this parameter reached a minimal value near a membrane potential of  $-50$  mV and then increased continuously to a "plateau" value at potentials greater than  $+50$  mV. The amplitude of this plateau was dependent on toxin concentration, reaching a maximum value equal to  $\sim$ 50% of the peak current; voltage-dependent reversal of the toxin's action limits the amplitude of the plateauing effect. The measured plateau effect was half-maximum at a toxin concentration of 12 nM, a value quite similar to the concentration producing half of the maximum slowing of Na channel inactivation. The results of Hill plots for these actions suggest that one toxin molecule binds to one Na channel. Thus, the binding of a single toxin molecule probably both produces the steady state currents and slows the Na channel inactivation. We propose that *Leiurus* toxin inhibits the conversion of the open state to inactivated states in a voltage-dependent manner, and thereby permits a fraction of the total Na permeability to remain at membrane potentials where inactivation is normally complete.

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## INTRODUCTION

The scorpion venom of *Leiurus quinquestriatus* strongly affects the excitability of various nerve and muscle preparations. Koppenhofer and Schmidt (1968*a, b*) found that the venom slowed the Na channel inactivation process in myelinated fibers and, at large depolarizations, produced incomplete Na channel inactivation, a finding later confirmed by Schmitt and Schmidt (1972), Conti et al. (1976), and Nonner (1979). In addition, both peak Na and K currents were reduced by the venom. Recently, Gillespie and Meves (1980) studied the effect of the same venom on the Na currents of the squid (*Loligo forbesi*) giant axon. They reported that the venom markedly increased the maintained, noninactivating Na conductance, which rose with increasing depolarization from a minimum at  $-20$  mV to a maximum at  $+40$  mV. However, they detected no significant slowing of the Na channel inactivation kinetics. These authors concluded that the maintained conductance is either due to the "reopening" of the inactivated Na channel during depolarization or to a separate population of Na channels.

Using a purified *Leiurus* toxin, Catterall (1979) showed in one figure that the toxin slowed the Na channel inactivation in frog skeletal muscle, but did not prevent channels from eventually inactivating. We are also interested in using purified *Leiurus* toxin to study its individual effects on the ionic currents of single myelinated nerve fibers. We reasoned that this approach would allow us to determine whether a single toxin can reproduce all or only some of the effects produced by crude venom and whether the steady state Na currents originate from a separate population of Na channels or from a reopening of previously inactivated Na channels. In this report, we describe a kinetic analysis of the toxin's actions on ionic currents to address such questions. In a subsequent paper, we will report the dynamic interactions between the toxin molecule and the Na channel, particularly at the very positive potential range.

## METHODS

*Leiurus Toxin Preparation*

*Leiurus quinquestriatus* scorpion venom was purchased from Sigma Chemical Co., St. Louis, MO. The crude venom was dissolved in 5 mM ammonium acetate, pH 7.0, for 2 h at  $4^{\circ}\text{C}$  and the insoluble material was centrifuged at  $35,000 g$  for 10 min. The *Leiurus* toxin II $\alpha$  (LQII $\alpha$ ) was purified from the supernatant by sequential chromatography on two cation exchangers (BioRex-70 column [Bio-Rad Corp., Richmond, CA] followed by CM-52 column). The details of the purification methods have been described previously (Wang and Strichartz, 1983). Judging from the elution profile, isoelectric point, and molecular weight, we believe that this toxin is identical to that purified by Catterall (1976). Purified toxin was dissolved in distilled water at 0.2–1.0 mg/ml and kept at  $-20^{\circ}\text{C}$  for a period of more than 1 yr, during which time no significant loss of toxin activity was detected.

*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, but series resistance compen-

sation was not employed in our measurement. 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 microcomputer (Horizon 2, North Star Computers, San Leandro, CA). The currents were calibrated by dividing the internodal ohmic potential by an assumed internodal resistance of 20 M $\Omega$ . 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. To remove K current, the internodes were cut in 0.12 M CsCl, unless otherwise noted. LQII $\alpha$  was dissolved in 2 ml TEA-Ringer containing 12 mM tetraethylammonium chloride (TEA Cl), 110 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 5 mM HEPES buffer, pH 7.2, and 1 mg bovine serum albumin (BSA)/ml solution. BSA was included in the solution to reduce the nonspecific adsorption of toxin by the nerve fiber, vaseline, and the nerve chamber. Without BSA, at least twice the concentration of toxin was required to give the same response as when BSA was present.

K currents were measured when the Na currents were blocked by 1  $\mu$ M tetrodotoxin (TTX) or saxitoxin (STX) and the internodes were cut in 0.12 M KCl. The nodes were bathed in normal Ringer without TEA Cl and the toxin was dissolved in the Ringer containing 1 mg BSA/ml solution.

TTX was obtained from Calbiochem-Behring, La Jolla, CA; STX was from the Food Research Laboratories, U.S. Food and Drug Administration, Cincinnati, OH. All other chemicals were reagent grade from commercial sources.

## RESULTS

### *General Descriptions of the Effects of LQII $\alpha$ on Na Currents*

The most obvious effect of LQII $\alpha$  on Na currents is the slowing of Na inactivation (Fig. 1). In control nerve fibers, the Na current rises quickly during channel activation and then declines to a small value within a few milliseconds because of channel inactivation, whereas in the toxin-treated nerve, the Na current declines much more slowly and a substantial amount of current is maintained, even at the end of an 80-ms pulse. Prolonged depolarization reveals that Na currents are maintained for as long as 800 ms (Fig. 1B), and that there is little further change in current amplitude between 100 ms and up to 4 s, measured at this voltage (-35 mV). The very small decay of the inward current observed in Fig. 1B between 100 and 800 ms could be due to a slow inactivation process (Fox, 1976), but probably not to Na<sup>+</sup> accumulation or depletion because the reversal potential is changed insignificantly for the maintained current (see Fig. 7A). Furthermore, in contrast to the peak Na current, the amplitude of this maintained current is not sensitive to the conditioning pulse that immediately precedes the current-measuring pulse (Fig. 1B). These phenomena are analyzed in detail below. The maintained currents determined after prolonged depolarization will be referred to in this report as steady state Na currents (or noninactivating currents). Both peak Na currents and steady state currents are blocked by STX with an apparent  $K_D$  of 1.1 nM, a value that is comparable to that of normal frog nerve fibers ( $K_D$  = 1.2 nM; Hille, 1968; Hahn and Strichartz, 1981).

The toxin has no effect on the time course of Na activation. Observed with a fast oscilloscope sweep speed, the rising phase of the Na current at any given membrane potential is the same in the control and toxin-treated nerve fibers, and the time to reach the peak Na current is not significantly changed. The Na

tail currents observed immediately after repolarization of the membrane are usually unchanged after *Leiurus* toxin treatment, but unfortunately, the settling time for our clamp is  $\sim 30\text{--}40\ \mu\text{s}$ , which prevents an accurate measurement of fast Na tail currents.

The relative peak Na permeability vs. depolarizing membrane test potential in the absence and presence of 200 nM *Leiurus* toxin is shown in Fig. 2. The two sets of data are very similar; the permeability reaches its maximum value at about +30 mV and is half this value at about -30 mV. As with the kinetics, this result

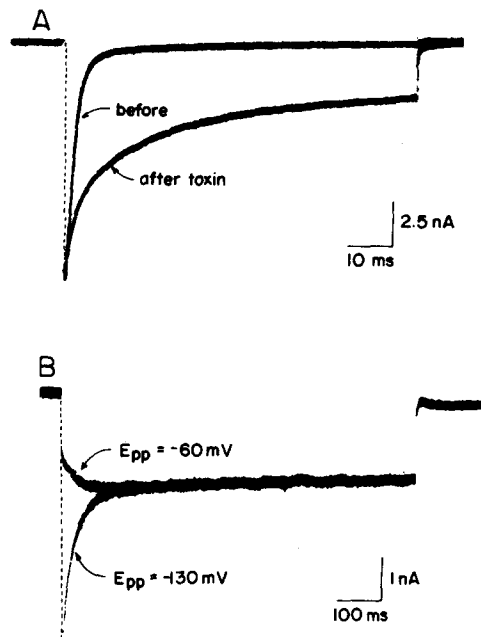


FIGURE 1. (A) Na currents recorded before and after 200 nM *Leiurus* toxin treatment for 1 min. Prepulse,  $-130\text{ mV}$ ; test pulse,  $-35\text{ mV}$ . (B) Na currents from a different nerve fiber recorded after 200 nM *Leiurus* toxin treatment for 30 min and washed with TEA-Ringer. Note the different time scales. The numbers indicated at the current traces are the amplitudes of a conditioning prepulse ( $E_{pp}$ ) of 50 ms duration; test pulse,  $-35\text{ mV}$ . Dashed lines fill in the rising phase, which was not recorded at these slow sweep speeds.

demonstrates that the activation process of the Na channel is not altered by the toxin.

In contrast to the results using crude venom, *Leiurus* toxin at concentrations of 200–400 nM does not affect the K currents in any way. In three separate experiments, the peak K current-voltage relationships were nearly identical. The kinetics of K currents also remain the same after the toxin treatment. In the remaining sections, we will describe in detail the effects of LQII $\alpha$  on Na channel inactivation at various toxin concentrations and will investigate the possible origins of the steady state Na currents.

*Effects of Toxin Concentrations on Na Current*

Families of Na currents, activated at different test potentials, were studied as a function of the LQII $\alpha$  toxin concentration. Fig. 3 shows that at all the concentrations tested, there was a small increase of the amplitude of peak Na currents, although the average change from many experiments shows no significant difference from control amplitudes (Wang and Strichartz, 1983). The peak Na currents normally deteriorate slowly over the course of experiments; however, this rundown varied considerably from fiber to fiber and was not studied systematically. The reversal potential of all TTX-sensitive currents remains unchanged (between 60 and 65 mV in this node), which indicates that the ion selectivity of Na channels for Na and K ions is not altered by this toxin (Hille,

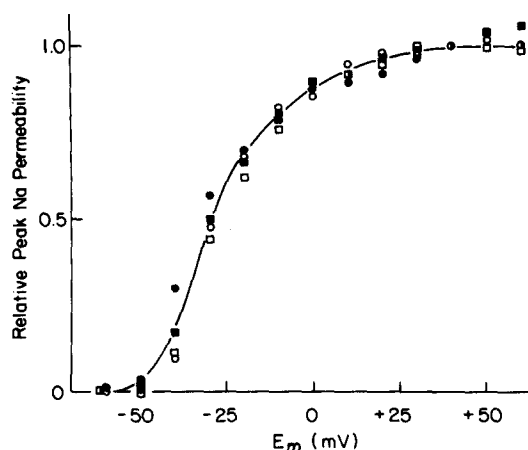


FIGURE 2. Relative Na permeabilities at various membrane potentials. Na peak currents were measured before (open symbols) and after (closed symbols) the treatment by 200 nM *Leiurus* toxin for 25 min in two separate experiments (circles or squares). The Na permeabilities were calculated according to the Goldman-Hodgkin-Katz equation and normalized to the value at  $E_m = 40$  mV. The line is drawn to fit all the data by eye. The average  $E_m$  at half relative peak Na permeability is  $-31.8 \pm 4.4$  mV (mean  $\pm$  SD,  $n = 4$ ) for control and  $-33.4 \pm 4.5$  mV (mean  $\pm$  SD,  $n = 4$ ) for toxin-treated fibers.

1972). In two experiments, the ion selectivity of Na channels was also measured by replacing all Na<sup>+</sup> in the Ringer with Li ions. The maximum peak inward current in Li<sup>+</sup>-Ringer was ~73% of that in Na<sup>+</sup>-Ringer, both in the control and the *Leiurus* toxin-treated nodes. The shift in the reversal potential by Li<sup>+</sup> replacement was <5 mV.

The effect of *Leiurus* toxin on the inactivation time course appeared at all the membrane potentials tested (even at +100 mV) and was saturated at a toxin concentration near 140 nM (Fig. 4). No further slowing of inactivation beyond that shown in Fig. 3D was detected when the toxin was increased to 400–800 nM. The toxin effects appeared rapidly at high concentrations and usually reached their final values within  $\leq 2$  min after LQII $\alpha$  addition. At low and

moderate concentrations (<30 nM), the toxin effects took ~5–10 min to reach steady state and were constant thereafter.

The effect of toxin on the Na inactivation could not be reversed in toxin-free solution alone. Extensive washing of the node with TEA-Ringer after the incubation did not reverse any of the effects during the course of study, which typically lasted from 60 to 90 min. However, during large conditioning depolarizations (approximately +100 mV), the effects of this toxin were very rapidly reversed ( $t_{1/2} < 100$  ms), but they recovered to their preconditioned levels in 1–2 min when the membrane was repolarized (Wang and Strichartz, 1984; see also

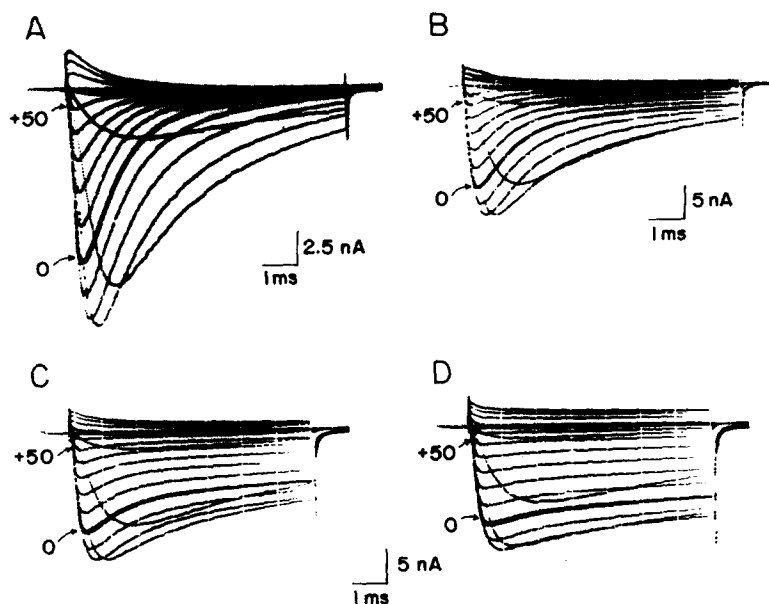


FIGURE 3. Na current families recorded before (A) and after (B–D) treatment by different *Leiurus* toxin concentrations. The toxin concentrations were 6.8 (B), 34 (C), and 136 (D) nM. Numbers indicated in current traces are corresponding membrane potentials. Note that the inactivation kinetics of both inward and outward currents are slowed and incomplete.

Mozhayeva et al., 1979, for *Buthus* toxins). Since the recovery was slowed and less complete when the node was perfused continuously with toxin-free Ringer, we believe that a toxin molecule actually dissociates from its binding site on the channel during the conditioning depolarization. The subsequent recovery occurs when the channel binds a toxin molecule from a nearby source. This source sink acts as a major buffer for toxin molecules, and its presence prevents the direct measurement of rate constants for the binding and dissociation of these scorpion toxins to nodal Na channels.

Depending on the binding capacity of this source sink and on its distance from the channel binding site, it could modulate the toxin concentration at the

receptor and thereby influence the dose-response relationship and the apparent  $K_D$ . Since we do not know the spatial relationship between this toxin buffer and the nodal membrane, we should regard any measured  $K_D$  values as representing only apparent and not true binding affinities for the toxin to the channel.

In order to quantitate the dependence of the slowing of Na inactivation on toxin concentration, we chose to measure the amplitude of the Na currents at the end of an 8-ms pulse. In untreated nodes, almost all currents have completely inactivated at this time for the test voltages we studied ( $E_m = 0$  mV; see Fig. 3A). We assumed initially that a toxin molecule interacts with an Na channel to reduce its probability of closing by inactivation and that the relative current present at 8 ms is proportional to the fraction of channels that are modified by the toxin. However, the decay of currents in the presence of LQII $\alpha$  has complex kinetics, with changes in both amplitudes and rate constants, and in choosing to quantify

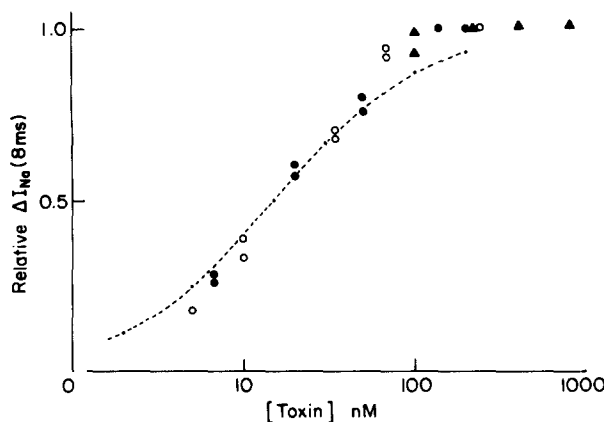


FIGURE 4. Dose-response curve for the effects on Na channel inactivation at various toxin concentrations. The increase in current amplitude,  $\Delta I_{Na}$ , measured at the end of an 8-ms test pulse to  $E_m = 0$  mV was normalized by the maximum value of the increase at saturating toxin concentrations of 140–800 nM. The dashed line is the dose-response relation, assuming that one *Leiurus* toxin binds to one Na channel with a  $K_D$  of 15 nM to produce the measured effect.

these effects by measuring the currents at 8 ms, we are admittedly simplifying this complex response.

The increase of the amplitude of the Na currents measured at 0 mV and 8 ms was normalized to the maximum amplitude, produced by the highest toxin concentration. The resulting dose-response curves are shown in Fig. 4; the half-maximal response occurs at  $\sim 15$  nM. The results of this analysis imply that there is a saturable, high-affinity receptor site for LQII $\alpha$  on Na channels that upon toxin binding is responsible for changing Na channel inactivation. The Hill plot from data like those of Fig. 4 has a slope value of  $1.3 \pm 0.1$  ( $\pm$  SD, three separate experiments), a value slightly larger than that for a one-toxin/one-Na-channel relationship. Fitting the curve of Fig. 4 with the assumption of one-toxin/one-

channel binding and with a  $K_D$  of 15 nM shows that the response at the low concentration is smaller than expected (Fig. 4, dashed line). One explanation for this smaller response at low toxin concentrations invokes the nonspecific adsorption of toxin to the chamber, to the nerve preparation, and to the vaseline seals. The dose-response results obtained from experiments done in the absence of BSA suggest that nonspecific toxin adsorption is indeed significant (see Methods). Thus, a fraction of toxin will be removed from the solution and the free toxin concentration will be lower than that actually applied, particularly at lower toxin concentrations. Such an effect will both artifactually elevate the apparent  $K_D$  and increase the molecularity calculated from Hill plots. Another possible explanation

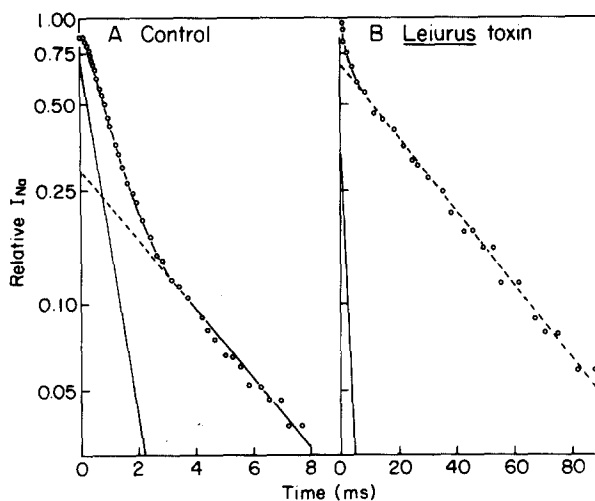


FIGURE 5. Time course of Na channel inactivation, at  $E_m = 0$  mV, before (A) and after (B) *Leiurus* toxin treatment (136 nM). The y-axis was calculated according to  $I_{Na}(t)/I_{Na}(\max)$ , where  $I_{Na}(\max)$  was estimated by extrapolating arbitrarily to the time that yielded the peak Na current. Steady state Na currents in *Leiurus* toxin-treated fibers were subtracted from the total currents to give  $I_{Na}(t)$ . The relative amplitudes of Na currents and inactivation time constants are shown in Table I. The lines are drawn to fit the two-exponential time courses resolved by eye.  $E_{pp} = -130$  mV for 50 ms.

for the lesser effect at low toxin concentrations is that the response measured is not directly proportional to the number of channels occupied by toxin. A more direct measurement, as taken by Okamoto et al. (1977), is to assay the fraction of Na current that is converted from the fast to the slow inactivating mode. Unfortunately, this method is not applicable to nodes because of the presence of multiple components of current decay, even after treatment by saturating concentrations of toxin (see the following section).

#### *Effects of Leiurus Toxin on the Inactivation Kinetics*

Na inactivation processes at all membrane potentials appear to be slowed by *Leiurus* toxin. Amphibian myelinated axons have two phases of inactivation, one



fast, one slow (Chiu, 1977; Neumcke et al., 1980, Nonner, 1980; Ochs et al., 1981). By comparing the time constants of the control and *Leiurus* toxin-treated fibers, we found that both the fast and slow inactivation kinetics of the inactivating current are slowed considerably over a wide range of membrane potentials (Fig. 5, A and B, Table I). The amplitude of the noninactivating current was measured at 100–140 ms, at least 2.5 times longer than the slowest time constant for inactivation processes. (With the hyperpolarizing conditioning prepulses used in these experiments, there are no inactivated Na channels before the depolarization begins, and thus no contamination of the current kinetics by channels opening slowly from initially inactivated states, as described in the following section.) The time constant for normal fast inactivation shows an apparent voltage dependence, falling, at +20 mV, to half the value measured at -20 mV, whereas the rate of slow inactivation is far less voltage dependent, if at all. This voltage dependence is preserved in the toxin-modified node, where the fast inactivation processes are slowed by a factor of ~3, on the average, and the slow ones by a factor of ~10

TABLE I  
*Na Channel Inactivation Kinetics Before and After Leiurus Toxin Treatment*

$E_m$	Control				Toxin-treated				
	$\tau_f$	$I_f$	$\tau_s$	$I_s$	$\tau_f$	$I_f$	$\tau_s$	$I_s$	$I_{steady\ state}$
mV	ms	%	ms	%	ms	%	ms	%	%
-20	1.3	54	4.0	46	3.7	18	38	59	23
-10	1.2	65	3.7	35	2.5	16	38	51	33
0	0.9	71	3.7	29	2.0	19	34	43	38
+10	0.8	78	4.1	22	2.5	25	40	34	41
+20	0.6	74	3.6	26	1.8	23	34	31	46

$E_m$ , test potential;  $\tau_f$  and  $\tau_s$ , fast and slow inactivation time constants, respectively;  $I_f$  and  $I_s$ , the percentage of the total Na current with fast inactivation ( $I_f$ ) and slow inactivation ( $I_s$ ) time courses. The fraction of the total Na current remaining after a 147-ms depolarization is shown in the last column,  $I_{steady\ state}$ . LQII $\alpha$  concentration, 136 nM.

(Table I). Although it appears that the slower inactivation process is slowed by a greater factor than is the faster inactivation process, the contribution to the slow process by channels that would normally inactivate rapidly cannot be quantitatively assessed. Results similar to those in Table I were found in four other experiments in the absence and presence of *Leiurus* toxin at saturating concentrations. The inactivation time course was not always fit closely by two exponentials, and errors could be present both in the extrapolated initial amplitudes and in the estimated time constants. However, we are confident about the qualitative conclusions of this analysis, for we can directly observe the reversibility of these changes in the inactivation kinetics when a strong depolarizing conditioning pulse is used to dissociate the toxin from its functional receptor (Wang and Strichartz, 1984).

Another interesting aspect of this analysis is that in the presence of *Leiurus* toxin, the relative amplitude of the fast inactivating component decreases dramatically from that of the control value, whereas the amplitude of the slow one

increases (Fig. 5, Table I). These changes of amplitude, along with those of the time constants (Table I), indicate that *Leiurus* toxin perturbs the state transitions that underlie Na channel inactivation of amphibian fibers in a complex manner. The results are distinctly different from those in squid axon (using unpurified venom, Gillespie and Meves, 1980) or tunicate eggs (Okamoto et al., 1977) and skeletal muscle (Catterall, 1979). In the first case, the venom appears to have no effect on the time course of remaining inactivation but only increases the steady state current; in the second and third cases, the toxin simply slows one single inactivation step without inducing a steady state current. Amphibian nodal currents suffer both a slowing and an incompleteness of inactivation.

#### *Kinetics of the Steady State Na Current*

One possible explanation for the existence of the steady state Na current during prolonged depolarization is that the inactivated Na channels reopen during this period, as suggested previously by Gillespie and Meves (1980). We found that the steady state current amplitudes are insensitive to "conditioning pulses," which precede the current-measuring test pulse and which establish the degree of inactivation in normal fibers (Fig. 1B). However, the Na currents during depolarization show multiphasic kinetics, and these depend on the prepulse potential in an unusual way. Fig. 6A shows typical current kinetics for a toxin-treated node. The pattern of membrane potentials includes a constant test pulse to +15 mV preceded by a conditioning prepulse to either -125 mV, which positions all the Na channels in the resting, activatable state, or to less negative potentials, up to +75 mV, which normally inactivates an increasing fraction of Na channels. A brief polarization to the holding potential (-100 mV for 1 ms) between the conditioning pulse and the test pulse was inserted to allow the activation process to resettle before the application of the test pulse, since some Na channels still are conducting at the very end of the conditioning pulse. With a depolarizing prepulse, the Na current during the test pulse is clearly multiphasic (Fig. 6A, traces *b* and *c*). The fast turn-on phase is indistinguishable from the normal activation time course when it is resolved at a high oscilloscope sweep speed. This fast component probably represents the fraction of Na channels that can be activated from the resting state and that follow normal activation kinetics. The current then decreases as these channels are slowly inactivated from the open state. However, the decline is usually small since it is soon dominated by another rising phase of Na current (see Fig. 6B, trace *b*). This slow turn-on phase of Na current rises to a steady state following a single exponential with a characteristic time of 22.4 ms (Fig. 6C,  $t_{1/2} = 15.5$  ms), which is much slower than that of the normal, nonexponential activation ( $t_{1/2} = 0.14$  ms at  $E_m = 15$  mV). The slow appearance of the steady state currents probably results from an opening of Na channels from their inactivated state, which was populated during the conditioning pulse. Similar kinetics are observed after different conditioning pulses. With a -5-mV conditioning pulse, there is a substantial steady state current already present at the end of 50 ms (Fig. 6A, trace *c*); however, the multiphasic appearance of Na current during the test pulse is still apparent. The slowly activated component of the Na current has the largest amplitude after prepulses

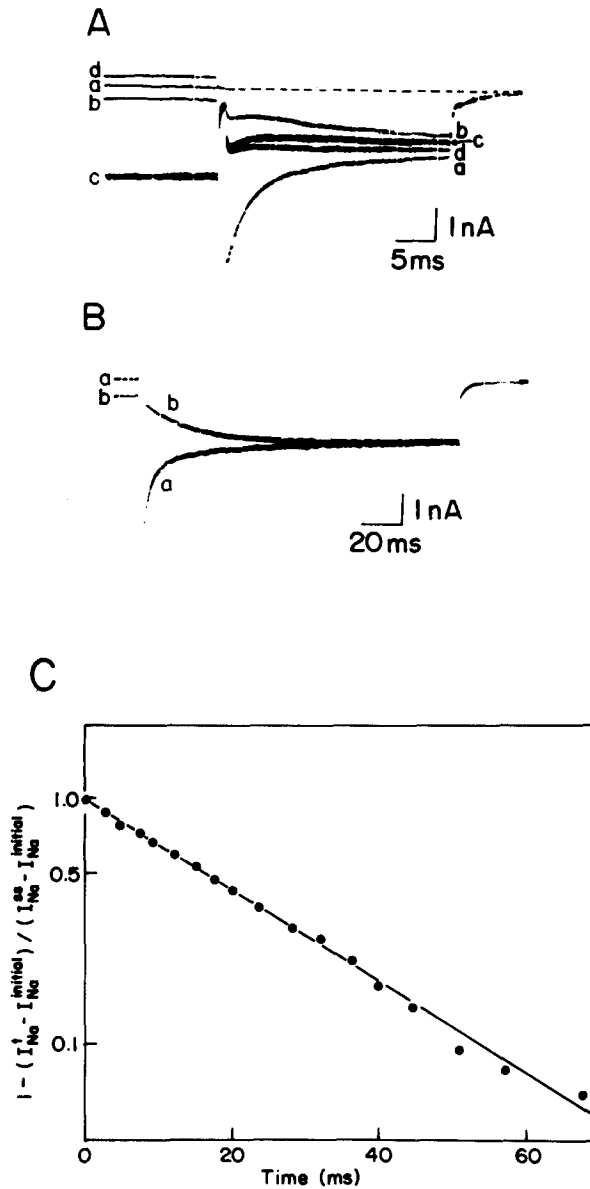


FIGURE 6. Kinetics of noninactivating Na currents. (A) Na current traces at  $E_{test} = 15$  mV after different conditioning prepulses to:  $-125$  (a),  $-45$  (b),  $-5$  (c), and  $+75$  (d) mV for 100 ms. The conditioning pulse was separated from the test pulse by a 1-ms repolarization to  $-100$  mV to “reset” the activation process. The current traces at the end of the conditioning prepulses are also shown. (B) The conditions were similar to those in A except that the sweep speeds were slower and only two current traces (a, b) are shown. (C) The rising phase of the steady state current was analyzed from current trace b in B. The half-time to reach 50% of the steady state Na current was 15.5 ms. Toxin concentration, 200 nM.

of intermediate voltage ( $-45$  to  $+15$  mV). More negative prepulses predispose Na channels to the resting state, from which they activate rapidly (Fig. 6A, trace *a*), and the most positive prepulses reduce the size of the slowly activated current, which implies that in toxin-modified nerves very large depolarizations do not effectively populate the inactivated states from which slow activation occurs. (This behavior is the opposite of that observed for the inactivation of normal channels, and occurs for two reasons: first, many channels are already in the

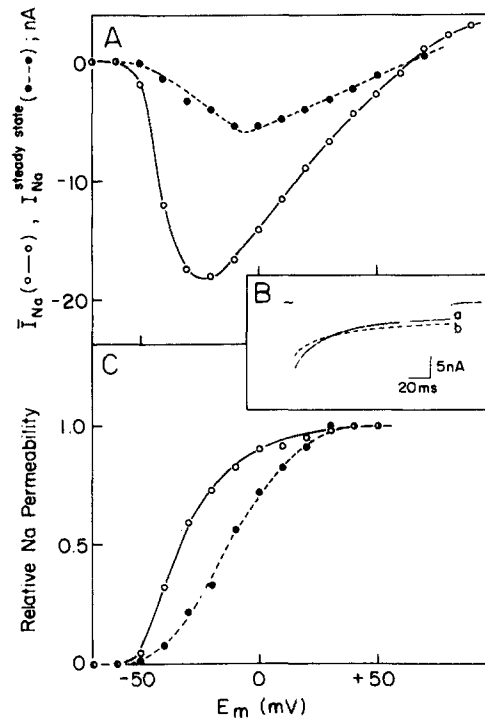


FIGURE 7. (A) The peak Na current (○) and the steady state Na current (●) amplitudes were measured at various membrane potentials in the presence of 136 nM *Leiurus* toxin. The steady state Na current amplitude was measured at 147 ms of the test pulse. (B) The cross-over of the peak and the steady state Na current traces at 0 mV (trace *b*) and  $-20$  mV (trace *a*) test potentials.  $E_{pp} = -130$  mV for 50 ms. (C) The relative peak (○) and steady state (●) Na permeability were calculated and normalized to the value at  $E_m = +40$  mV.

activated state at  $+75$  mV and cannot contribute additional conductance at the  $+15$  mV test pulse; and second, such large conditioning depolarizations reverse the binding of the toxin and thereby reduce the amplitude of currents with modified kinetics [see below and Fig. 9].) All of the slowly rising current traces eventually approach the same steady state level, as shown in Fig. 6B, which indicates that, regardless of history, the same final equilibria define the same fraction of noninactivating channels.

*Amplitude of the Steady State Na Currents Depends on the Test Pulse Potential*

Both the steady state current amplitude and the peak current amplitude as a function of various test membrane potentials are shown in Fig. 7A. The inward steady state current reaches its maximum value near  $-10$  mV, whereas the peak Na current reaches its near  $-30$  mV. Both peak and steady state currents, however, have the same reversal potential, in this case  $+65$  mV. The ratio of the steady state current to the peak Na current amplitude increases from 12% at  $-40$  mV to up to 50% at  $+40$  mV, which corresponds to a shift in the voltage dependence of Na permeability (Fig. 7C). The peak Na permeability reaches its half-maximal value around  $-33$  mV, whereas the steady state permeability reaches its at around  $-13$  mV. Fig. 7B illustrates this fact kinetically: the peak current at  $-20$  mV is larger than that at  $0$  mV, and vice versa for the steady state current; the current traces cross over after  $\sim 40$  ms of depolarization.

Altogether, these results demonstrate that the slow activation reactions producing the steady state Na current are energetically distinct from those processes producing the normal peak Na currents, which implies that the channel opens from different precursor states, namely, a normally inactivated state and a resting state, respectively. The character of "inactivated" channels is modified greatly by the toxin, as evidenced both by their ability to reopen, and by their altered response to conditioning pulses, described next.

*Effect of Conditioning Pulses on the Peak Na Current*

The relative amplitudes of peak Na currents in amphibian nodes of Ranvier normally decline monotonically with increasingly positive prepulse potentials, as channels are converted to an inactivated state from which they cannot directly open (Dodge and Frankenhaeuser, 1958). However, this relationship is changed dramatically by *Leiurus* toxin. The peak Na currents of the control and *Leiurus* toxin-treated fibers at one test potential and several prepulse potentials are shown in Fig. 8, which also includes the pulse pattern for testing this function. The relative peak Na currents are plotted against their respective prepulse potentials in Fig. 9. In control nerve fibers, almost all Na channels are inactivated by a prepulse of  $-50$  mV for 50 ms (Fig. 9A, open circles). In contrast, in fibers exposed to *Leiurus* toxin (136 nM), a significant fraction of Na channels is still activatable at the end of an identical prepulse (Fig. 9A). This fraction increases continuously with depolarizing prepulse potentials until it reaches an apparent plateau value around  $+50$  mV.

The channels that are still activatable after large depolarizing prepulses show a prepulse voltage dependence that is very similar to the voltage dependence of the steady state current amplitude (Fig. 9B, solid line), which suggests that the origin of this component is similar to that of the current maintained at the end of the conditioning pulse. It is noteworthy that the noninactivated channels close with normal, fast kinetics when the membrane is repolarized and are reactivated with normal kinetics upon subsequent depolarization (Fig. 8, B and C).

The measurements in Figs. 8 and 9 do not truly represent  $h_{\infty}$  values in a conventional manner. They are contaminated by a secondary effect of long

depolarizations to potentials higher than +20 mV, the functional reversal of the effects of the toxin (Wang and Strichartz, 1984). To limit this reversal, we chose a conditioning pulse having a constant 50-ms duration. If we take the liberty of correcting for the reduction of steady state current caused by toxin dissociation, the corresponding relative  $I_{Na}$  values in Fig. 9A for 136 nM *Leiurus* toxin will be ~0.67, 0.75, and 0.9 for conditioning pulses to +50, +70, and +100 mV, respectively (Fig. 9A, X's). These corrected data reveal that the apparent voltage dependence of Na channel inactivation is inverted by the toxin—i.e., the higher

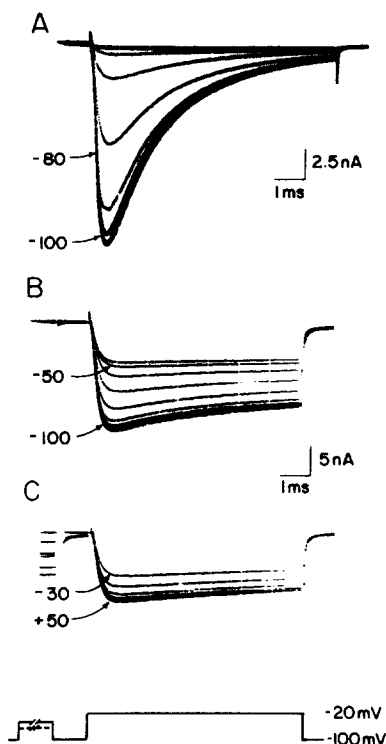


FIGURE 8. Na current traces at various conditioning prepulses before (A) and after (B and C) 136 nM *Leiurus* toxin treatment. The pulse programs are shown at the bottom. Note that in B and C, a 1-ms gap was used since significant Na currents at the end of the 50-ms conditioning pulse are present, shown clearly on the left-hand side of C. Numbers indicated are prepulse potentials in millivolts.

the voltage, the smaller the extent of inactivation—and that this inverted voltage dependence is a continuous function of potential such that, at sufficiently positive voltages, all channels will be poised in a recruitable, noninactivated state.

The amplitude of the noninactivatable current is dependent on the concentration of *Leiurus* toxin under the conditions described in Fig. 8. Higher concentrations of *Leiurus* toxin produce a larger fraction of noninactivatable channels, but this reaches a saturating value of 40–60% of the peak current at ~140 nM toxin concentration (Fig. 9A). The maximal noninactivating component, measured at

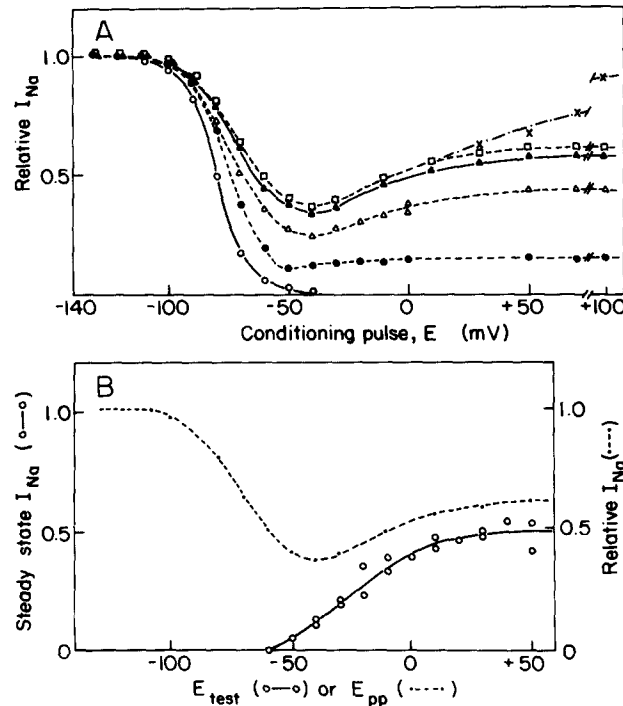


FIGURE 9. (A) The relative peak Na currents after various conditioning prepulses. The relative peak Na current amplitudes shown in Fig. 8 (A-C) were normalized by the maximum peak Na current amplitude in each panel and plotted against their conditioning prepulse potential.  $\circ$ , control nerve fiber;  $\square$ , *Leiurus* toxin at 136 nM;  $\blacktriangle$ , toxin at 68 nM;  $\triangle$ , toxin at 34 nM;  $\bullet$ , toxin at 6.8 nM;  $\times$ 's represent currents adjusted for the voltage-dependent reversal of toxin action at various conditioning potentials (see text). Adjustment was made using rates of reversal in nodes treated with a high concentration of toxin (200 nM), so the corrections represent a lower limit for this phenomenon, although no reversal was detectable during depolarizations more negative than +20 mV. (B) Fractions of the steady state currents at various membrane potentials and their relationship to the relative peak Na currents at various conditioning pulses. The open circles plot the relative steady state Na current measured as the ratio of the Na current at 147 ms to the peak Na current, at each test pulse. *Leiurus* toxin concentration, 136 nM. The dashed line represents the relative peak Na currents after various conditioning prepulses, and is taken from A ( $\square$ ).

$E_m = +50$  mV, is plotted against the *Leiurus* toxin concentration in Fig. 10. From this result it is obvious that both the slowing of inactivation (Figs. 3 and 4) and the amplitude of the noninactivating component (Figs. 9 and 10) are saturated by *Leiurus* toxin with a half-maximal response at 12–15 nM, which is consistent with the interpretation that the same binding site is responsible for both effects. On this basis, we disfavor the notion that the steady state conductance is due to a separate population of Na channels.

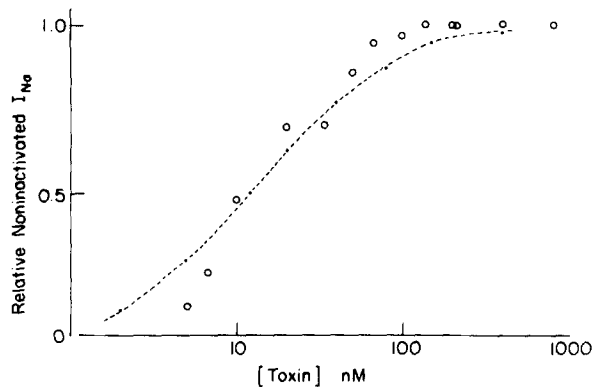


FIGURE 10. Dose-response curve for noninactivatable channels modified by LQII $\alpha$ . The amplitudes of peak  $I_{Na}$ , for  $E_{pp} = +50$  mV, were normalized to the values at a *Leiurus* toxin concentration of 200 nM and plotted against various *Leiurus* toxin concentrations. Data were obtained from experiments exactly as in Fig. 9A. The dashed line is the dose-response relation assuming that one *Leiurus* toxin binds to one Na channel with a  $K_D$  of 12 nM to produce the measured change in relative peak  $I_{Na}$ .

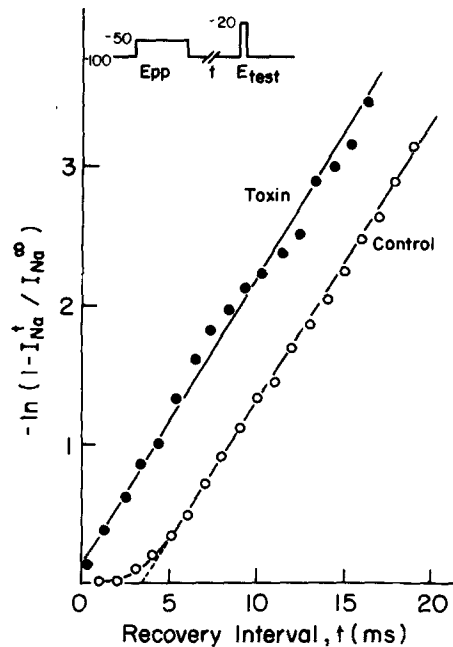


FIGURE 11. Recovery of Na channels from inactivation after membrane repolarization. The growth of Na currents after 50-ms depolarizations in a normal (O) and 200 nM toxin-treated (●) nerve fiber is graphed. The fraction of peak  $I_{Na}$  at various recovery times was normalized by peak current of  $I_{Na}^{\infty}$ , measured at 50 ms recovery time, and plotted logarithmically against time after repolarization. Note that in the *Leiurus* toxin-treated nerve fiber, the delay of 3.5 ms is absent. The pulse program is shown at the top of the figure. Numbers indicated are membrane potentials in millivolts.



*Recovery of Na Channels from Inactivation*

Inactivated Na channels return to their resting, activatable state after membrane repolarization. This transition in the amphibian node of Ranvier is characterized by a single exponential following a delay, as described previously by Chiu (1977). The recovery kinetics are modified by *Leiurus* toxin (Fig. 11). In the *Leiurus* toxin-treated nerve fibers, the normal delay observed in control fibers is absent; instead, a small, noninactivatable current appears immediately ( $<0.1$  ms) after membrane repolarization. The remaining inactivation is removed exponentially with a time constant of  $\tau_R = 4.7$  ms, which is similar to that of the control ( $\tau_R = 5$  ms). Thus, in the presence of *Leiurus* toxin, the inactivated Na channels can re-equilibrate to the resting state after repolarization with the normal rate but with no delay.

## DISCUSSION

In this report, we have demonstrated that purified *Leiurus* toxin (LQII $\alpha$ ) acts specifically on Na channel inactivation. We found no significant effects of this toxin on peak Na current amplitudes, on the Na activation time course, or on the shape of the peak Na current-voltage relationship, which shows that the normal activation process of all Na channels is unchanged. K channels were also unaffected.

Na channel inactivation in amphibian myelinated nerve fibers is complicated and proceeds in multiple steps. Two or more inactivated states have been postulated (Chiu, 1977; Nonner, 1980; Sigworth, 1981), and different inactivation schemes have been suggested. Also unsettled is the possibility of multiple open states or even multiple types of Na channels present in a single node (e.g., Ochs et al., 1981; Sigworth, 1981; Benoit et al., 1985). Because of this complexity, in this discussion we will focus on two of the kinetic schemes selected from a broad spectrum of descriptions of inactivation in the node. This discussion by no means excludes other models, such as the model proposed by Chandler and Meves (1970) and Gillespie and Meves (1980) (see below), who suggested that inactivated channels in squid axons can reopen to a new distinct open state.

*Action of Leiurus Toxin on Na Channel Inactivation in the Node*

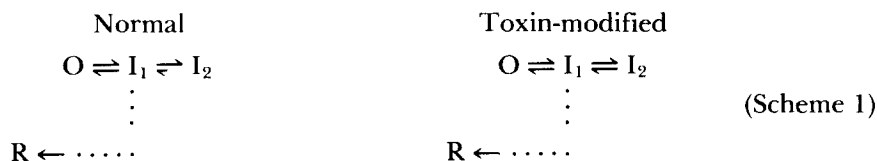
Our results show that the Na conductance declines much more slowly in the presence of the toxin than in its absence. In addition, a steady state Na current appears during prolonged depolarizations. This steady state Na current, although it has a very similar sensitivity to STX and TTX, differs from the peak Na current in its voltage dependence and in the time course of appearance. The simplest explanation for the noninactivating steady state currents is that some of the channels that open during the initial depolarization maintain a finite probability of being open during a prolonged depolarization. After subtracting the steady state Na current, the inactivation time course usually still can be fitted by two exponentials. Both the fast and the slow time constants are altered by the toxin. The relative amplitude of the fast component decreases dramatically, while the relative amplitude of the slow component increases. These results are similar to those reported by Mozhayeva et al. (1980), using a scorpion toxin purified

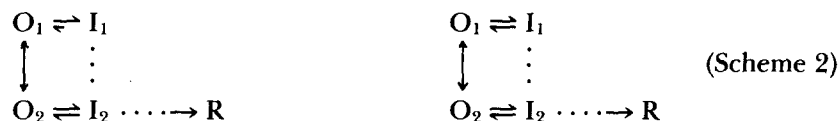
from *Buthus eupeus*. These authors, however, proposed that the faster component of decay arises from the normal inactivation of unmodified Na channels, whereas the slower component and the steady state current represent their toxin-modified counterparts. This is probably a valid analysis at the potential they analyzed ( $E_m = +80$  mV), since the bound toxin molecules appear to dissociate from the channels at this potential (Mozhayeva et al., 1979, 1980). However, over the membrane potential range at which we chose to analyze inactivation ( $E_m = -20$  to  $+20$  mV), we have observed no dissociation of *Leiurus* toxin (Wang and Strichartz, 1984), but the fast inactivation component persists, even at saturating toxin concentrations. Furthermore, even the fastest-decaying current was slowed by toxin treatment and no normal fast inactivation processes were ever detectable at high toxin concentrations. Our results, therefore, are inconsistent with the notion that the fast inactivation component represents the behavior of unmodified Na channels in the *Leiurus* toxin-treated node.

Since the normal inactivation process does not "grade" the Na channel conductance (Conti et al., 1976; Patlak and Horn, 1982), but apparently acts in an all-or-none fashion, we interpret these results to support the notion that discrete, multiple inactivated states are present both in the control and in the toxin-treated nerve fibers and that it is the free energy levels of these states and their transitions that are altered by the toxin (cf. Nonner, 1980).

#### *Kinetic Models for Toxin-modified Na Channels*

Any model of Na channel kinetics in toxin-modified axons must account for three observations: (a) the slowing of the normal inactivation process, (b) the appearance of a slowly activated steady state Na current, with a saturating, positive voltage dependence (cf. Fig. 9), and (c) the absence of the normal delay in the recovery from Na channel inactivation, with little change in the rate constant for this process. In this discussion, we consider the qualitative predictions from simple modifications of two previously published kinetic schemes for Na inactivation. Scheme 1 illustrates Chiu's sequential three-state model (Chiu, 1977). In this model, the toxin-induced changes of inactivation can be accounted for by increasing the free energies of the first and second inactivated states, as well as the transition energies for the  $O \rightarrow I_1$  and, in particular, the  $I_1 \rightarrow I_2$  reactions. These altered energetics will selectively populate O at the expense of the inactivated channels, whose formation is also slowed. (The first-order rate constants for the transitions among the O,  $I_1$ , and  $I_2$  states do not equal the time constants of current decay [inactivation], since these measured values and their corresponding amplitudes are functions of all the relaxation processes.) For example, Schmidtmayer (1985) found that the effects of an  $\alpha$ -type toxin, *Ane- monia sulcata* toxin II, on Na channel inactivation can be simulated adequately by Chiu's three-state model, thus demonstrating the validity of this general scheme for these toxin-modified kinetics.





Under normal conditions of depolarization, the second inactivation process in Scheme 1 is essentially irreversible, for  $I_2$  is an absorbing, nonconducting state, and after a long pulse most channels will be in  $I_2$ . However, the  $I_2$  state can reverse to  $I_1$  in depolarized, toxin-modified channels. Recovery from inactivation corresponds to the repopulation of resting states, R, after repolarization (dotted line, Scheme 1), a process that normally has a delay as  $I_2$  converts to  $I_1$ , but has no delay after toxin exposure, presumably because less of the  $I_2$  state is formed and this is converted to  $I_1$  relatively rapidly. The slow rise of steady state  $I_{Na}$  corresponds to the sequential, voltage-dependent conversion of  $I_2$  to  $I_1$  and thence to O. In theory, this voltage dependence may be derived from two sources: the binding of toxins to a channel may confer a novel voltage-dependent transition, or the inhibition of intrinsic reactions by toxin may reveal additional, cryptic, voltage-dependent processes. This latter possibility would correspond in Scheme 1 to the  $I_2 \rightarrow I_1$  reaction, which is normally masked by the rapid and irreversible conversion of  $I_1$  to  $I_2$  at membrane potentials larger than  $-50$  mV.

Alternatively, the toxin-induced changes can be explained by a parallel inactivation scheme, like that previously proposed to account for multiple open and inactivated states (Scheme 2; Sigworth, 1981). According to this model, the different inactivated states can be reached by two parallel routes from different open states, i.e.,  $O_1$  (open)  $\rightarrow$   $I_1$  (first inactivated) and  $O_2$  (open)  $\rightarrow$   $I_2$  (second inactivated). One irreversible inactivation reaction ( $O_1 \rightarrow I_2$ ) competes for  $O_1$  with the conversion to the second open state; inactivation of the second open state ( $O_2$ ) is somewhat reversible. For this scheme, we postulate that *Leiurus* toxin strongly inhibits the  $O_1 \rightarrow I_1$  transitions so that the  $I_1$  state is no longer a trapping state in the toxin-modified channel. The slowing of inactivation during a depolarization and the deletion of the delay in removing inactivation after repolarization (Scheme 2, dotted line) are explained as they were for Scheme 1.

The slowly activated steady state current then corresponds to either the  $I_2 \rightarrow O_2$  or  $I_1 \rightarrow O_1$  reactions. As in the explanation offered above, the positive voltage dependence of the amplitude of steady state  $I_{Na}$  may arise uniquely from toxin-modified channels or be revealed by kinetic alterations per se (e.g.,  $I_1 \rightarrow O_1$ , which is normally insignificant). With regard to this last point, we favor the second explanation because the apparent voltage dependence of current decay during inactivation is not altered by LQII $\alpha$ , but rather the rate constants are increased by relatively constant factors (Table I). Either of the two schemes presented above can account, qualitatively, for our observed results, and at this time we find no evidence to distinguish between them. We believe that future experiments using other toxins and single channel analysis may resolve a simple, unique kinetic model for modified Na channels.

#### *Comparison with Previous Results*

Although the crude venom of *Leiurus* also slows Na inactivation (Koppenhofer and Schmidt, 1968*a, b*; Schmitt and Schmidt, 1972; Nonner, 1979), there are

some marked differences between the effects of the venom and that of the purified toxin. (Toxin LQII $\alpha$  accounts for 2–4% of the total protein mass of *Leiurus* venom and is the major protein component affecting Na channels [Wang and Strichartz, 1983].) In myelinated nerve fibers, the venom reduces the maximum Na and K currents but the purified toxin has no such effect. The venom also produces an apparent shift of the  $h_{\infty}$  curve in the hyperpolarized direction, whereas no such shift was observed with the purified *Leiurus* toxin. These differences can be attributed to other neurotoxins or nonprotein contaminants that are present in the crude venom. Nevertheless, the similarities are far greater than the differences in the effects produced by the venom and the isolated toxin. The inactivation time courses, both in the venom- and toxin-treated nodes, can be fitted by two exponentials, and the maintained currents are present even after 10 s depolarization. The maintained currents in the venom-treated node also show slow turn-on kinetics, although this phenomenon was not characterized in detail previously (Koppenhofer and Schmidt, 1968*a, b*; see also Siemen and Vogel, 1983, for *Buthus* venom). Furthermore, the larger the depolarization, the larger the amplitude of the maintained currents elicited by the venom or by the toxin. These results demonstrate that the main effects of *Leiurus* venom are due to the LQII $\alpha$  toxin component.

In the squid giant axon, the crude venom has no significant effect on the time course of inactivation, but significantly increases the steady state Na conductance during a maintained depolarization. On the basis of measurements of enhanced steady state Na conductance, and of the occasional slowing of Na "tail currents" after repolarization, Gillespie and Meves (1980) proposed that a second open Na channel state exists as  $h_2$  in the kinetic scheme:  $h_1$  (open)  $\rightarrow$  inactive  $\rightarrow h_2$  (open). According to this model, the transition of inactive  $\rightarrow h_2$  (open) is favored by the venom, whereas the  $h_1$  (open)  $\leftrightarrow$  inactive transition is not altered. The reopening process differs from the initial activation process in both kinetics and voltage dependence. This conclusion is also consistent with our kinetic data, although we envision no new distinct open state (see previous discussion). With certain modifications, particularly in the number of inactivated states, this model may also explain the action of *Leiurus* toxin in the node of Ranvier.

Several purified sea anemone toxins also affect Na channel inactivation in the frog, when present in the  $10^{-6}$  M concentration range (Bergman et al., 1976; Neumcke et al., 1980; Ulbricht and Schmidt-mayer, 1981). The inactivation time courses were considerably slowed and steady state currents were present during a prolonged depolarization. In general, the fast inactivation process is affected little or not at all by these toxins, but the slow inactivation one is dramatically slowed. These primary effects of sea anemone toxins on Na channels are comparable to those of *Leiurus* toxin despite the differences in their affinities and molecular structures. The molecular mass of sea anemone toxins is 2,500–5,000 daltons, which is considerably smaller than the 7,000 daltons of most scorpion toxins (for review, see Catterall, 1980), but sea anemone toxins apparently compete with *Leiurus* toxins for a common receptor site on the Na channel (Catterall and Beress, 1978).

Recently, Warashina and Fujita (1983) reported that in crayfish axons, the time course of Na channel inactivation, which was adequately fitted with one

single exponential, was clearly slowed by a purified sea anemone toxin. They also observed that less inactivation occurred at the more positive potentials, a finding qualitatively comparable to this report, and they postulated, similarly, that inactivated Na channels can reopen at more positive potentials. From all of these similarities, it is likely that a common mechanism of action is involved after these two classes of toxins bind to Na channels of either amphibian nodes, crayfish axons, or other excitable membranes.

#### *Rates of Action and Equilibrium Affinity of Leiurus Toxin*

We examined the toxin concentration dependence of the modified Na currents in order to determine the  $K_D$  for toxin action on amphibian nodes. However, our results are qualified by two observations: the apparent equilibrium  $K_D$  is inconsistent with the extremely slow rate of reversal upon washing, and the parameter that we measured as the response (the current amplitude at 8 ms at 0 mV) may not be a direct measure of the number of modified channels. Nevertheless, we believe that the apparent  $K_D$ , so measured, is close to the true equilibrium  $K_D$  for two reasons. First, essentially the same  $K_D$  value was obtained by measuring the fraction of channels that remained activatable after a pulse of 50 ms duration to +50 mV (Fig. 10) as was obtained by measuring the fraction of conducting channels after an 8-ms depolarization to 0 mV (Fig. 4). Second, the equilibrium binding of radiolabeled *Leiurus* toxin to amphibian muscle shows a dissociation constant of 14 nM (Catterall, 1979), in close agreement with our calculated values. It seems improbable that these separate values are in merely fortuitous agreement.

Despite the apparent validity of the measured equilibrium binding, we do not believe that the observed rates of toxin-related actions reveal the true rate constants for LQII $\alpha$  binding or dissociation from functional sites on the Na channel. First-order kinetics are inconsistent with the measured rates and  $K_D$ . For example, if the time constant for the appearance of modified channels were 10 min at a toxin concentration equal to the  $K_D$ , 15 nM, then the calculated  $k_{on}$  would be  $6.6 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$ , a reasonable value (cf. Ulbricht, 1981). However, the corresponding  $k_{off}$  should be  $0.1 \text{ min}^{-1}$ , but no apparent reversal occurs over periods as long as 180 min. In preliminary experiments (Wang and Strichartz, 1984), we have shown that the effects of LQII $\alpha$  toxin are reversed by very large depolarizing conditioning pulses and then recover to their preconditioning values within 90 s when there is no free toxin in solution. We interpret this result to reveal a sink source for scorpion toxin near the node of Ranvier. The sink source must have a binding capacity, far in excess of the number of channels in one node, for it can release toxin molecules to the washed node rapidly, but is not measurably depleted over a 2–3-h period in toxin-free solution. The diffusion of toxin molecules to and away from the nodal membrane is governed by this source sink. Delays in the onset and reversal of the effects of anemone toxin in frog node have been previously reported (Ulbricht and Schmidtayer, 1981; Ulbricht, 1983), although no such lags were seen with the smaller cationic organic molecules TTX and STX (Ulbricht and Wagner, 1975; Hahin and Strichartz, 1981).

Two different structures are possible candidates for the source sink of peptide

Na channel toxins, which are basic proteins with pI values near 10. The nodal gap is filled with an anionic material, partially analyzed as sulfated glycans, which are avidly bound by a variety of metal cations, including Na ions (Langley and Landon, 1967; Landon and Langley, 1971). This gap substance fills the continuous space between the Schwann cell covering the fiber down to the nodal axolemma (Bertholt, 1978; Landon, 1982). Experiments in our laboratory show that sulfate groups on ion exchange resins bind LQII $\alpha$  extremely tightly (unpublished observation).

The other candidate structure for the sink source is the negatively charged carbohydrate moieties bound covalently to the Na channel protein itself. Analysis from the channel of *Electrophorus electricus* provides estimates of up to 100 negatively charged sialic acid residues on each individual channel peptide (Miller et al., 1983), constituting a dense cation-binding matrix located 20–30 Å from the phospholipid head groups. This matrix is perhaps more intimately associated with the channel than is the gap substance and may directly influence the binding of toxin molecules to their receptor sites.

Our analyses at best approximation suggest that one toxin binds to one Na channel to produce all the observed effects in toad node. A one-to-one relationship has been reported previously by Okamoto et al. (1977) from electrophysiological experiments on tunicate eggs, probably using the same *Leiurus* toxin, and by Mozhayeva et al. (1979) on frog nodes using purified *Buthus* toxins. Furthermore, it has also been concluded from both electrophysiological and radiolabeled toxin-binding studies that one STX or TTX molecule binds to one Na channel in axons (Hille, 1968; Levinson and Meves, 1975; Strichartz et al., 1979). Since all Na channels in frog or toad nerve are blocked by STX or TTX, by binding with high affinity to a single class of receptors, we believe that *Leiurus* toxin binds monomolecularly to the same channels in this preparation and that the stoichiometry of STX receptors to LQII $\alpha$  receptors is unity.

Appreciation is expressed to Thomas Rando for helpful discussions and to Rachel Abrams for excellent secretarial service.

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