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Purification and Physiological Characterization of Neurotoxins from Venoms of the Scorpions *Centruroides sculpturatus* and *Leiurus quinquestriatus*

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SUMMARY

Several different toxins having specific effects on the kinetics of sodium channels have been isolated from the venoms of two scorpions. A combination of two steps of ion-exchange chromatography has been used to purify these toxins, whose sizes and purities have been assayed by gel filtration, urea/sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and isoelectric focusing. The actions of the toxins and their relative potencies have been determined by studying the modifications they produce in action potential shape, using the sucrose-gap method, and in ionic current kinetics, measured under voltage-clamp, both assays performed on myelinated axons of frogs and toads. The venom of *Leiurus quinquestriatus* scorpions yielded two active neurotoxins; the major neurotoxin has a mass of approximately 7000 daltons. This major toxin affected the sodium channel inactivation process exclusively, slowing the rates of inactivation as well as preventing complete inactivation from occurring in some of the channels. Such slowed and incomplete sodium inactivation resulted in action potentials that were prolonged, from their usual duration of 5–8 msec to hundreds of milliseconds or even seconds. Five toxins were isolated from the venom of *Centruroides sculpturatus* Ewing scorpions, all of which also had masses of approximately 7000–7500 daltons. Four of these toxins acted primarily on the activation process of sodium channels, producing a novel increase in sodium permeability upon repolarization of the nerve membrane following a depolarizing pulse, as previously described for the crude venom [Cahalan, M. D. *J. Physiol. (Lond.)* **244**:511–534 (1975)]. These toxins also caused repetitive firing of action potentials in single axons in response to one stimulating pulse, as well as spontaneous impulse firing. A fifth neurotoxin from *C. sculpturatus* venom had effects similar to those of the *L. quinquestriatus* toxins, slowing and preventing complete sodium inactivation. The effect of this toxin was slowly removed during external perfusion by Ringer's solution.

INTRODUCTION

The sodium channels that are responsible for action potentials in most excitable membranes are characterized by voltage-dependent transitions among ion-conducting and nonconducting configurations. In the resting membrane the channels are distributed between a closed configuration, which can be activated to an ion-conducting or open state by membrane depolarization, and an inactivated configuration, which cannot be opened by depolarization. Channels which open during depolarization eventually assume an inactivated configuration, but will equilibrate to the closed, activatable configuration following membrane repolarization.

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The description of these configuration changes, or of channel "gating," remains largely at the phenomenological level despite their detailed characterization in the 30 years since their original designation by Hodgkin and Huxley (2). Although knowledge of the mechanisms of gating is spare, it is clear that a variety of drugs act by modifying the gating of ion channels. Among these are the toxins from scorpion venoms, which have profound effects on the inactivation and activation phenomena. These toxins bind with high affinity and very slow reversibility to sodium channels in nerve and muscle membranes. Miranda *et al.* (3), Rochat and co-workers (4), and Watt and his colleagues (5, 6) have been isolating and studying the biochemistry of scorpion toxins for many years. Recently a neurotoxin purified from the scorpion *Leiurus quinquestriatus* has been applied by Catterall in a series of experiments to potentiate the channel-activating action of other drugs (7), to localize

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sodium channels *in situ* (8), and to begin to characterize the protein subunits of the sodium channel (9).

In this paper we report the purification of isotoxins from two scorpions, and the characterization of their physiological action on excitable membranes at nodes of *Ranvier* in myelinated nerve. The neurotoxins occur in two classes, one which affects the inactivation process of sodium channels, the other which affects primarily the activation process. *L. quinquestriatus* venom contains only the first class of toxins, whereas the venom of *Centruroides sculpturatus* Ewing scorpion contains both classes. Our experiments rank the potency of the toxins and show that toxins from each class can act simultaneously on the same sodium channel.

METHODS

Sources of scorpion venoms. C. sculpturatus venom was purchased from L. Honetschlager (Mesa, Ariz.) and was a gift of Dr. W. Culp, Department of Biochemistry, Dartmouth Medical School (Hanover, N. H.). *L. quinquestriatus* venom was purchased from Sigma Chemical Company (St. Louis, Mo.).

Chemical reagents. BSA[2] was purchased from Sigma Chemical Company; CsCl and ammonium acetate from Fischer Scientific Company (Springfield, N. J.). TEA·Cl was purchased from Eastman Chemical Company (Rochester, N. Y.) and the stock was further recrystallized. Sephadex G-25 and G-50 (fine) were obtained from Pharmacia (Piscataway, N. J.). All other chemical reagents were of analytical grade from commercial sources.

Cation exchangers. Bio-Rex 70 was purchased from Bio-Rad Laboratories (Rockville Centre, N. Y.). Carboxymethyl-cellulose 52 (CM-52) was from Whatman (Clifton, N. J.). These resins were prepared and equilibrated to the desired concentration of ammonium acetate buffer as described by the manufacturer.

Protein contents. Protein contents were measured according to the method of Lowry *et al.* (10), using BSA as standard.

Sucrose-gap assay. Compound action potentials of desheathed sciatic nerves from the frog *Rana pipiens* were measured as described (11). Sucrose (0.18 M) was used to flush a 5-mm section of nerve to increase the amplitude of compound action potentials and to ensure monophasic signal. The volume of the test chamber containing the toxins was 0.5 ml. Washing was usually performed by exchanging 4 volumes of frog Ringer's solution or TEA-Ringer's solution. The compound action potentials were in the range of 30–60 mV, depending on the season and the size of the frog. The nerve was stimulated supramaximally using a 50-μsec square pulse of current at frequencies below 0.1 Hz. Stimuli were supplied from a Grass Instrument Company (Quincy, Mass.) Model S44 stimulator, and action potentials were measured and recorded on a Tektronix (Beaverton, Ore.) storage oscilloscope.

Voltage clamp of single myelinated fibers. Single fibers from sciatic nerves of toad (*Bufo marinus*) or frog (*R. pipiens*) were dissected and mounted in a Lucite chamber as described (12). The ends of the fibers were cut in 0.12 M CsCl: this isotonic solution exchanged with axoplasmic fluid and blocked the potassium channels internally (13). The volume of the test chamber (Pool A) was about 0.1 ml. The test solution of 2- to 3-ml volume was flushed through Pool A to exchange completely the solution bathing the node within 20 sec. Test solution usually contained 12 mM TEA-Cl, which blocks potassium channels extracellularly (14). All of the fibers were voltage-clamped according to the method of Dodge and Frankenhaeuser (15) at a temerature of 8°. Membrane currents were recorded on a storage oscilloscope and photographed. The values of membrane currents were calculated by assuming a longitudinal axoplasm resistance of 40 Mohm. The sizes of the currents appear to be comparable with those previously reported by Sigworth (16). The capacitive and leak currents were subtracted by using an analogue simulator with two time-constant capacitive elements (17). The bathing Ringer's solution contained 110 mM NaCl, 2.5 mM KCl, and 2.0 mM $CaCl_2$ in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.0. TEA-Ringer's solution contained, in addition, 12 mM TEA·Cl.

Purification of scorpion toxins from the venom of C. sculpturatus. C. sculpturatus crude venom was dissolved in 5 mM ammonium acetate (pH 7.0) for 2 hr at 4°. The suspension was centrifuged at 20,000 × *g* for 20 min at 4° to remove the insoluble material. The supernatant, about 20 ml, was loaded onto a Bio-Rex 70 column (1.5 × 25 cm) previously equilibrated with 10 mM ammonium acetate (pH 7.0). Proteins adsorbed to the column were eluted with a linear gradient made by mixing equal volumes (370 ml each) of 10 mM and 250 mM ammonium acetate (pH 7.0). The individual active peaks, assayed by the sucrose-gap method, were pooled, lyophilized, and resuspended in 5 ml of distilled water. This solution was loaded onto a CM-52 cellulose column (1.5 × 20 cm) equilibrated with 10 mM ammonium acetate (pH 7.0). The adsorbed proteins were eluted with a linear gradient of ammonium acetate (pH 7.0) from 10 mM to a final concentration of 400–600 mM, depending upon which peaks were applied. Active peaks were assayed, pooled, lyophilized, and resuspended in 2 ml of distilled water. Unless otherwise noted, all procedures were conducted at room temperature (20–22°). The purified scorpion toxins were stored in this condition at −20° for several months without losing their biological activities.

Purification of scorpion toxins from the venom of L. quinquestriatus. The purification procedures of the *Leiurus* scorpion toxins were similar to those for *Centruroides* toxins just described. The detailed procedures and gradient ranges are shown in the appropriate figure legends.

SDS-urea/PAGE. Acrylamide (15%) (bis/acrylamide, 0.8:30) containing 0.1% sodium lauryl sulfate and 6 M urea in 0.1 M sodium phosphate (pH 7.2) was polymerized by ammonium persulfate and TEMED. Acrylamide stacking gel (5%) was made above the resolving gel. The running buffer was 0.1 M sodium phosphate (pH 7.2)/0.1% SDS. Samples were dissolved in 1% SDS, 1% 2-mercaptoethanol, 0.01% bromphenol blue, 6 M urea, and 10 mM sodium phosphate (pH 7.2). After electrophoresis, slab gels were stained in 0.1% Coomassie blue/50% tri-

[2] The abbreviations used are: BSA, bovine serum albumin; TEA, tetraethylammonium; SDS, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylenediamine; PAGE, polyacrylamide gel electrophoresis.

chloroacetic acid for 1 hr and destained in 7% acetic acid overnight.

Isoelectric focusing of scorpion toxins. Isoelectric focusing gel electrophoresis was performed as described by Catterall (18). A mixture of 7.5% (w/v) acrylamide, 0.25% (w/v) bisacrylamide, 2.2% ampholine 9–11, and 0.4% ampholine 3–10 was polymerized by 0.03% ammonium persulfate (w/v) and 0.1% TEMED (v/v). Samples in 10% sucrose and 0.1% ampholine 9–11 were applied to the top of gels (0.3 × 10 cm) and overlayered with 5% sucrose and 0.1% ampholine 9–11. The anode buffer was 10 mM acetic acid applied to the upper reservoir, and 100 mM NaOH was the cathode buffer in the lower reservoir. The gels were run at a constant voltage of 150 V for 2–3 hr. Under these conditions cytochrome *c* migrates about 4–5 cm from the top. Gels were fixed by 10% trichloroacetic acid and the protein bands could be visualized as white precipitates. A few of the protein precipitates, however, tended to diffuse out of the gels during this treatment. Staining with Coomassie blue did not improve the resolution.

RESULTS

Purification of scorpion neurotoxins from C. sculpturatus. Lyophilized crude *C. sculpturatus* venom contained some insoluble mucus which could be removed by centrifugation. The soluble material remaining in the supernatant contained almost all of the proteins in the crude venom. More than 80% of the absorbance measured at 280 nm (A_{280}) in the supernatant could be eluted at low ionic strength from a Bio-Rex 70 column (Fig. 1A). At least four biologically active peaks eluted between 75 and 175 mM ammonium acetate were detected by the sucrose-gap assay. The physiological effects of the active peaks are described in the next section. There was some biological activity in the high-salt (1 M ammonium acetate) eluate; however, multiple small peaks were found after further resolving this fraction on a linear gradient of 0.2 M–1 M ammonium acetate. Because of the small amount of these proteins, further purification was not attempted for these high-salt fractions.

Each peak, labeled *I–IV* in Fig. 1A, was further purified in a CM-52 cellulose column. With no exception, the active component(s) from each peak (I–IV) was bound much more strongly to the CM-52 resin than to the Bio-Rex resin and was eluted at a higher salt concentration (Fig. 1B–E). It appears that, by the combination of these two cation exchangers, the neurotoxins can be separated and detected both biologically and chemically, even with only 90 mg of crude venom as starting material. In fact, the sensitivity of the sucrose-gap biological assay is far greater than that for optical detection at 280 nm (see below).

Five major active components were purified, each containing 0.3%–1.8% of the protein from the crude venom. The size of these five neurotoxins was found to be less than that of α-bungarotoxin (8000 daltons) by gel filtration (Sephadex G-25 and G-50 method; data not shown). As judged by SDS-urea/PAGE, all of the neurotoxins purified from *C. sculpturatus* have similar molecular masses, 7000–7500 daltons (Fig. 2A, Lanes 3–7). We could detect no peptides smaller than this molecular weight range.

Under isoelectric focusing gel electrophoresis conditions using ampholine 9–11, a single band was observed from each of the preparations of Iα, IIα, IIIα, and IIIβ. Centruroides toxin IVα could not be visualized under the conditions described under Methods; the reasons for this phenomenon are not clear, but perhaps it is due to the high solubility of this neurotoxin in trichloroacetic acid. The purification scheme and the percentage of each toxin derived from *C. sculpturatus* venom are presented in Table 1A.

Purification of scorpion neurotoxins from L. quinquestriatus. Procedures similar to those used for *Centruroides* purification were used for *Leiurus* toxin purification. Two active components were found after resolving the crude venom on a Bio-Rex 70 column (Fig. 3A). Peak I contained little material and was not purified further. Peak II contained about 9% of the protein from crude venom and was further resolved into two major peaks (Fig. 3B) by a CM-52 cellulose column. Only Peak IIα had strong effects on the sodium conductance of nerves. The purification scheme is shown in Table 1B. Peak IIα had a molecular mass of about 7000 daltons, as measured in SDS-urea/PAGE (Fig. 2A, Lane *1*).

A single band was visualized in acrylamide gel isoelectric focusing experiments using ampholine 9–11. The protein of *Leiurus* IIβ also migrated as a single band, slightly faster than the *Leiurus* IIα neurotoxin. According to Catterall (18), the pI for *Leiurus* IIα was about 9.8, similar to the result we observed. This protein was found to be nearly excluded from the Sephadex G-25 resin and therefore its size corresponded to at least 6000 daltons. The results of this *Leiurus* toxin purification are very similar to those reported by Catterall (18), who separated toxins on a single column (Amberlite CG-50) followed by electrophoretic analysis to assess the purity of each fraction in the active peak. Only the pure toxin fractions (i.e., non-overlapped fractions) were pooled and used by Catterall. We found that by using another cation exchanger, such as CM-52 cellulose resin, it was possible to separate the overlapped fractions (with molecular weight around 9000 daltons; see Fig. 2A, Lane *2*) after Bio-Res 70 chromatography and to achieve a higher toxin yield. About 2–4% of the crude venom protein was accounted for by the Peak IIα neurotoxin.

Effects of purified scorpion toxins on sciatic nerve. The effects of purified scorpion toxins on membrane potentials of the sciatic nerve were detected by the sucrose-gap method. Figure 4A illustrates the effects of the *Centruroides* toxin Peak Iα on the compound action potential. Changes in shape of the compound action potential appeared within 200 sec after the addition of the toxin and reached a steady state after 10–20 min (Fig. 4B). These changes consisted of prolonged action potentials with long, noisy plateaus which could last for more than 20 sec; normal compound action potentials in TEA-Ringer's solution have a duration of less than 10 msec. The effects could not be removed by extensive washing with TEA-Ringer's solution. The modified action potentials were sensitive to saxitoxin, which at 100 nM blocked 90% of the peak amplitude of the compound action potentials and markedly reduced the plateau. The modified impulses were also reduced by local anesthetics such as lidocaine. All other neurotoxins purified from *Centru-*

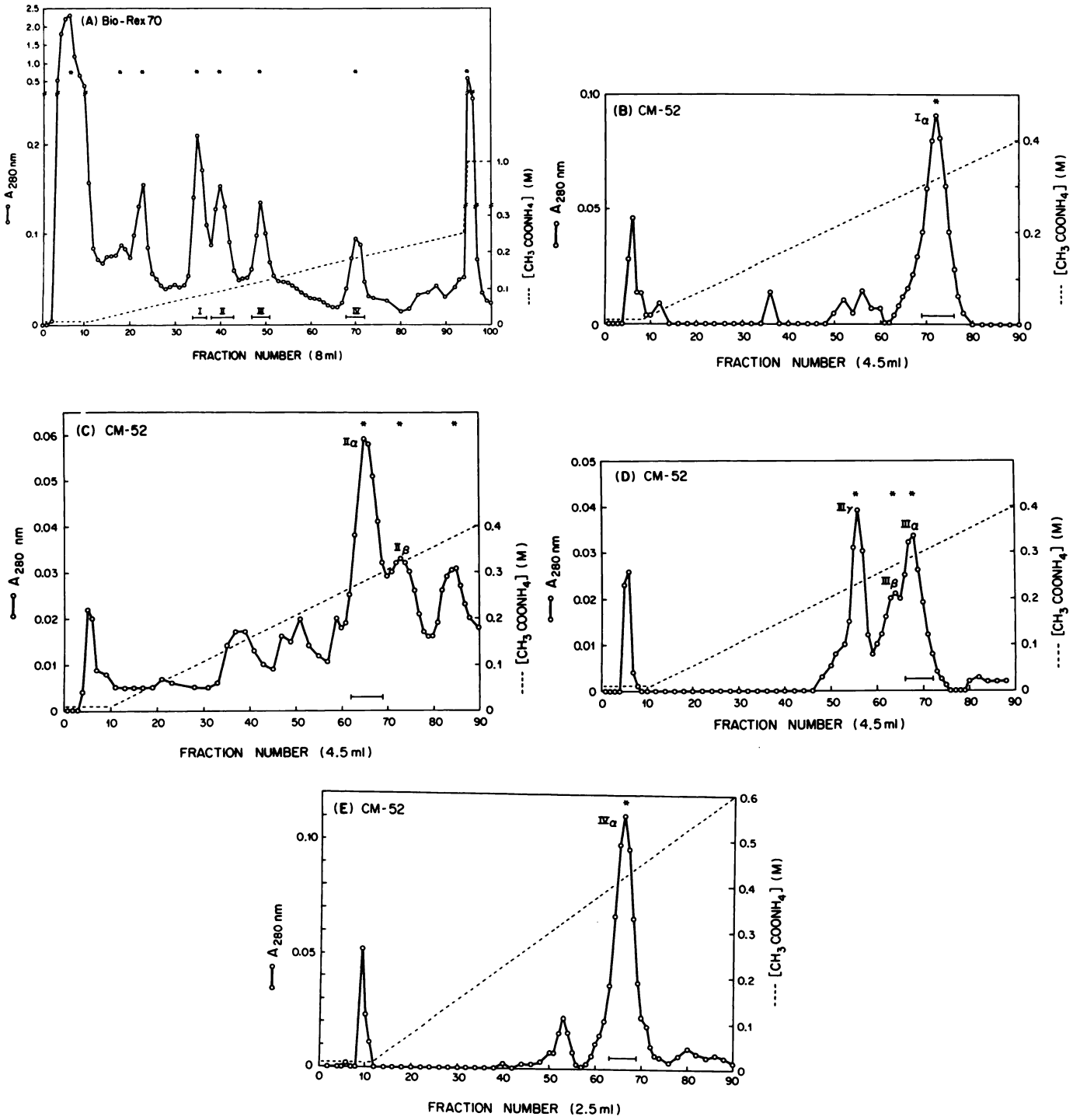


FIG. 1. *Chromatography of Centruroides sculpturatus fractions*

A. Chromatography of *C. sculpturatus* venom on Bio-Rex 70. Crude *C. sculpturatus* venom (103 mg) was extracted in 40 ml of 10 mM NH_4CH_3COO (pH 7.0) for 2 hr and centrifuged at 20,000 × *g* for 20 min. Approximately 40 ml of clear supernatant were loaded on a Bio-Rex column (1.5 × 25 cm) previously equilibrated with 10 mM NH_4CH_3COO solution. *Asterisks* indicate fractions which were bioassayed. Only the biologically active fractions are numbered (*I–IV*).

B–E. Chromatography of *C. sculpturatus* toxins on CM-52. The active fractions (Peaks *I–IV*) eluted from the Bio-Rex 70 column of A were each pooled, lyophilized, and resuspended in 5 ml of distilled water. These solutions were loaded on separate CM-52 columns (1.5 × 20 cm) equilibrated with 10 mM NH_4CH_3COO (pH 7.0). The proteins were eluted with a linear gradient of NH_4CH_3COO as indicated. As in A, fractions indicated by *asterisks* were bioassayed, and active fractions (numbered, and spanned by the horizontal bars) were again separately pooled, lyophilized, and resuspended in 2–3 ml of distilled water.

roides have physiological effects on compound action potentials similar to those of Peak Iα. These results imply that purified scorpion toxins can modify the gating of sodium channels; the detailed mechanism of each toxin was studied by the voltage-clamp method, described in the next section.

The sucrose-gap method is a very sensitive method for screening the active fractions during purification proce-

dures; as routinely performed, a 1:1000 dilution of the active fractions (corresponding to less than 0.1 μg of protein per milliliter, or less than 0.001 absorbance unit at 280 nm) can be detected by this method.

Figure 4C shows the effects of the purified *Leiurus* toxin IIα on compound action potentials. Similarly to the purified *Centruroides* toxins, the *Leiurus* toxin IIα also prolonged the compound action potentials with a long plateau which lasted from tens of seconds to minutes (in this case 2 min). Again, these toxin effects were not reversed by extensive washing with Ringer's solution. The replacement of normal Ringer by TEA-Ringer's

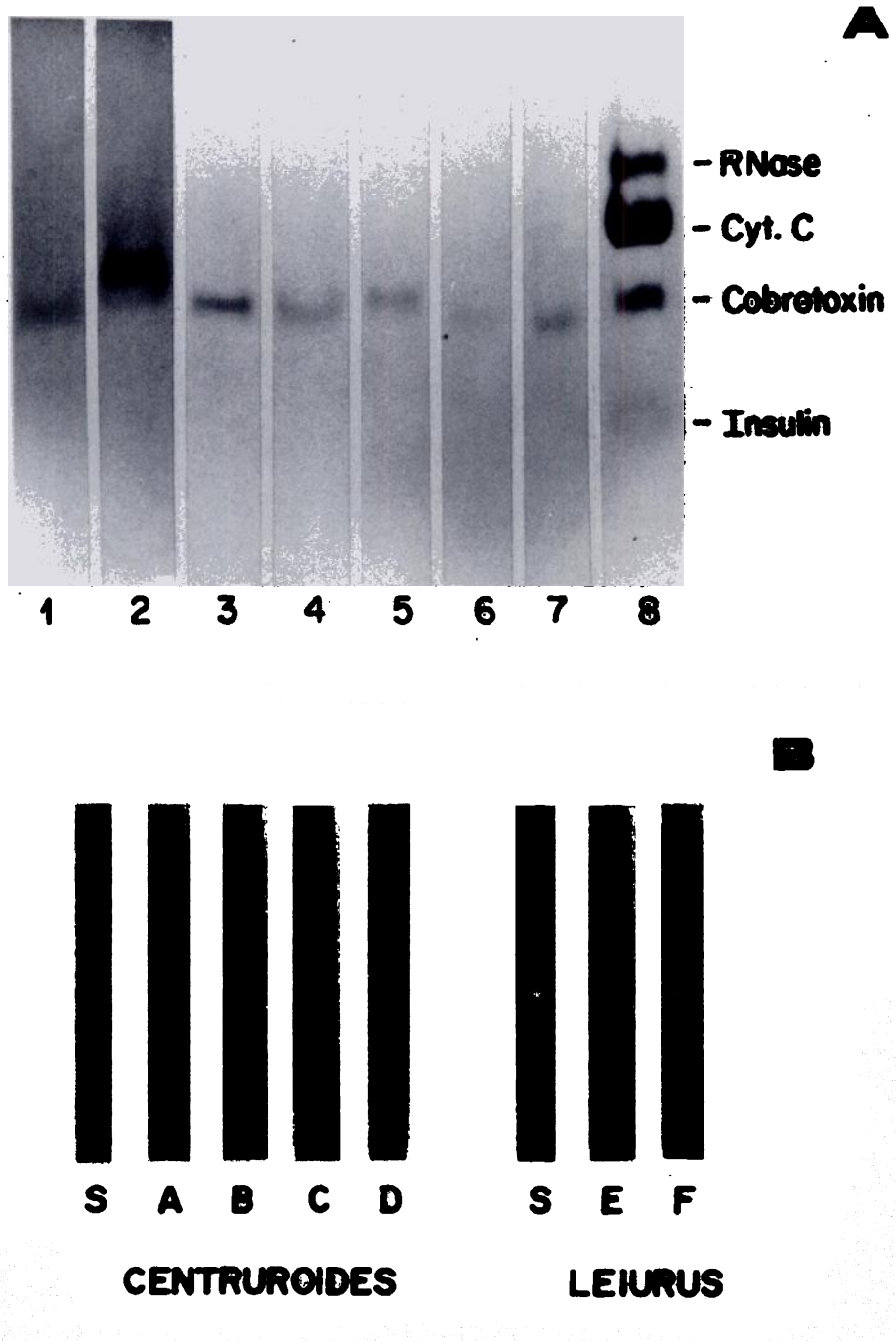


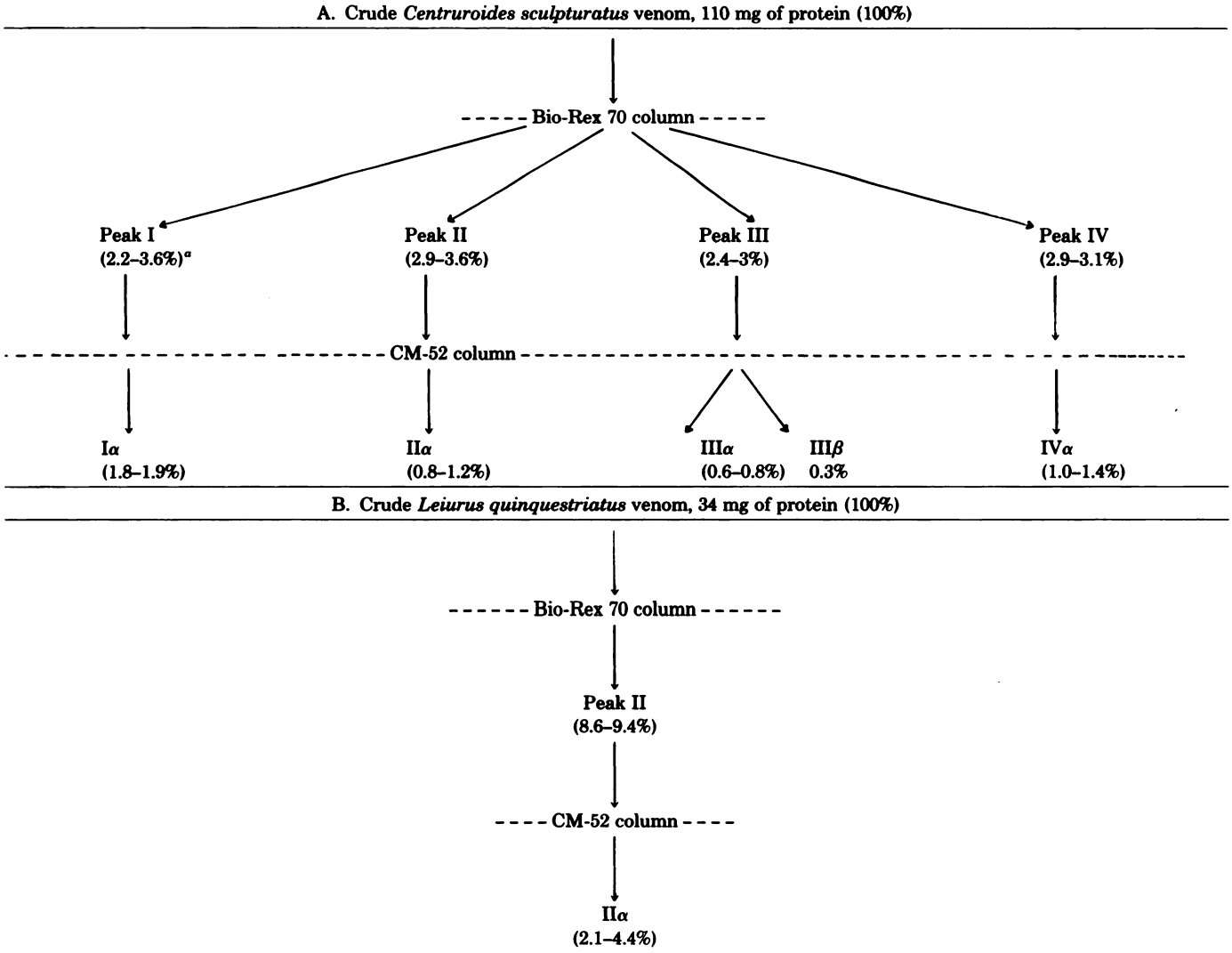
FIG. 2. *Electrophoresis of purified scorpion neurotoxins*

A. SDS/urea gel electrophoresis of purified scorpion neurotoxins; 15% polyacrylamide gels were prepared as described under Methods. Toxins and their protein content in each lane were as follows: *1*, *Leiurus* IIα, 15 μg; *2*, *Leiurus* IIβ, 35 μg; *3*, *Centruroides* IVα, 20 μg; *4*, *Centruroides* IIIα, 7 μg; *5*, *Centruroides* IIIβ, 9 μg; *6*, *Centruroides* IIα, 2 μg; *7*, *Centruroides* Iα, 10 μg; *8*, molecular weight standards (RNase = 14,300; cytochrome *c* = 12,300; cobrotoxin = 7,820; insulin = 3,000 daltons).

B. Isoelectric focusing of purified neurotoxins. Separate focusing of *Centruroides* and *Leiurus* toxins, each including cytochrome *c* as a standard (*S*). The other gels correspond to toxins (with the mass that was chromatographed) as follows: *Centruroides sculpturatus*: *A*, Iα, 40 μg; *B*, IIα, 60 μg; *C*, IIIα, 66 μg; *D*, IIIβ, 70 μg (toxin IVα could not be fixed by trichloroacetic acid and visualized in the gel). *Leiurus quinquestriatus*: *E*, IIα, 60 μg; *F*, IIβ, 40 μg.

TABLE 1

Outline of purification procedures



[a] The percentage of the original protein recovered in each toxin after the cation exchange columns are shown in parentheses. The ranges of the recovery in three separate experiments are listed.

solution potentiates the toxin's effect and prolongs the compound action potentials even more (Fig. 4D), probably by blocking the outward potassium current which normally facilitates membrane repolarization of amphibian myelinated nerve fibers. Thus, all neurotoxins from both *Centruroides* and *Leiurus* have similar effects on the compound action potential. The resolution of detailed mechanisms of action of each toxin requires the kinetic analysis of sodium currents which can be obtained by the voltage-clamp method, described next.

Effects of purified scorpion toxins on single myelinated fibers. Two different effects on action potentials from isolated single myelinated fibers were found among the five purified *Centruroides* toxins. Peaks Iα, IIα, IIIα, and IIIβ each produced a train of repetitive action potentials in response to a single stimulating pulse, followed by a noisy plateau. Figure 5B shows this effect from toxin Iα. The amplitude of the first action potential following the 300-μsec stimulus is always bigger than that of subsequent impulses; the number of action potentials generated after a single stimulus could be greater than 30. The toxins also frequently induced spontaneous trains of action potentials, as shown in Fig. 5C, in which toxin IIα was applied. This kind of effect could be detected within 3 min after the addition of toxin Iα, IIα, IIIα, and IIIβ in the presence of 12 mM TEA-Ringer's solution. TEA potentiates the ability of these toxins to produce repetitive impulse firing, by selectively blocking the potassium channels of the nodal membrane. This reduces the outward current that repolarizes the membrane and affects the potentiation in two ways, both of which are indirect. First, the action potentials are longer in TEA, providing a longer integrated depolarization, which increases the slow inward current induced by the toxins upon repolarization (see next section). Second, when potassium channels are blocked, the membrane repolarizes to a less negative value and the size of the toxin-induced current needed to reach threshold is also smaller. As with normal

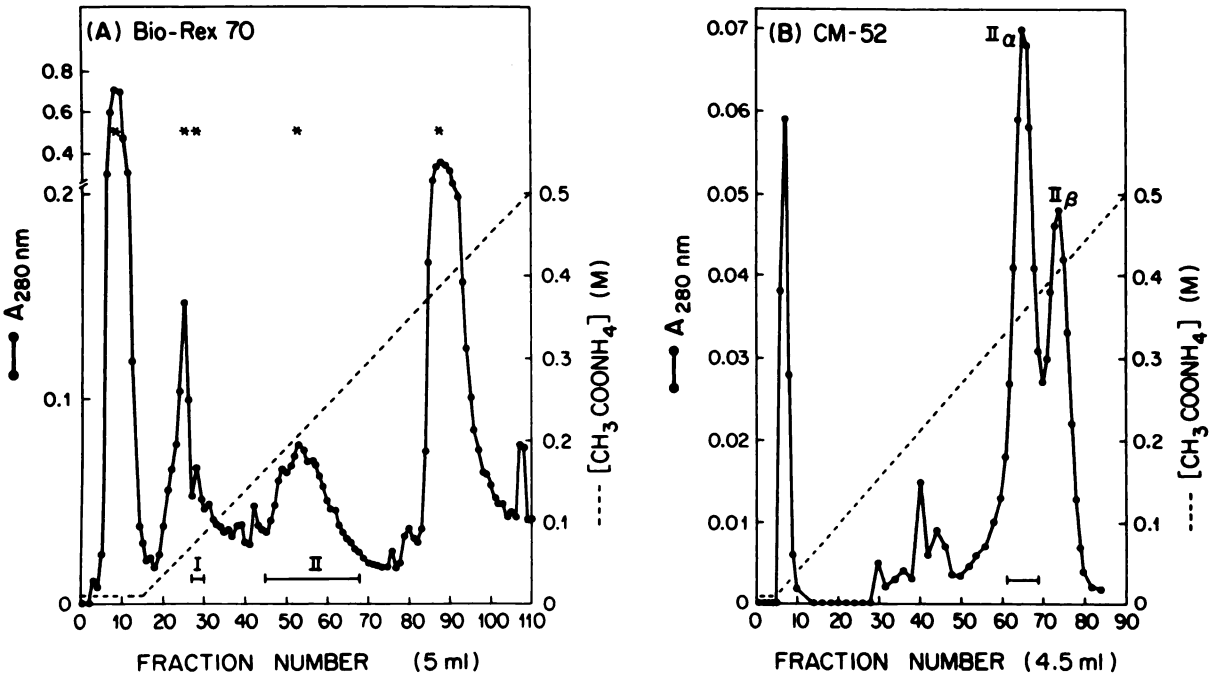


FIG. 3. *Chromatography of Leiurus quinquestriatus venom*

Chromatography of *L. quinquestriatus* venom (33 mg) on Bio-Rex 70. The venom extraction was prepared as described in Fig. 1A. Fractions of Peak II were pooled, lyophilized, resuspended in 2 ml of distilled water, and loaded onto a CM-52 column (B). Proteins were eluted from the columns by an ammonium acetate salt gradient. Active fractions of Peaks IIα and IIβ were separately pooled, lyophilized, and resuspended in distilled water. The column sizes were the same as in Fig. 1. *Asterisks* indicate fractions which were bioassayed.

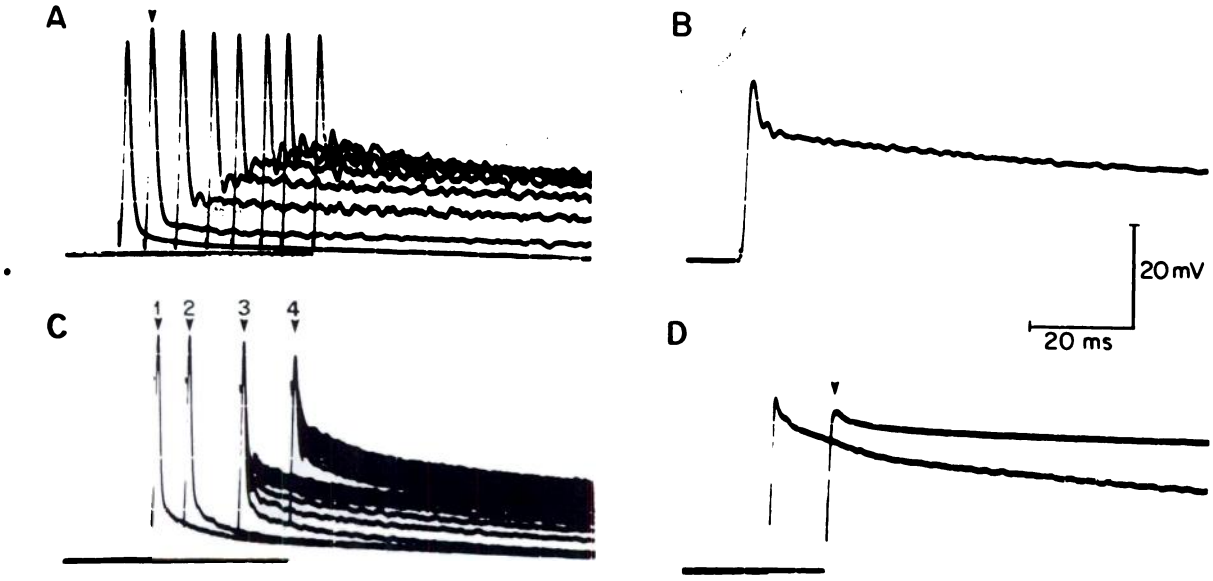


FIG. 4. *Effects of Centruroides toxin and Leiurus toxin on sciatic nerve*

Compound action potentials were measured using the sucrose-gap method. *Centruroides* toxin Iα was tested at 2 μg of protein per milliliter of TEA-Ringer's solution. A, Sequential oscilloscope sweeps recording the action potentials are displaced to the right by increments and are stimulated every 200 sec (sweep speed and amplitude as shown below Trace B). The left-most trace is the action potential before toxin addition, which occurs just before the *arrowhead*. B, Action potential at steady state, 30 min after *Centruroides sculpturatus* toxin addition. The toxin was active at concentrations as low as 0.2 μg of protein per milliliter of TEA-Ringer's solution. C, Effects of *Leiurus* toxin IIα on compound action potentials (*arrow 1* indicates the addition of Fraction 65 of Fig. 3B at 1:10,000 dilution; the toxin concentration was increased 10-fold at *arrow 2*). The appearance of the plateau potential just after the stimulated impulse peak is shown by the overlapping traces at position 3 and, later, at position 4. D, The action potential recorded after a 20-min exposure to normal Ringer's solution containing *Leiurus* toxin IIα (shown to the *left*) is converted to one which repolarizes much more slowly (under the *arrowhead*) when the nerve is bathed by TEA-Ringer's solution, which results in a selective reduction of outward potassium currents.

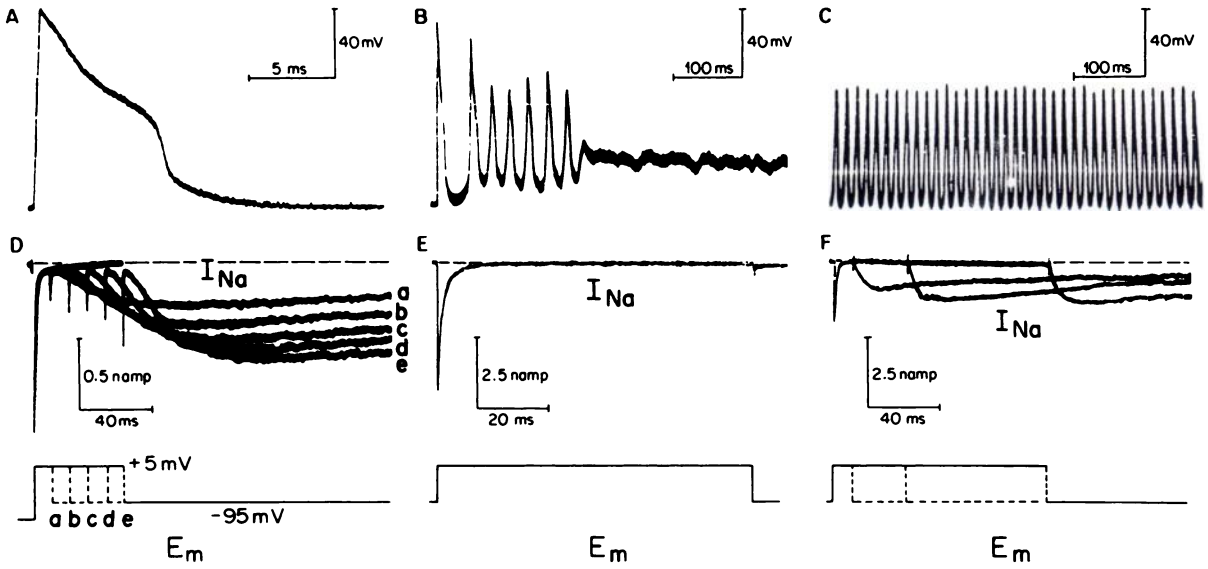


FIG. 5. *Effects of Centruroides toxins Iα or IIα on single myelinated fibers from toad (A–D) or frog (E, F)*

A, Action potentials stimulated by one 300-μsec pulse. In TEA-Ringer's solution without or (B) with *Centruroides* toxin Iα (1.4 μM). C, Spontaneous action potentials caused by *Centruroides* toxin IIα (200 nM); no stimuli were applied. D, E, Records of voltage-clamp currents; zero current is shown by the *broken horizontal line*; lower traces show the applied membrane potentials. D, *Centruroides* toxin IIα (200 nM) induced an unusual inward sodium current following the membrane repolarization to −95 mV after brief depolarizations to +5 mV for 10–50 msec (noted by letters *a–e* on the lower trace; the current traces corresponding to depolarizations of different duration are identically lettered). (Note that the peak of the transient inward sodium current which occurs during the beginning of these brief depolarizations lies off the figure and that its activation component was not recorded by the storage oscilloscope.) E, Normal sodium current from frog node in TEA-Ringer's solution during a 90-msec long depolarization from −95 mV to +5 mV. F, Sodium currents from frog node treated with *Centruroides* toxin Iα (1.4 μM) during and after depolarizations to +5 mV of 10-, 40-, and 120-msec duration. The turn-on kinetics of the toxin-induced current in toad nerve fibers (D) are markedly slower than those in frog (F). Original photos have been retouched to restore faint lines.

channels (14), there appears to be no direct interaction between TEA and toxin-modified sodium channels.

Actions of the isolated toxins on nodal sodium currents measured under voltage clamp. The voltage-clamp method permits a more direct assessment of pharmacological mechanisms. Under voltage-clamp conditions, measured membrane ionic currents reflect changes in ion permeability which are due to the changes in the configurations of the voltage-dependent ion channels. In normal, toxin-free nodes the sodium current shows a multiphasic response to constant membrane depolarization (Fig. 5E). Initially the current (I_{Na}) grows rapidly as sodium channels are "activated" from a closed state at resting potentials to an ion-conducting open state. The current then spontaneously "inactivates" as the channels are converted to another closed state, apparently different from that which predominates at rest. In myelinated fibers of frog (19) and toad[3] this "inactivation" reaction is described by the sum of two exponential functions representing a fast and a slow inactivation process. Upon repolarization, any current remaining during the depolarization is rapidly terminated as the reversal of the activation reaction returns channels to the resting, nonconducting conformation.

At rest, the distribution of channels between closed states (which will open in response to depolarizations) and inactivated states (which cannot be opened by depolarization) is determined by the resting membrane potential. The fraction of channels in the inactivated form at steady state ($1 - h_\infty$) is greater in more depolarized membranes, smaller in hyperpolarized membranes (cf. Fig. 11). In the original description of the sodium conductance kinetics by Hodgkin and Huxley (20), the activation and inactivation reactions were proposed to occur simultaneously and independently. More recent reports have concluded that these processes are closely coupled, even occurring sequentially in certain tissues (21). However, the molecular nature of the interaction between the conformationally altered regions of the channel controlling the activation and inactivation steps remains unspecified.

The kinetics of the sodium conductance change are altered by scorpion toxins. Toxins Iα, IIα, IIIα, and IIIβ from *Centruroides* reduce the sodium current flowing during depolarization within 30 sec of their addition. However, there is little change in the relationship between the relative peak I_{Na} and the depolarization potential (Fig. 6A, for toxin IIα), indicating that sodium channels are activated normally by depolarization. The sodium current kinetics during maintained depolarizations, analyzed in records with faster time resolution than those shown in Fig. 5, are almost indistinguishable from those measured before toxin addition. Evidently the activation and inactivation processes measured during an initial depolarization are modified little by these toxins. Nevertheless, within 10 min after their addition, the toxins IIα, IIIα, and IIIβ induce a new inward current which begins to grow only after membrane repolarization following an initial depolarization (Fig. 5D). This "induced current" is described by the product of two exponentials,

[3] G. K. Wang, unpublished observation.

with a time constant for its increase (turn-on) of 24 msec and a time constant of decay of 850 msec at −95 mV (Fig. 5D). The magnitude of the induced current is dependent on both the duration and the amplitude of the preceding depolarization step. As a rule, longer (up to 250 msec; see Fig. 5D) and larger depolarizations (up to +50 mV) produce larger induced currents upon repolarization with little change of the turn-on and decay kinetics. These results are comparable to the previous findings of Cahalan (22), using the crude venom of *C. sculpturatus* and are thus consistent with his proposal that *C. sculpturatus* venom acts by a very slowly reversible shifting of the voltage dependence of sodium activation, by as much as 50 mV in a negative direction, during depolarization, so that upon repolarization a fraction of the channels remains activated for relatively long times, producing the "induced current." According to Cahalan (22), the slow turn-on of channels is due to removal of sodium inactivation at the negative holding potentials, thus revealing the channels whose activation process is modified by the venom. If this is so, then the time constant for the growth of induced currents should always equal that for the removal of sodium channel inactivation. But, as described in the next paragraph, these two parameters do not always have the same values.

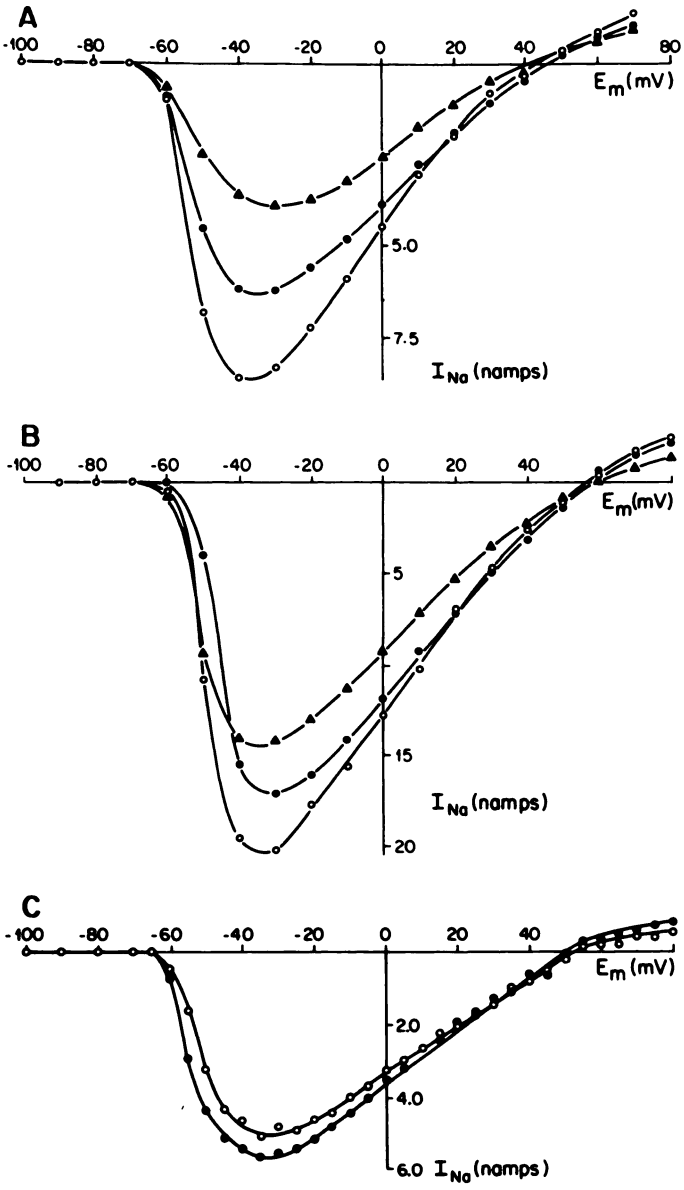


FIG. 6. *Effects of individual toxins on the relationship between the peak sodium current (I_{Na}) and depolarizing membrane potential (E_m).*

A, I_{Na} versus E_m in TEA-Ringer's solution (○) and after exposure to *Centruroides* toxin IIα (20 nM) for 16 min (●) and for 28 min (▲). B, I_{Na} in TEA-Ringer's solution before the addition of *Centruroides* toxin IVα (○) and after exposure to 500 nM *Centruroides* toxin IVα for 3 min (●) and for 25 min (▲). C, Peak currents in TEA-Ringer's solution before the addition of *Leiurus* toxin IIα (○) and after exposure to the toxin for 12 min (●). Note that the peak sodium currents are slightly increased. All currents from nerve fibers of *Bufo marinus*, voltage-clamped at 8°.

The turn-on constant of 12 msec and the decline time constant of 250 msec of the currents induced in frog nerves by toxins Iα–IIIβ are almost identical with the parameters reported by Cahalan (22) using crude *C. sculpturatus* venom on nodes of *R. pipiens* frogs, but are significantly faster than the kinetic parameters of the currents induced by toxins IIα–IIIβ in *B. marinus* nodes (Fig. 5D). The turn-on time constant of the induced current in *Bufo* nerves was 24 msec at −95 mV, but the time constant for removal of inactivation in these nerves was 14 msec (measured by varying the duration of a repolarization step to −95 mV, separating an inactivating conditioning pulse and a test depolarization), almost one-half that for the turn-on process of the current. Thus, in *R. pipiens* nerves there is close agreement between the rate of appearance of toxin-induced current and the rate of removal of sodium inactivation, but such agreement is not observed in nerves from the toad *B. marinus*. Surprisingly, *Centruroides* toxin Iα does not produce any induced-current in single myelinated fibers from the toad *B. marinus*, even at a concentration of 20 μM. In fibers from *R. Pipiens*, *Centruroides* toxins Iα–IIIβ were able to produce an induced current, although a relatively high concentration of toxin Iα (1–5 μM) was required (Fig. 5F). Thus, the potency of different toxins varied over a wide range, and some species were more sensitive than others.

In contrast to toxins Iα–IIIβ, toxin IVα from *Centruroides* produced neither spontaneous repetitive firing nor an induced current. Instead, this toxin prolonged the action potential of a single fiber from 5 msec to hundreds of milliseconds. A long, noisy plateau followed the steep repolarization of the action potential (Fig. 7A). Under voltage clamp, *Centruroides* toxin IVα had three effects on I_{Na}: peak current amplitudes were reduced (Fig. 6B), a "non-inactivating" component of the sodium current appeared having a time-constant of decay longer than 5 secs (data not shown), and the kinetics of inactivation of the remaining transient current were slowed (Fig. 7B–D).

Although the decay of currents during inactivation is a multiexponential process (19), a half-time for this decay has been calculated for the current corresponding to a +5 mV depolarization in Fig. 7, as shown by the *open arrowheads* pointing downward. The control half-time equaled 1.2 msec and the value after *C. sculpturatus* toxin IVα equaled 1.7 msec. No induced current appeared

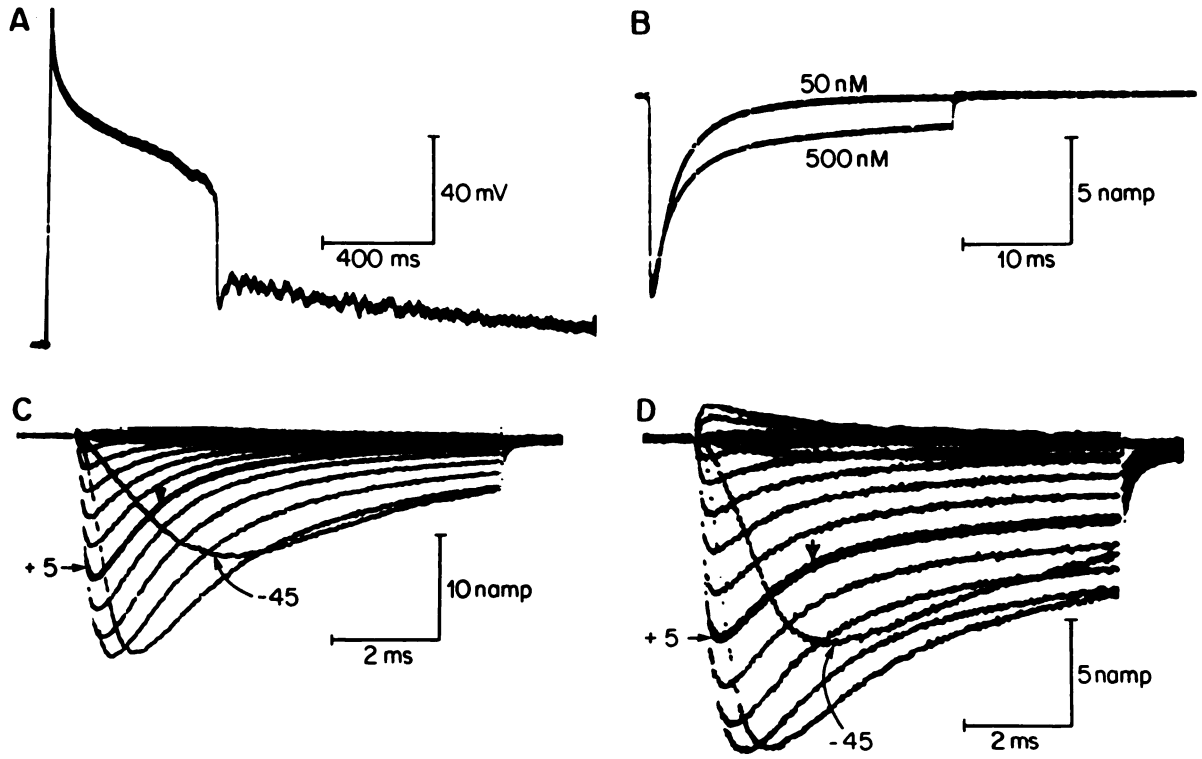


FIG. 7. *Effects of Centruroides toxin IVα on single myelinated fibers from Bufo marinus*

A, Action potential of fiber treated with *Centruroides* toxin IVα at 500 nM in TEA-Ringer's solution. B, Under voltage clamp, the sodium currents are shown in the presence of two different concentrations of *Centruroides* toxin IVα. C, Families of sodium currents in TEA-Ringer's solution before and (D) after exposure to 500 nM toxin for 25 min. Each current trace is produced by a step depolarization; depolarizing steps ranged in amplitude from −65 to +65 mV in 10-mV increments and were applied at a frequency of 1 Hz. Peak currents are decreased (note change in scale), and inactivation is slowed and removed by the toxin. Half-times for the inactivating components of the currents during a depolarization to +5 mV are indicated by the *open arrowheads* pointing downward to these current traces and are equal to 1.2 msec for untreated nodes (C) and 1.7 msec in *Centruroides* toxin IVα-treated nodes (D).

upon repolarization. The effect of this toxin in slowing sodium inactivation was at least partially reversed by four washings with TEA-Ringer's solution (3 ml each time, data not shown). These results demonstrate that there are two different classes of toxins in *C. sculpturatus* venom. One (*Centruroides* toxin IVα) affects primarily the inactivation processes during a maintained depolarization; the other (*Centruroides* toxins Iα, IIα, IIIα, and IIIβ) affects the activation process after a maintained depolarization. Both classes of toxins reduce the amplitude of peak sodium currents.[4]

Sodium channels can be modified simultaneously by both classes of *Centruroides* toxins. When single fibers were first exposed to *Centruroides* toxin IVα, which slowed the inactivation processes, the subsequent addition of *Centruroides* toxin IIα resulted in a further reduction of peak I_{Na} and the development of an induced current (Fig. 8). However, these effects in the presence of *Centruroides* toxin IVα were different in three respects than those produced by *Centruroides* toxin IIα alone. First, the rapidly inactivating currents during the maintained depolarization were selectively reduced, while the maintained currents were essentially unchanged (Fig. 8A). Apparently, noninactivating channels modified by *Centruroides* toxin IVα are much less sensitive to the inhibitory actions of the inducer toxins than are the inactivating channels. An identical result occurred when *Centruroides* toxin IIα was present with *Leiurus* toxin IIα, and the order of addition of the toxins did not change this effect (23). Second, the current induced upon repolarization by *Centruroides* toxin IIα appeared within less than 1 min after toxin addition, whereas in the absence of *Centruroides* toxin IIα this current was detectable after 3 min at the earliest (Fig. 9). Third, the kinetics of the induced current in the presence of the two toxins are different, particularly the turn-on phase. The current with two toxins reaches its peak value immediately or shortly after repolarization (Fig. 8B), whereas with *Centruroides* toxin IIα alone the current took tens of milliseconds to reach its maximal amplitude (Fig. 5D). The kinetics and the amplitude of the induced current are exquisitely sensitive to the membrane potential of repolarization (Fig. 8B), reflecting the dual actions of toxins

[4] Small shifts in the peak I_{Na}-E_m curves along the voltage axis were seen consistently in nodes treated by *Leiurus* and *Centruroides* toxins. With *Leiurus* toxin IIα shifts of less than 10 mV in the negative direction were observed in 7 of 9 analyzed experiments; the remaining 2 experimental results showed no shift. With *Centruroides* toxins IIα and IVα, shifts in the voltage dependence of peak I_{Na} were also seen in each set of two analyzed experimental records. Because the voltage clamp was not compensated for series resistance artifacts, and because a detailed kinetic analysis of the effects of scorpion toxins will be included in a subsequent paper, we will not discuss the significance of the small shifts at this time.

which modify both activation and inactivation parameters of sodium channels (see Discussion). These results using *Centruroides* toxins IIα and IVα are identical with ones we have reported previously for the simultaneous additive effects of *Centruroides* toxin IIα and *Leiurus* toxin IIα (23).

Leiurus toxin IIα, like the *Centruroides* toxin IVα, prolonged the action potential to several hundred milliseconds (Fig. 10). Under voltage clamp, the toxin slowed the sodium channel inactivation processes and produced a noninactivating sodium current, similar to the effects of *Centruroides* toxin IVα (Fig. 10B and C). As in the analysis for *Centruroides* toxin IVα, calculated half-times for the complex inactivation processes at +5 mV increased from 1.36 msec in control to greater than 1.7 msec in the *Leiurus* toxin IIα-treated node, calculated after the estimated noninactivating current was accounted for. The toxin concentration needed to produce the maximal slowing of inactivation was about 200 nM; further increases of the toxin concentration up to 800 nM did not significantly change the rate of inactivation.

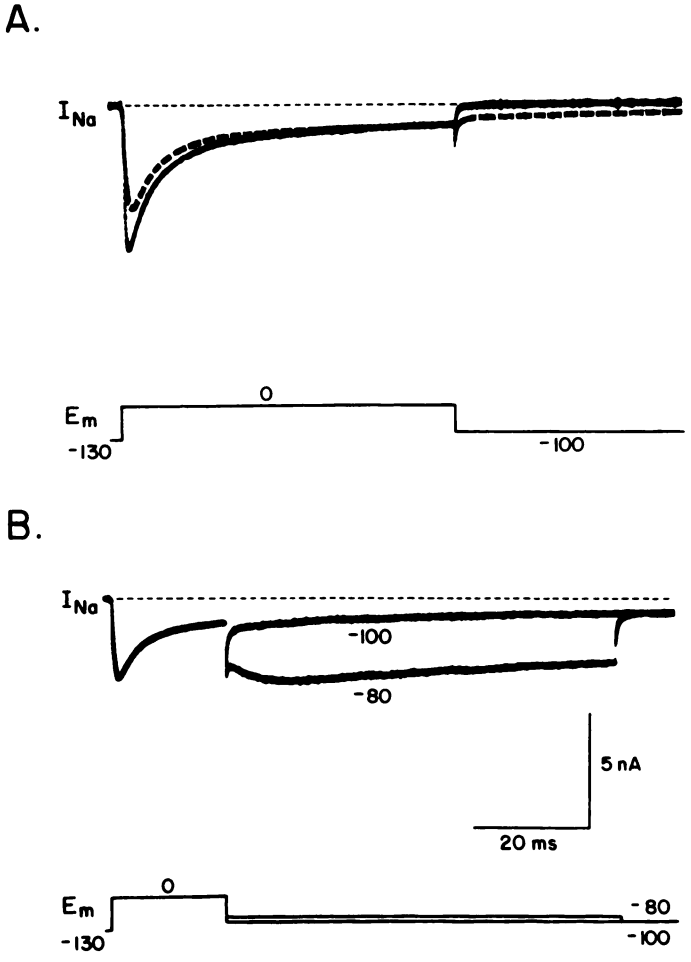


FIG. 8. *Effects of Centruroides toxin IIα on I_{Na} in a fiber treated with Centruroides toxin IVα*

A, A myelinated fiber from *Bufo marinus* was treated with 770 nM *Centruroides* toxin IVα for 25 min. The sodium currents were then measured before (*solid line*) and 1 min after the addition of 460 nM *Centruroides* toxin IIα (*broken line*). Note the induced sodium current that appears after the depolarization. B, Five minutes later the induced sodium current remains and is highly sensitive to the repolarized membrane potential. Numbers under each current trace denote the membrane potential in millivolts. The *thin broken line* indicates the baseline of zero current.

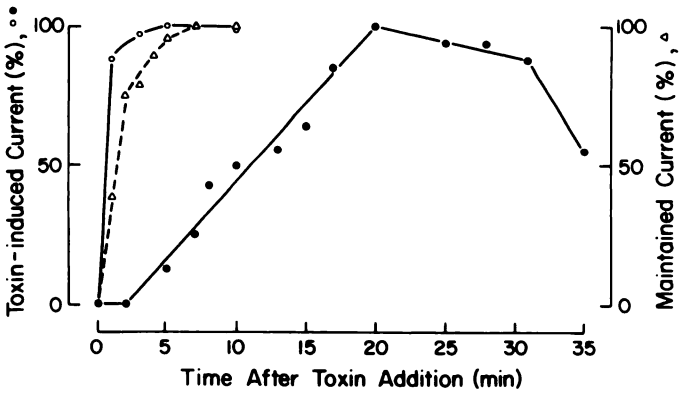


FIG. 9. *Time course of the appearance of induced current and of the maintained, non-inactivating current after the addition of different scorpion toxins*

After addition of toxin CS IIα (200 nM) at time $t = 0$, the peak induced current was measured upon repolarization to −95 mV from a 20-msec depolarization to +5 mV, and is graphed here as a percentage of the maximal induced current: induced current in a node treated with CS IIα alone (●) and in a node that had been exposed to *Leiurus* toxin IIα (200 nM) for 15 min before the addition of the *Centruroides* toxin (○). Induced currents in both situations declined in time after reaching a maximal value. △, Normalized amplitude of the maintained current at the end of a 40-msec depolarization to +5 mV after addition of *Leiurus* toxin IIα at 400 nM; the rate of appearance of the maintained current produced by 200 nM *Leiurus* toxin IIα was similar, demonstrating that the rate-limiting reaction that inhibits sodium channel inactivation is not diffusion of the toxin molecules to their receptor sites.

The size of the maintained current produced by *Leiurus* toxin relative to the peak current varied among different preparations. In nine experiments the relative current at the end of a depolarization to +5 mV of 20- or 50-msec duration was 0.20 ± 0.10 (mean ± standard error). This measurement includes the presence of a long 50- to 100-msec hyperpolarizing pulse (to −125 mV) just preceding the test depolarization which removes the resting inactivation of unmodified sodium channels; in the absence of such a prepulse, more of the unmodified channels are already inactivated at rest and the relative size of the maintained current can be as large as 80–90% of the peak current. Saturating concentrations of *Leiurus* toxin IIα do not prevent inactivation completely, indicating that total receptor occupancy of this ligand cannot totally inhibit the processes of inactivation.

The peak current-voltage relation with *Leiurus* toxin IIα is shown in Fig. 6C; there is a small shift in the curve (of questionable significance; see above) and, interestingly, there is often a slight increase of the amplitude of peak I_{Na} in the presence of *Leiurus* toxin IIα. This phenomenon is regularly obtained in about one-half of the experiments and is distinctly different from the effects of crude *Leiurus* venom (24, 25) and purified *Centruroides* toxin IVα (Fig. 6B and C) on peak I_{Na}.

The shape of the sodium inactivation function, h_∞, relating the fraction of potentially activatable channels (measured as peak I_{Na}) to the conditioning prepulse potential or to the resting membrane potential, was al-

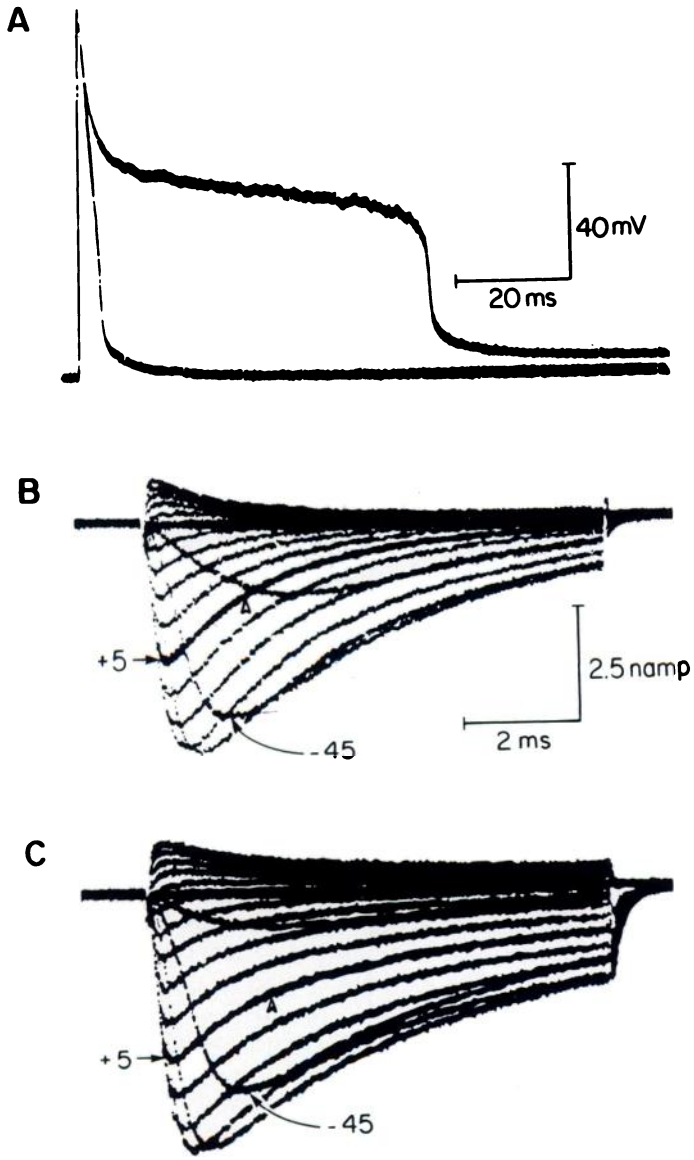


FIG. 10. *Effects of Leiurus toxin IIα on single myelinated fibers from Bufo marinus*

A, The duration of the action potential is increased from 3.5 msec before toxin exposure to almost 60 msec after exposure to *Leiurus* toxin IIα, 0.4 μg/ml (57 nM). B, A family of sodium currents in TEA-Ringer's solution before toxin addition and (C) after addition of *Leiurus* toxin at 1.4 μg/ml (200 nM). Peak currents are slightly increased, but the rate of inactivation is markedly slowed. The half-time for inactivation of currents during a pulse to +5 mV, shown by the *open*, upward-pointing *arrowheads* is 1.36 msec in the control node and greater than 1.7 msec in the toxin-treated node.

tered in nodes treated with *Leiurus* toxin IIα (Fig. 11). In normal nodes the channels are totally converted to an inactive form by depolarizing conditioning pulses (more than −50 mV), but in the modified nodes some of the channels do not inactivate, even at potentials of +50 mV (Fig. 11), although for prepulse conditioning potentials below −50 mV the h_∞ function in modified nodes is almost normal. A very similar change in the h_∞ function is produced by *Centruroides* toxin IVα (data not shown). Such changes have also been reported for crude *Leiurus* venom (24, 26) and for a toxin (called *V*) isolated from *C. sculpturatus* Ewing and investigated by Meves *et al.* (27).

Table 2 summarizes the physiological effects exerted by the purified neurotoxins from *C. sculpturatus* and *L. quinquestriatus* on single myelinated fibers. The potency of each toxin is rather difficult to determine by the voltage-clamp method, since the effects are usually irreversible and the choice of which parameter to relate to toxin concentration is still somewhat arbitrary. Our preliminary results indicate that *Leiurus* toxin IIα is more potent than *Centruroides* toxin IVα and, unlike the effects of *Centruroides* toxin IVα, those of *Leiurus* toxin IIα cannot be removed by washing. The other *Centruroides* toxins have potencies in the following order: toxin IIα > toxins IIIα = toxin IIIβ > toxin Iα.

DISCUSSION

The results demonstrate the existence of multiple neurotoxins affecting sodium channels in the venoms of *C. sculpturatus* and *L. quinquestriatus* scorpions. Since the sucrose-gap assay method is sufficiently sensitive to detect the neurotoxins at concentrations of less than 0.1 μg/ml, it is not necessary to have large quantities of scorpion venoms for purification; 100 mg of *C. sculpturatus* and 35 mg of *L. quinquestriatus* venoms are sufficient. At least five toxins from the former and one toxin from the latter venom were isolated, and other minor active components were detected but not purified further. All of the isolated neurotoxins appear to have molecular masses of 7000–7500 daltons, and there is no evidence of their further dissociation into smaller polypeptides as judged by their migration in SDS/PAGE and chromatography on Sephadex G-25 or G-50.

By analyzing their effects on the kinetics of sodium currents under voltage-clamp conditions, we have separated the toxins into two distinct classes. Those which selectively slowed and prevented sodium channel inactivation included both *Leiurus* toxin IIα and *Centruroides* toxin IVα. We call these toxins "stabilizers" because, by hindering inactivation, they stabilize channels in their ion-conducting state. The other toxins, all from *Centruroides* venom, acted to induce a slowly developing inward current after membrane repolarization following a depolarization. Because of this phenomenon, we refer to these toxins as current "inducers," after the original description by Cahalan (22).

The actions of the isolated neurotoxins were very similar to those of the crude venom, with few exceptions. Like *Leiurus* venom (24, 25, 28), the *Leiurus* toxin IIα slowed the channel inactivation reactions and prevented complete inactivation, resulting in a maintained current during a prolonged depolarization. In 200 nM *Leiurus* toxin the relative size of the maintained current as compared with the peak sodium current appeared to vary from node to node, but was approximately 20% of the maximal peak current that could be activated. At saturating concentrations of *Leiurus* toxin IIα (200 nM), no sodium current that decayed with normal inactivation kinetics could be detected (Fig. 10), yet the fraction of current which did not inactivate at all was still only 20%, the remaining 80% showing slowed inactivation kinetics. There are two explanations for this result: either two

TABLE 2

Summary of the physiological effects of purified scorpion toxins on single myelinated fibers of Bufo marinus

	Scorpion toxin[a]					
	Leiurus quinquestriatus	*Centruroides sculpturatus*				
	IIα	Iα	IIα	IIIα	IIIβ	IVα
Changes in I_{Na}	0[b]	–	–	–	–	–
Maintained current at the end of 40-msec pulse	+	0	0	0	0	+
Toxin-induced inward current	0	0/+[c]	+	+	+	0
Repetitive action potentials after stimulus	0	+	+	+	+	0
Minimal effective concentrations[d]	20 nM	1 μM	20 nM	50 nM	50 nM	50 nM

[a] –, Reduced; +, increased; 0, no change.

[b] No significant changes or a slight increase in I_{Na} measured 5–10 min after addition of the toxin.

[c] When myelinated fibers from *Rana pipiens* but not *Bufo marinus* were used.

[d] Minimal effective concentrations of toxins on slowing I_{Na} inactivation or producing repetitive action potentials after a single stimulus. Toxin concentrations less than 20 nM were not tested.

populations of sodium channels exist in amphibian nodes, having different responses to bound *Leiurus* toxin, or the kinetics of channel inactivation represent a complex, multistep process such that inhibition by toxin of a single step cannot prevent inactivation from occurring completely. Previous analyses of I_{Na} inactivation (19) and of gating current changes (29) have documented the multiphasic behavior of normal sodium channel inactivation, supporting the second general explanation, but until we carefully analyze the dose-response behavior for a single toxin species we cannot exclude some contribution from the first possibility.

Unlike crude *Leiurus* venom, which reduces the magnitude of the peak sodium current measured at all potentials, the purified *Leiurus* toxin actually increased the peak sodium current in about one-half of the experiments, in some cases by as much as 10–20%. This increase was not accompanied by any detectable change in the activation kinetics of sodium current, and therefore must be either due to an increase in the fraction of channels which is available for opening in response to depolarization or to the slowing of the inactivation process. In contrast, the maximal sodium conductance was always reduced by the stabilizer *Centruroides* toxin IVα, unlike the *Leiurus* toxin IIα, showing that the "stabilizer" action of a toxin alone is not sufficient to ensure the maintenance or increase of the sodium conductance. The reduction in maximal sodium conductance following exposure to crude *Leiurus* venom must, therefore, be due to some component other than the toxins we have isolated.

The purified *Centruroides* toxins, except for toxin IVα, produced physiological effects similar to those produced by crude venom. *Centruroides* toxins Iα–IIIβ produced a slowly developing inward current when the membrane was repolarized following depolarization. The magnitude of this current increased with larger and longer preceding

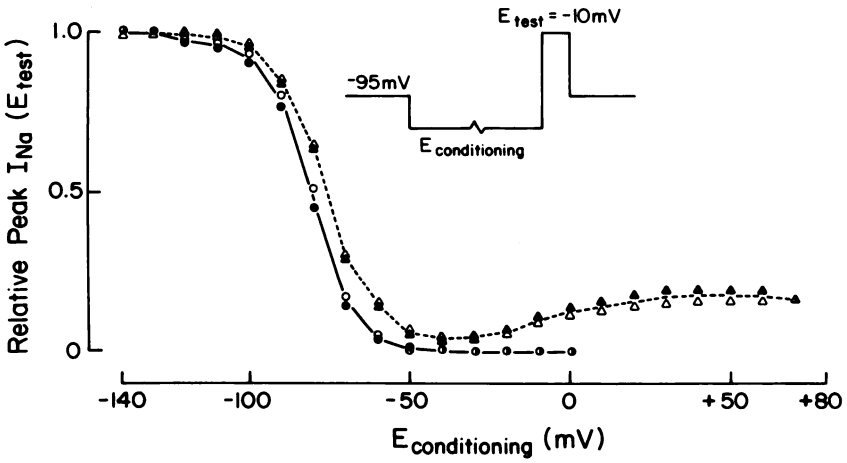


FIG. 11. *Relative peak sodium currents after long conditioning pulses*

The peak sodium currents during a test depolarization to −10 mV were measured after 100-msec long conditioning pulses (see *inset*) and normalized by the maximal peak current. The ordinate thus normalized is a measure of the fraction of channels which are not in the inactivated state at the end of the conditioning prepulse. Peak currents from the same nerve fiber are shown before (○, 15 min; ●, 5 min) and after (△, 17 min; ▲, 35 min) exposure to *Leiurus* toxin IIα (200 nM).

depolarization, as described by Cahalan (22) for crude venom. The induced current could not be detected earlier than 2–3 min after the beginning of exposure to *Centruroides* toxin, although a different action of the toxin, the reduction of sodium currents during depolarizing pulses, occurred within 30 sec of toxin addition, as it does with crude venom. The sum of the peak sodium conductance during a depolarization plus the induced sodium conductance after that depolarization does not equal the original peak conductance before the addition of inducer toxin; a fraction of the channels is removed irretrievably by inducer *Centruroides* toxins. The time course of the inactivation process was not significantly changed by the "inducer" toxins alone, and we have never observed a maintained current in the presence of inducer toxin during a prolonged depolarization. Thus, these toxins appear to act exclusively on the activation processes of sodium channels.

It is not clear why the crude venom studied by Cahalan (22) did not produce the toxin IVα effect of slowing inactivation and producing a maintained current during a prolonged depolarization. Perhaps it was because the effects of toxin IVα are reversible. In Cahalan's study the venom solution was incubated with the myelinated nerve for 3–5 min and subsequently exchanged for venom-free Ringer's solution. This short incubation time followed by washing the node with a venom-free Ringer's solution would limit the binding of toxin IVα to its site on the sodium channel.

Our results with *Centruroides* toxins are comparable to those recently reported by Meves *et al.* (27). They also found different actions of the multiple neurotoxins on frog node, although the detailed effects of their toxins differ somewhat from our findings. For example, the toxins which they found to produce an induced current also modified sodium channel inactivation, whereas we never observed such an effect. Also, several of their "inducer" toxins did not inhibit peak I_{Na} at concentrations of 0.33–3.3 μg/ml (corresponding to 50–500 nM concentrations of proteins with a molecular mass of 7000 daltons), although we always observed such an inhibition with *Centruroides* toxin IIα at 200 nM. There is no obvious explanation for these differences, but we do not know that both laboratories are studying identical toxins of the same purity.

The kinetics of sodium currents measured in the presence of the two types of *Centruroides* scorpion toxins clearly demonstrate that both types can bind to their respective sites and modify channel gating simultaneously. Stabilizers inhibit inactivation and inducers selectively modify activation. Identical results were obtained in a previous study in which we added irreversible *Leiurus* stabilizer toxins and *Centruroides* inducer toxins sequentially (23). Although separate binding sites exist for the two classes of toxins, there is good evidence that the binding of a toxin molecule at one site modifies toxin binding or the associated response at the other site. This evidence includes the accelerated appearance of induced current in the presence of stabilizer toxins (Fig. 9; ref. 23) and the selective reduction of rapidly inactivating currents by inducer toxins when stabilizers have elicited a maintained current (Fig. 8). The pharmacological results showing separate but interacting binding sites for scorpion toxins in frog nerve may be a manifestation of the separate yet interactive nature of the activation and inactivation processes in normal sodium channels.

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