11-1981

Detergent Extraction of a Presumptive Gating Component from the Voltage-Dependent Sodium Channel

W J Culp
Dartmouth College

D T McKenzie
Dartmouth College

Proc. Natl. Acad. Sci. USA
Vol. 78, No. 11, pp. 7171–7175, November 1981
Neurobiology

Detergent extraction of a presumptive gating component from the voltage-dependent sodium channel

(*Leiurus* neurotoxin)

W. J. CULP AND D. T. MCKENZIE

Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755

Communicated by W. H. Stockmayer, May 29, 1981

ABSTRACT **A physiologically characterized radiolabeled neurotoxin complex obtained from venom of the scorpion *Leiurus quinquestriatus* has been used to identify detergent-solubilized presumptive sodium channel components in sucrose gradients. This toxin-binding component is found in extracts prepared from three sources of excitable membrane but appears to be absent from similar extracts prepared from nonexcitable membrane or from *Torpedo californica* membrane. Procedures that destroy the physiological activity of the *Leiurus* neurotoxin lead to a corresponding loss of toxin binding to the putative sodium channel component. The major component recognized by the *Leiurus* toxin sediments at 6.5 S. Scatchard analysis of quantitative binding experiments carried out in sucrose gradients shows approximately linear plots and indicates that the toxin recognizes a relatively small number of sites with a dissociation constant near 10 nM. Once formed, the channel element–toxin complex is quite stable. Experiments show diphasic dissociation kinetics with half-times near 70 hr and greater than 200 hr.**

Electrophysiological experiments have provided a quantitative description of the time and field dependence of gating processes that regulate voltage-dependent sodium channels. In contrast there is, as yet, little information on the structure and organization of these channels. One approach to providing such information is that of using physiologically characterized animal neurotoxins as selective affinity ligands for the identification of sodium channel components. Two neurotoxins that have proven to be of particular value in this regard are tetrodotoxin (TTX) and saxitoxin (STX). Although structurally different, these molecules have very similar physiological activities, binding to voltage-dependent sodium channels with high affinity to produce a selective sodium conductance block (1). This interaction effectively inhibits ion transport through the channel but does not appear to alter either the kinetics or voltage dependence of the asymmetry currents associated with channel gating (2, 3). These toxins also appear to show considerable biochemical specificity, recognizing the sodium channel to the relative exclusion of other membrane proteins (4). STX and TTX have been used as affinity ligands for the identification and partial purification of a sodium channel component solubilized from eel electroplaque (5) and rat skeletal muscle (6). The solubilized toxin-binding component has a molecular weight of 200,000–300,000 and a sedimentation coefficient near 9 S. A rapid loss of high-affinity toxin-binding activity occurs in the presence of excess detergent or during procedures that strip bound lipid from the solubilized channel component; thus purification of this component in an active form appears possible only if exogenous lipid is added to the solubilization and chromatography buffers (7).

A second group of neurotoxins that may be of significant value in biochemical studies on the sodium channel are those obtained from scorpion venoms. Scorpion toxins selectively alter channel gating without substantially changing channel conductance. Venoms from two species are of particular interest. *Centruroides sculpturatus* neurotoxins selectively alter the voltage dependence of sodium activation, or "m" gating (8), and neurotoxins isolated from *Leiurus quinquestriatus* characteristically slow the kinetics of sodium inactivation (9, 10).

Of the scorpion neurotoxins, those from *Leiurus* have been the most extensively employed in biochemical studies on the sodium channel. Work on this venom by Miranda *et al.* (11) led to the isolation of five neurotoxic components. These toxins appear to be structurally related in that they have molecular weights near 6600, lack methionine, and show regions of amino acid sequence homology (12). More recently, Catteral (13) reported the purification of a single neurotoxin from *Leiurus* venom. This component seems likely to be identical with or related to one of the toxins described by Miranda *et al.*, because it also is reported to have a molecular weight near 6600 and does not contain methionine.

These toxins have been used in significant experiments demonstrating high-affinity voltage-dependent binding to a toxin site on the sodium channel of neuroblastoma (14) and muscle (15) cells and rat synaptosomes (16, 17). Recently, a photoactivatable derivative of this toxin has been used to label covalently two presumptive sodium channel components with molecular weights near 250,000 and 35,000 (18).

We have used physiologically characterized radioiodinated *Leiurus* toxin in developing procedures that allow sodium channel components to be solubilized from excitable membranes in a form in which they retain high-affinity saturable scorpion neurotoxin-binding activity. These procedures and a preliminary characterization of the solubilized channel component are presented here.

MATERIALS AND METHODS

Electroplaque Membrane Preparation. *Electrophorus electricus* eels (Aquarium Foods, Ardsley, NY) were used as a source of excitable membrane. The electric organ was removed, diced into approximately half-inch (1-cm) cubes, and serially washed in three 750-ml volumes of ice-cold Ringer's solution (169 mM NaCl/5 mM KCl/3 mM $CaCl_2$/1.5 mM MgCl/1.5 mM $NaPO_4$, pH 7.2). The tissue was then disrupted in a fourth 750-ml volume of cold Ringer's solution containing Trasylol at 20 units/ml and pepstatin at 0.2 μg/ml (P-Ringer's solution), using a Polytron (Brinkmann, Instruments), and filtered through two layers of sterile gauze. This homogenate was centrifuged at 20,000 rpm for 20 min in a Sorvall SS-34 rotor. The pelleted membrane fraction was resuspended in 10 vol of fresh P-Ringer's solution and centrifuged for 20 min. The membrane pellets were then resuspended in P-Ringer's solution made 1.0

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; P-Ringer's solution, Ringer's solution containing Trasylol and pepstatin.

M in NaCl and centrifuged as above. A final wash was carried out with normal P-Ringer's solution, and the membranes were stored in liquid N_2 in 2-ml tissue culture freezing vials until use. All procedures were carried out at 3°C. Activity gradually declined during storage, but a preparation was generally useful for about 3 months.

Detergent Extraction of Electroplaque Membrane. A small amount, usually about 0.5 g, of frozen membrane was removed from storage, weighed, and homogenized with 5 vol of 0.05% Triton X-100 in phosphate-buffered saline at 0°C in a loose-fitting glass homogenizer. (Phosphate-buffered saline contains 138 mM NaCl, 3 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8.15 mM Na_2HPO_4, 1.5 mM KH_2PO_4.) This homogenate was centrifuged for 5 min in an Eppendorf microcentrifuge (Brinkmann Instruments). The pellet was then homogenized in 0.5% Triton X-100 in phosphate-buffered saline, incubated at 3°C for 30 min, and centrifuged in the microcentrifuge for 15 min. The clarified supernatant was removed and used immediately.

Sucrose Gradient Centrifugation. Linear 4.3-ml gradients of 5–20% sucrose in phosphate-buffered saline containing 0.05% Triton X-100 were poured 12–20 hr prior to use. Samples of detergent extracts were then incubated for 30 min at 0°C with the indicated concentration of labeled neurotoxin. A 100- to 200-μl sample of this mixture was layered on top of the gradient and centrifuged at 55,000 rpm for 12 hr at 3°C in a Beckman SW-60 rotor. Gradients were fractionated by using a needle-puncture device (Hoefer Instruments, San Francisco, CA), and the distribution of radioactivity in the gradient was determined by measuring the fractions in a Beckman Gamma-4000 counter.

Ion-Exchange Chromatography of *Leiurus* Neurotoxins. Typically, 40 mg of lyophilized *L. quinquestriatus* venom (Sigma) was extracted three times at room temperature with 2.0 vol of water with centrifugation. The combined supernatants were applied to a 1.5 × 15 cm column of Bio-Rex 70 (Bio-Rad) equilibrated in 0.02 M NH_4HCO_3/0.005 M $NaPO_4$, pH 7.2. After unbound protein was eluted from the column with equilibration buffer, the bound proteins were eluted with a linear NH_4HCO_3 gradient developed between 0.02 M and 0.75 M in 0.005 M $NaPO_4$. As assessed electrophysiologically, four peaks of neurotoxicity were eluted at conductivities of 10, 13, 15, and 18 mS (0.5 M NH_4HCO_3 = 25.5 mS). The neurotoxin fraction used in this work is that eluting at 13 mS.

Radioiodination of *Leiurus* Neurotoxin. Iodination was performed by using a modification of the coupled glucose oxidase–lactoperoxidase method (19). Generally, 15 μg of lyophilized neurotoxin was dissolved in 30 μl of 0.075 M $NaPO_4$, pH 7.2. To this solution was added 4 μl of 500 μM NaI, 15 μl of ^{125}I (1.5 mCi, 50 μM NaI; Amersham–Searle; 1 Ci = 3.7×10^{10} becquerels), 5 μl of 5% glucose in water, and 4 μl of Enzymobead suspension (Bio-Rad). The mixture was incubated for 3 min at room temperature. Radiolabeled toxin was then separated from free iodine by chromatography on a 1-ml column of Sephadex G-10 (Pharmacia) in 0.1% Lubrol-PX in 0.1 M NH_4HCO_3/0.005 M $NaPO_4$, pH 7.2. Breakthrough fractions were pooled, made 10 μM in NaI, and rechromatographed on a second Sephadex G-10 column. Radiolabeled neurotoxin was routinely assayed for activity by intracellular recording from frog skeletal muscle fibers.

A major difficulty in the use of radiolabeled toxin derivatives lies in determining the extent to which the labeling reactions inactivate or modify the binding properties of the toxin in these experiments. The assessment of biological activity is based on the increase in the duration of the frog muscle action potential. Blind experiments on a dilution series of a single toxin sample indicate that an activity loss of 20% or less would not be detected. Toxin concentration has been estimated on the basis of preliminary amino acid composition data and 280 nm absorbance. The concentration values shown here may be in error by roughly a factor of 2. Given these uncertainties, specific radioactivity of the neurotoxin was generally near 75,000–100,000 Ci/mol.

Physiological Inactivation of *Leiurus* Neurotoxin. Radiolabeled *Leiurus* neurotoxin was incubated with a 10-fold molar excess of chloramine-T for 5 min at room temperature and then reisolated by chromatography on a 1.0-ml Sephadex G-10 column in 0.1 M NH_4HCO_3/0.005 M $NaPO_4$, pH 7.2, in 0.01% Lubrol-PX.

RESULTS

Extraction of salt-washed electroplaque membrane as described in *Materials and Methods* leads to the solubilization of membrane components with *Leiurus* neurotoxin binding activity. Sucrose gradient centrifugation has been used to identify and quantitate this binding. As shown in Fig. 1, there is a major toxin-binding component sedimenting at 6.5 S and a minor component at 9 S. The 6.5S component appears to be characteristic of excitable membranes and has been observed, although in lesser amounts, in extracts prepared from rat sciatic nerve and frog skeletal muscle membrane. Conversely, this component has not been detected in equivalent extracts prepared from erythrocyte, spleen, liver, or *Torpedo californica* electroplaque membrane. Fig. 1 shows the profile obtained with an extract of liver membrane.

Electrophysiology experiments have demonstrated that the neurotoxic activity of *Leiurus* toxin is irreversibly destroyed by oxidizing agents such as H_2O_2 or chloramine-T (unpublished observations). This loss of physiological activity is associated with a similar loss of binding activity as monitored in sucrose gradient experiments (Fig. 1). Similarly, nontoxic venom proteins purified by ion-exchange chromatography and radiolabeled as described in *Materials and Methods* show no detectable binding (Fig. 1).

Given the physiological specificity of the *Leiurus* toxins, these observations are consistent with the idea that the 6.5S

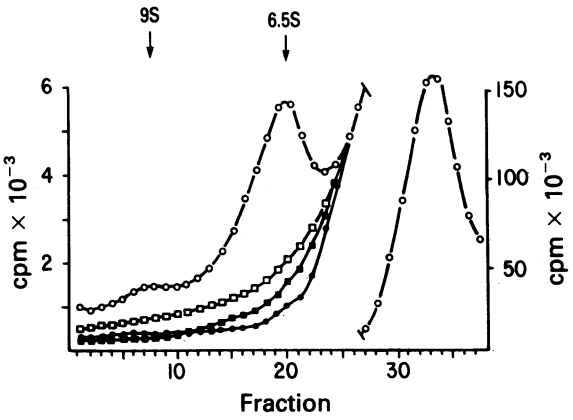

FIG. 1. *Leiurus* neurotoxin binding to solubilized membrane components. Membrane extracts were prepared, and radiolabeled toxin (20 μM), nontoxic venom proteins isolated during ion-exchange chromatography and radiolabeled (0.5 μg/ml), or inactivated radiolabeled neurotoxin (40 nM) was incubated with a 200-μl sample of the indicated extract at 4°C for 30 min. The incubation mixture was then subjected to sucrose gradient centrifugation. Gradients were fractionated to give a total of 36–38 fractions. ○, *Leiurus* neurotoxin with eel electroplaque membrane extract; □, *Leiurus* neurotoxin with liver membrane extract; ■, nontoxic venom protein with eel electroplaque membrane extract; ●, inactivated *Leiurus* neurotoxin with eel electroplaque membrane extract.

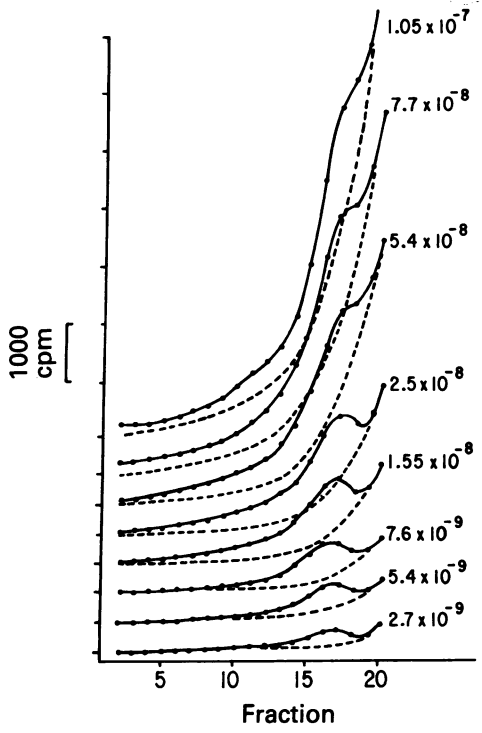

FIG. 2. Dose response for *Leiurus* neurotoxin binding to solubilized components of electroplaque membrane. Procedures and conditions as described in *Materials and Methods* and the legend to Fig. 1 except that the incubation mixtures contained the indicated molar concentration of neurotoxin.

toxin-binding activity represents a component of the voltage-dependent sodium channel. Of the membranes tested, this activity is present only in those that are excitable, and binding appears to be a property only of physiologically active toxin; inactivated toxin and nontoxic components fail to bind.

Quantitative sucrose gradient experiments have been carried out to determine the number and toxin affinity of the solubilized protein. Fig. 2 shows a series of sucrose gradients of extracts incubated with the indicated concentration of neurotoxin. Discernible binding in the 6.5S region is present at 3 nM and increases as the toxin concentration of the initial incubation is raised. At high concentrations the binding peak begins to disappear into the count profile of presumably unbound toxin entering the gradient by diffusion-sedimentation.

These results are consistent with the concentration-dependent saturation of a small number of high-affinity toxin-binding sites. This can be seen more clearly when the amount of toxin binding observed in each region of the gradient is plotted against the concentration of toxin present in the initial incubation. Fig. 3 presents data from three fractions, 8, 16, and 20, of these gradients. The amount of toxin in fraction 20 is linearly dependent on the concentration of toxin in the initial incubation. In contrast, fractions 8 and 16 show a characteristic biphasic dose response consistent with the presence of a component showing high-affinity toxin binding that is superimposed on a linear background of nonspecific binding or toxin entering the gradient by a diffusion–sedimentation mechanism. Nonspecific binding in these fractions has been estimated by drawing a line through zero and having the slope observed at high toxin concentrations. Plots similar to those shown for fractions 8 and 16 were generated for each of the gradient fractions. These were used to provide an estimate of the distribution of the nonspecifically bound and unbound toxin. This estimate is shown

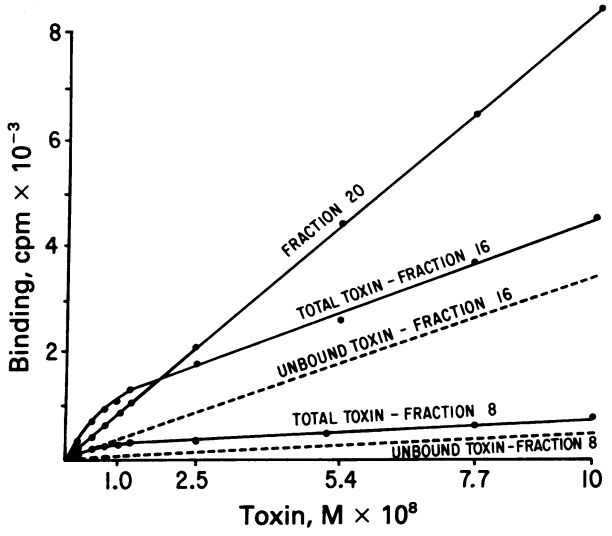

FIG. 3. Estimation of the distribution of saturable and nonsaturable toxin binding in sucrose gradients. Radioactivity contained in fractions 8, 16, and 20 of the gradients shown in Fig. 2 was plotted versus the concentration of neurotoxin in the initial incubation mixture. The broken lines were drawn through the zero intercept with a slope the same as that of the dose–response curve at high toxin concentrations. This was taken as an estimate of the amount of nonspecifically bound or unbound radioactivity present in the fraction as a function of the toxin concentration of the initial incubation mixture.

by the broken lines underneath each of the gradient profiles in Fig. 2.

Specific binding has been taken as the difference between the observed total binding and the estimated nonsaturable binding. In Fig. 4 this value is presented as a function of the toxin concentration of the initial incubation mixture. The specific binding is clearly saturable over the range of toxin concentrations tested.

Other experiments have demonstrated that unlabeled neurotoxin is an effective competitor of radiolabeled neurotoxin. Exposing detergent extracts to 25 nM labeled neurotoxin in the presence of 100 nM unlabeled neurotoxin produced a decrease in the amount of specifically bound radioactivity ranging between 70% and 85% in four experiments. These results support

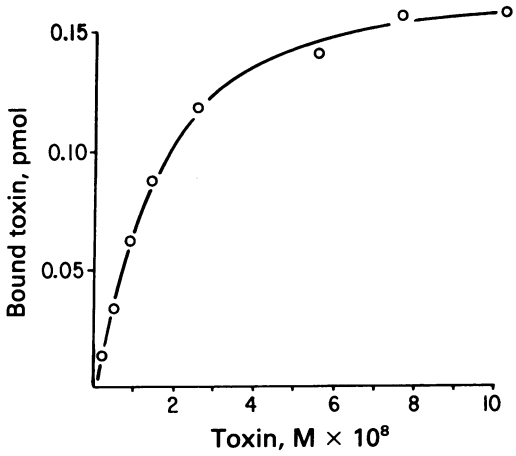

FIG. 4. Concentration dependence of specific *Leiurus* neurotoxin binding. The area between the total count profile (solid line) and the estimated unbound toxin profile (broken line) of the gradients shown in Fig. 2 was determined and plotted against the concentration of neurotoxin in the initial incubation mixture.

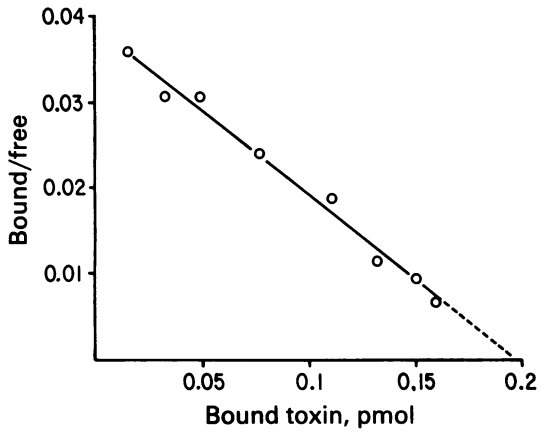

FIG. 5. Scatchard analysis of *Leiurus* toxin binding to solubilized sodium channel components. Bound toxin was determined as described in the text and the legend to Fig. 4. Free toxin is taken as total toxin added minus bound toxin. The calculated dissociation constant K_d is approximately 10 nM.

the idea that radiolabeled toxin is recognizing a binding site normally occupied by native neurotoxin.

Scatchard analysis of the data presented in Fig. 3 gives an approximately linear plot (Fig. 5) and is consistent with the presence of a single class of *Leiurus* neurotoxin binding sites with a toxin affinity near 10 nM. These active presumptive channel components are present in the electroplaque extract at a concentration near 0.1 nM. On the basis of the protein concentration of the extracts, and assuming that the population of extracted membrane proteins has a mean molecular weight of 100,000, this value indicates that approximately 1 protein molecule in 10,000 shows toxin-binding activity.

Once formed, the toxin–channel complex appears to be quite stable. The time course of this dissociation has been measured

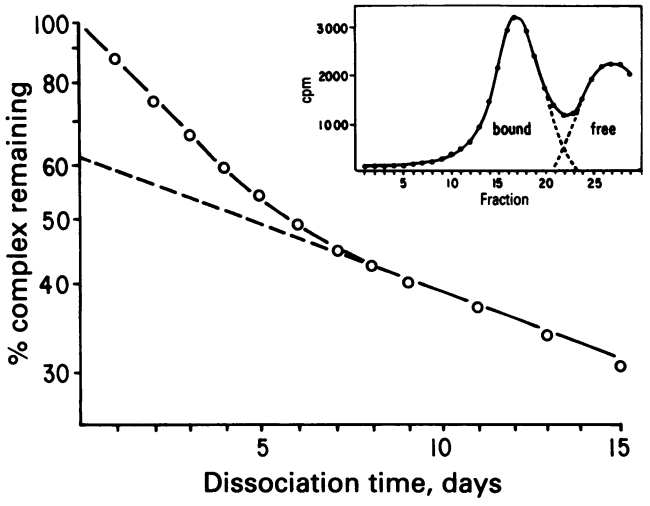

FIG. 6. Dissociation time course of *Leiurus* toxin–channel component complex. Fractions from preparative sucrose gradients containing 6.5S complex were pooled, diluted 1:2, and recentrifuged on 15–25% sucrose gradients to remove residual free toxin. Fractions containing toxin complex were pooled and stored at 3°C for the indicated period. Prior to analysis, a sample was removed, diluted 1:3, and centrifuged at 3°C for 11 hr on a 10–30% sucrose gradient at 56,000 rpm in a Beckman SW-60 rotor. The number of cpm remaining in the 6S complex and those released were determined by measuring radioactivity of the gradient fractions in a gamma counter (see *Inset*). All gradients and diluting buffers contained 0.05% Triton X-100 in phosphate-buffered saline.

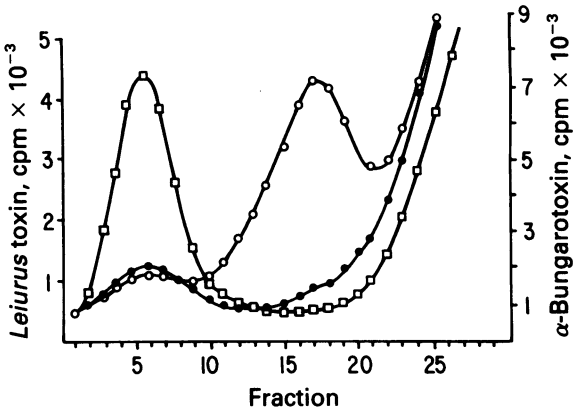

FIG. 7. *Leiurus* neurotoxin binding to the 9S binding component. Extracts of *Torpedo* and *Electrophorus* membrane were prepared as described in *Materials and Methods*. *Leiurus* toxin binding and sucrose gradient centrifugation procedures are as described in *Materials and Methods* and the legend to Fig. 1. α-Bungarotoxin binding was determined after incubation of the *Torpedo* membrane extract with 50 nM ^{125}I-labeled α-bungarotoxin for 30 min. Similar experiments monitoring α-bungarotoxin binding to eel electroplaque extracts give essentially identical results and are not shown here. ○, ^{125}I-Labeled *Leiurus* neurotoxin plus *Electrophorus* electroplaque extract; ●, ^{125}I-labeled *Leiurus* neurotoxin plus *Torpedo* electroplaque extract; □, ^{125}I-labeled α-bungarotoxin plus *Torpedo* electroplaque extract.

by isolation of the complex from preparative gradients and then resedimentation of the complex after various periods of incubation. Bound and unbound toxin can be estimated in a fairly quantitative manner from the count profile in these gradients (see *Inset* of Fig. 6). The dissociation process shows biphasic kinetics, in which roughly 40% of the toxin is unbound with a half-time of approximately 70 hr and the remainder at a much slower rate, the half-time being greater than 200 hr. These results suggest two bound forms of toxin and may reflect a binding heterogeneity not revealed by the Scatchard analysis shown in Fig. 4.

The profile shown in Fig. 1 also indicates a binding component that sediments near 9S. As indicated above, membrane extracts prepared from electroplaque tissue of *T. californica* lack the 6.5S component. However, these extracts do contain a 9S binding activity. Work to be reported elsewhere demonstrates that at least one *Leiurus* neurotoxin is an effective agonist for nicotinic acetycholine receptors in BC3H-1 cell monolayers. The 9S binding component observed in eel and torpedo extracts cosediments with definitive acetylcholine receptors as measured by α-bungarotoxin binding (Fig. 7) and may be due to the recognition of solubilized acetylcholine receptors by *Leiurus* neurotoxin.

DISCUSSION

As discussed earlier, *Leiurus* neurotoxins show saturable high-affinity binding to excitable membranes (14–17). This binding is modulated by the transmembrane potential such that depolarization produces a decrease in the toxin affinity of the membrane component (14). Voltage clamp experiments to be published elsewhere show that all of the identified *Leiurus* neurotoxins are physiologically indistinguishable, each slowing sodium inactivation. In agreement with the binding experiments, electrophysiological measurements indicate that the toxin binding is quite stable at normal resting potentials but becomes more reversible when the membrane potential is held at less negative voltages. This effect is observed to some degree with all of the isolated *Leiurus* toxins and thus appears to be a general property of these molecules (unpublished). Taken to-

gether, these findings support the hypothesis that the *Leiurus* toxins bind to a membrane component associated with the gating of voltage-dependent sodium channels.

The experiments presented here demonstrate that the *Leiurus* neurotoxin binding element, presumably a part of the voltage-dependent sodium channel, can be solubilized in a form in which high-affinity toxin binding is preserved. This presumptive channel element has a sedimentation coefficient near 6.5 S and toxin affinity with a K_d near 10 nM. The toxin channel element interaction is quite stable and shows nonlinear dissociation kinetics with at least two rate constants, both in excess of 50 hr.

An important question relevant to sodium channel structure is whether the channel element described here is related to that which binds TTX and STX. These activities appear to be distinctly different in at least two respects. First, the TTX/STX-binding element is substantially larger, sedimenting near 9 S (5, 6). Second, the TTX/STX-binding activity is unstable in the presence of excess detergent or during procedures that may deplete the solubilized protein of bound lipid. In contrast, the *Leiurus* neurotoxin binding activity described here is stable to extraction in 5% Triton X-100 and survives isolation from preparative sucrose gradients in the absence of exogenous lipids.

There are two possible general explanations for the observed differences. The first is that the two binding activities reflect the existence of two distinct channel components, one sedimenting near 9 S, the second near 6.5 S. The second is that the 6.5S *Leiurus* binding activity represents a subunit of the 9S complex and that scorpion toxin binding is retained under conditions that lead to the dissocation of the 9S complex and loss of TTX/STX-binding activity.

The sucrose gradients shown here do in fact contain a small amount of *Leiurus* neurotoxin bound to a 9S component. The conclusion that this may represent interaction with a sodium channel element is complicated by the observation that the *Leiurus* neurotoxins may also recognize acetylcholine receptor. Evidence that at least one *Leiurus* neurotoxin shows acetylcholine receptor agonist activity will be presented in a separate communication. These observations suggest the need for caution in the interpretation of experiments employing *Leiurus* neurotoxins as selective affinity ligands for voltage-dependent sodium channels.

The invaluable technical support of Deborah A. Mayka and Laura N. Dietch is gratefully acknowledged. The authors thank Dr. Stanley Froehner for providing the purified α-bungarotoxin used in some of the experiments described here and Dr. Froehner and Dr. Robert Jackson for their comments on the manuscript. This work was supported by the Muscular Dystrophy Association of America and the U.S. Public Health Service, Grants NS-00058 and NS-12067.

1. Kao, C. Y. (1966) *Pharmacol. Rev.* **18**, 997–1049.
2. Armstrong, C. M. & Bezanilla, F. (1974) *J. Gen. Physiol.* **63**, 533–552.
3. Keynes, R. D. & Rojas, E. (1974) *J. Physiol. (London)* **239**, 393–434.
4. Ritchie, J. M. & Rogart, R. B. (1977) *Rev. Physiol. Biochem. Pharmacol.* **79**, 1–51.
5. Agnew, W. S., Levinson, S. R., Brabson, J. S. & Raftery, M. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2606–2610.
7. Agnew, W. E. & Raftery, M. A. (1979) *Biochemistry* **18**, 1912–1919.
8. Cahalan, M. D. (1975) *J. Physiol. (London)* **244**, 511–534.
9. Koppenhofer, E. & Schmidt, H. (1968) *Pflugers Arch.* **303**, 133–149.
10. Koppenhofer, E. & Schmidt, H. (1968) *Pflugers Arch.* **303**, 150–161.
11. Miranda, F., Kupeyan, C., Rochat, H., Rochat, C. & Lissitzky, S. (1970) *Eur. J. Biochem.* **16**, 514–523.
12. Rochat, H., Rochat, C., Kupeyan, C., Miranda, F., Lissitzky, S. & Edman, P. (1970) *FEBS Lett.* **10**, 349–351.
13. Catterall, W. A. (1976) *J. Biol. Chem.* **251**, 5528–5536.
14. Catterall, W. A. (1977) *J. Biol. Chem.* **252**, 8660–8668.
15. Catterall, W. A. (1979) *J. Gen. Physiol.* **74**, 375–392.
16. Ray, R., Morrow, C. S. & Catterall, W. A. (1978) *J. Biol. Chem.* **253**, 7307–7313.
17. Beneski, D. A. & Catterall, W. A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 639–642.
18. Jover, E., Martins-Moutat, N., Couraud, F. & Rochat, H. (1978) *Biochem. Biophys. Res. Commun.* **85**, 377–382.
19. Karonen, S. L., Morsky, P., Siren, M. & Seuderling, U. (1975) *Anal. Biochem.* **67**, 1–10.