Purification of a Toxic Protein from Scorpion Venom Which Activates the Action Potential Na\(^+\) Ionophore

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Venom of the scorpion *Leiurus quinquestriatus* acts cooperatively with the alkaloids veratridine, aconitine, and batrachotoxin in activating the action potential Na\(^+\) ionophore. A small (7, 6700), basic (pI \(
\approx
\) 8.9), toxic polypeptide purified approximately 80-fold from this venom by ion exchange chromatography appears homogeneous by gel electrophoresis and isoelectric focusing and, like whole venom, acts cooperatively with the alkaloids veratridine, aconitine, and batrachotoxin to activate the action potential Na\(^+\) ionophore. The action of the scorpion toxin is slowly reversible. Concentration-response curves suggest interaction with a single class of sites with \(K_D = 1.3\) to 2.4 nM. The scorpion toxin is a poor activator of the Na\(^+\) ionophore when tested alone. However, treatment of cells sequentially with scorpion toxin followed by veratridine activates as well as treatment with both simultaneously suggesting that scorpion toxin binds in the absence of veratridine but does not activate the Na\(^+\) ionophore unless veratridine is present. In contrast, scorpion toxin causes 3- to 20-fold decreases in apparent \(K_D\) for aconitine, veratridine, and batrachotoxin. The effect of the toxin is inhibited competitively by divalent cations and noncompetitively by tetrodotoxin (\(K_I = 4 \text{nM}\)).

Several neurotoxins cause repetitive action potentials and persistent depolarization of nerves. This group of toxins includes the alkaloids veratridine (1, 2), batrachotoxin (3), and aconitine (4, 5), grayanotoxin (6), and the polypeptide toxins of scorpion venom (7-11), and coelenterate nematocysts (12). Since the action of these toxins is blocked by tetrodotoxin, a specific inhibitor of the action potential Na\(^+\) current (13, 14), their effects have been ascribed to activation of the action potential Na\(^+\) ionophore. These toxins, therefore, are potentially important tools in studying the mechanism of action potential generation.

Voltage clamp studies of three species of scorpion venom have been carried out (7, 9, 10). Different effects of the various species of venom have been reported including inhibition of Na\(^+\) current inactivation (7, 9), modification of Na\(^+\) current activation (10), and suppression of K\(^+\) current (7, 9, 10). Toxins have been purified from five scorpion venoms (10-17). They are basic proteins, have molecular weights of approximately 7,000, and lack methionine (15, 16). Recently, one of these toxins has been studied using the voltage clamp procedure (18) in invertebrate giant axons. At concentrations of 0.25 to 4 \(\mu\)M, the toxin caused inhibition of Na\(^+\) current inactivation, slowing of Na\(^+\) current activation, and suppression of K\(^+\) current in axons of one or more of the three species tested.

Clonal lines of mouse neuroblastoma cells grown in vitro are electrically excitable (19, 20). The Na\(^+\)-dependent portion of the action potential is inhibited by tetrodotoxin at low concentration, suggesting that an action potential Na\(^+\) ionophore identical with that in nerve axons is present in these cells (21, 22). Variant cell clones have been obtained which specifically lack the depolarizing phase of the action potential (22, 23). Treatment of electrically excitable neuroblastoma cells with veratridine, batrachotoxin, aconitine, or scorpion venom results in a marked increase in Na\(^+\) permeability detectable by measurements of passive \(^{22}\text{Na}^+\) uptake (24, 25). Two kinds of evidence indicate that this increase in Na\(^+\) permeability reflects ion transport activity of the action potential Na\(^+\) ionophore: (a) it is completely inhibited by low concentrations of tetrodotoxin (24) and (b) variant neuroblastoma clones specifically lacking the depolarizing phase of the action potential do not respond to these toxins (24). Using this approach, we have found that the alkaloid toxins, veratridine, batrachotoxin, and aconitine compete for a single site (25, 26) while scorpion venom acts at a separate site (25). These two sites appear cooperatively coupled (25) suggesting that these neurotoxins activate the action potential Na\(^+\) ionophore by an allosteric mechanism. In this report, we describe a convenient procedure for purification of the active protein from venom of the scorpion *Leiurus quinquestriatus* and examine the activation of the action potential Na\(^+\) ionophore by this protein.

**EXPERIMENTAL PROCEDURE**

**Materials**—Chemicals were obtained from the following sources: veratridine and electrophoresis grade acrylamide from Aldrich; aconitine from K and K; tetrodotoxin from Calbiochem; ouabain and scorpion (*Leiurus quinquestriatus*) venom from Sigma; \(^{4,5}\text{H}\)leucine from New England Nuclear; Amberlite CG-50 from Mallinckrodt was purified by the method of Hirs (27) before use;
enzyme grade urea from Schwarz/Mann; especially pure sodium dodecyl sulfate from BDH Chemicals; and ampholines from LKB.

Batrachotoxin was kindly provided by Drs. J. Daly and B. Witkop (Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health). Stock solutions of batrachotoxin and aconitine were prepared in ethanol at 50 to 100 times the desired final concentration and then diluted into assay medium at 36°C immediately prior to use.

Cell Cultures—Clone N18 of mouse neuroblastoma C1300 was used for all studies. Cells were grown essentially as previously described (25).

Measurements of "Na⁺ Uptake.—Measurement of the rapid rate of "Na⁺ uptake caused by treatment of cells with scorpion toxin and either veratridine, batrachotoxin, or aconitine required modification of the procedures previously described (26). Washout of "Na⁺ at 0°C in the wash medium previously described ([Na⁺] = 150 mM; [K⁺] = 5.5 mM) had a halftime of 1.5 min after treatment of cells with veratridine and scorpion toxin (Fig. 1). Thus, significant loss of "Na⁺ would have occurred during the usual 15-s wash period. Replacement of NaCl by choline chloride increased the halftime to 2.5 min, and replacement of both NaCl and KCl by choline chloride increased the halftime to 10 min. A wash medium consisting of 165 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM CaCl₂, and 0.8 mM MgSO₄ was used in subsequent experiments.

Since the toxins under study act slowly, cells were equilibrated with toxins for a short initial rate of "Na⁺ uptake was measured. This incubation was carried out at 36°C in Na⁺-free medium so that the increase in Na⁺ permeability caused by the toxins would not cause uptake of nonradioactive Na⁺ during toxin treatment. The medium used for toxin incubation consisted of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM MgSO₄, 5.4 mM KCl, and 1 mg/ml of bovine serum albumin.

After equilibration with toxins, the initial rate of "Na⁺ uptake was determined in a medium consisting of 120 mM choline chloride, 10 mM NaCl, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 5 mM ouabain, 0.8 mM MgSO₄, and 5.4 mM KCl. Under these conditions, the uptake of "Na⁺ was linear with time for 1 to 2 min (Fig. 2A). Unless otherwise indicated, uptake was measured for 0.5 min. The initial rate of "Na⁺ uptake provides a measure of Na⁺ permeability only if the rate of movement of counterions (K⁺ efflux, Cl⁻ influx) is sufficient to balance the charge carried in by Na⁺. Since the rate of "Na⁺ uptake is linear with increasing [Na⁺] up to 15 to 20 mM in the presence of saturating concentrations of veratridine and scorpion toxin (Fig. 2B), this condition is fulfilled at Na⁺ concentrations less than 15 mM. A concentration of 10 mM was used in subsequent experiments.

Uptake of "Na⁺ was terminated by washing three times with 3 ml of the wash medium described above. Cells were suspended in 0.4 M NaOH and radioactivity determined as described previously (26). Results are presented as nanomoles of "Na⁺ taken up per min of assay time per mg of cell protein (determined by the method of Lowry et al. (28)).

Data Analysis—The data for activation of action potential Na⁺ ionophore activity were analyzed by letting V₀ represent the velocity at infinite activator concentrations and fitting velocities (v) at different activator concentrations (A) to a hyperbolic saturation curve of the form v = V₀A/(Kₐ + A) where Kₐ is taken as the apparent dissociation constant of the activator. Values of Kₐ and V₀ were obtained by fitting the experimental data directly to the hyperbolic form of the above equation using an iterative least-squares curve-fitting algorithm. If either Kₐ or V₀ appeared constant under different experimental conditions, a simultaneous fit of data under all conditions was made assuming a constant Kₐ or V₀. The best fit values of Kₐ and V₀ are given in the figure legends.

Gel Electrophoresis—Gel electrophoresis at pH 4.5 in the β-alanine/acetate buffer system of Reisfeld et al. (29) was carried out in the presence of urea as described by Swank and Munkres (32). Gels (5 x 75 mm) were prepared by ammonium persulfate-catalyzed polymerization of a solution consisting of 18% (w/v) acrylamide, 0.47% (w/v) N,N'-methylenebisacrylamide, 0.1 M H₃PO₄ (adjusted to pH 6.8 with Tris base), 0.1% (w/v) sodium dodecyl sulfate, 0.075% (w/v) N,N',N'-tetramethylglycine, and 8.0 M urea. Samples (100 μl) were incubated at 100°C for 10 min in 1% (w/v) sodium dodecyl sulfate, 1% (v/v) mercaptoethanol, and 0.01 M Tris/phosphate (pH 6.8), and subjected to electrophoresis toward the anode at 1 mA/gel with 0.25% Tris/phosphate (pH 6.8) and 0.1% (w/v) sodium dodecyl sulfate as the electrode buffer.

Gels run in either electrophoretic system were fixed and stained by immersion in 0.25% Coomassie blue in 10% (v/v) acetic acid, 20% (v/v) methanol and destained in a dialysis destainer in 10% acetic acid, 20% methanol.

Isoelectric Focusing—Isoelectric focusing was performed in poly-
acrylamide gels essentially as described by Wrigley (33). Gels (5 × 75 mm) were prepared by ammonium persulfate-catalyzed polymerization of a solution consisting of 7.5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.05% (v/v) N,N',N'-tetramethylethylenediamine, 2.2% (v/v) Ampholine 9-11, and 0.4% (v/v) Ampholine 7-9. Samples (100 μl) in 10% (w/v) sucrose, 0.1% (v/v) Ampholine 7-9 were overlaid with 100 μl of 5% (w/v) sucrose, 0.1% (v/v) Ampholine 7-9, and the tubes were filled with anode buffer (10 mM sodium acetate). Gels (5 × 75 bands could be visualized as white precipitates. In some experiments, cold water was circulated. An electric field of 150 V was applied for 2.2% (v/v) Ampholine 9-11, and 0.4% (v/v) Ampholine 7-9. Samples (100 μl) in 10% (w/v) sucrose, 0.1% (v/v) Ampholine 7-9 were overlaid with 100 μl of 5% (w/v) sucrose, 0.1% (v/v) Ampholine 7-9, and the tubes were filled with anode buffer (10 mM sodium acetate). The gels were immersed in cathode buffer (100 mM NaOH) in a standard disc gel apparatus equipped with a water jacket through which cold water was circulated. An electric field of 150 V was applied for 2.5 h. During this time the cytochrome c used as a marker migrated to the middle or end of the peak (Fig. 5B). The stain intensity in the fastest moving band correlates well with the amount of toxin activity present in individual fractions throughout the peak.

Fractions which stimulated 22Na+ uptake and contained only one protein band in gel electrophoresis were pooled for further analysis. Since samples of approximately 10 μg were run on these gels under conditions where 0.2 μg can be observed by staining procedures, contaminants comprising 2% of the protein of an individual fraction could have been observed. To minimize contamination of the preparation by the two proteins in the beginning of the peak, the first three fractions having only one protein band on gels were usually not included in the pooled fractions. In the experiment illustrated in Fig. 4, Fractions 175 to 190 were pooled yielding 247 μg of purified toxin. We have found it necessary to analyze individual fractions by gel electrophoresis in each preparation to determine which fractions to include for further study.

The preparations were analyzed by two other procedures to test for purity. The protein migrated as a single band in sodium dodecyl sulfate gel electrophoresis in the presence or absence of urea (Fig. 5D). This is not a particularly sensitive test of purity, however, since most of the protein of the H2O extract of venom migrates in the same area of the gel and the resolution is poor. The protein (30 μg) also forms a single band in acrylamide gel isoelectric focusing experiments using Ampholine 9-11 (Fig. 5F). Scorpion venom (1 mg) was run under identical conditions (Fig. 5E). Only a small fraction of the input protein fraction in the pI 9 to 11 gradient giving six bands in the range of pH 9.5 to 10. Thus gel isoelectric focusing provides a sensitive test of the purity of the preparation. The single band from the purified toxin coincides with the most basic of the

![Figure 3](http://www.jbc.org.) Increase of 22Na+ uptake by scorpion venom and purified scorpion toxin in the presence of aconitine, veratridine, or batrachotoxin. N18 cells were incubated for 30 min at 36°C with the indicated concentrations of scorpion venom (A) or purified scorpion toxin (B) alone (●) or with 0.1 μM batrachotoxin (Δ), 20 μM veratridine (○), or 30 μM aconitine (□). Initial rates of 22Na+ uptake were then measured as described under “Experimental Procedure.”
bands from the venom. Its isoelectric point, determined by comparison with the positions of RNase (pI = 9.4), cytochrome c (pI = 10.1), and lysozyme (pI = 11.1) is approximately 9.8. The staining conditions used in isoelectric focusing detect 0.3 µg of protein and we therefore conclude that the preparation contains no contaminants which comprise as much as 1% of the protein.

Migration of proteins in sodium dodecyl sulfate gels is a logarithmic function of their molecular weight (31). In calibrating our gels, we have used an approach similar to that described by Swank and Munkes (32) for determination of molecular weight of proteins less than 10,000. A calibration curve was generated in gels containing urea using 13 small molecular weight of proteins less than 10,000. A calibration curve was generated in gels containing urea using 13 small molecules with known molecular weights as standards. The calibration curve was constructed using the equation log (M) = 4.8 - 1.7 (RF). The purified protein had an RF of 0.57 in these experiments corresponding to a molecular weight of approximately 6,700.

Preliminary amino acid analyses also indicate that the protein lacks histidine. The apparent lack of two amino acids provides further evidence for the purity of the preparation.

The purified protein retains the ability to interact cooperatively with aconitine and batrachotoxin as well as veratridine (Fig. 3B). Comparison of the midpoints of the concentration-response curves for venom (0.8 µg/ml) and purified toxin (9 ng/ml) suggest that the activity has been purified almost 100-fold.

In contrast to the venom, the pure toxin has little, if any, capacity to increase 22Na+ uptake when tested alone (Fig. 3). Thus, it is likely that this property of venom requires another component removed in the purification.

Fig. 6 illustrates concentration-response curves for toxin in the presence of 2, 6, or 20 µM veratridine. The data are fit well by theoretical curves drawn for a common Kd (9.4 ng/ml) and Vmax values of 15.8, 29.8, and 50 nmol/min/mg. Similar results were obtained when toxin titration experiments were conducted in the presence of varying batrachotoxin and aconitine concentrations. Thus, the alkaloid toxins affect the maximum rate of 22Na+ uptake but do not affect the affinity for scorpion toxin.

The time dependence of scorpion toxin action in the presence of 20 µM veratridine is illustrated in Fig. 7A. At each concentration tested, the rate of 22Na+ uptake increases without a lag, reaches a maximum, and then declines. In order to test if the presence of veratridine is necessary for scorpion toxin action, cells were incubated with scorpion toxin for various times (Fig. 7B). 22Na+ uptake was inhibited by 1 µM tetrodotoxin in all fractions.
permeability are not observed unless veratridine or another prolonged incubation in toxin (Fig. 6). Na+-free medium containing either 256 rig/ml of scorpion toxin acts in the absence of veratridine but its effects on Na' concentration of veratridine (200 PM) was added in the assay presence of veratridine. Titration curves of Na+ uptake after treatments are presented in Fig. 8. They are indistinguishable. Similar results were obtained in experiments in which a high concentration of scorpion toxin for 30 min under these two sets of experimental conditions. In the experiments illustrated in Fig. 9A, cells were incubated in Na+-free medium containing either 200 ng/ml of scorpion toxintoxin, 200 µM veratridine, or toxin plus veratridine for varying times, then incubated with toxin plus veratridine for 5 min and finally assayed. Exposure to Na+-free solution or to scorpion toxin alone had little effect on the response to a subsequent 5 min treatment with toxin. Exposure to veratridine had a small effect while exposure to toxin plus veratridine caused a rapid loss of response to a subsequent treatment with toxin. These results suggest that activation of the ion transport activity of...
the ionophore is necessary for loss of responsiveness to occur. Accordingly, K⁺ levels in cells were measured to determine whether loss of intracellular K⁺ due to an increase in K⁺ permeability caused the loss of responsiveness by reducing the membrane potential and, therefore, the driving force for inward movement of ²⁺Na. Intracellular levels of K⁺ were reduced during exposure to high concentrations of toxin plus veratridine (Fig. 9B) but not during exposure to toxin alone. The loss of intracellular K⁺ was inhibited by tetrodotoxin (Fig. 9B) indicating that the efflux was mediated directly by the action potential Na⁺ ionophore. The specificity of the Na⁺ ionophore has been studied in voltage clamp experiments on nerve axons (Ref. 35 and references therein). The ratio of Na⁺ conductance to K⁺ conductance of the Na⁺ ionophore is approximately 12. The ratio of half-times for equilibration of ²⁺Na⁺ (approximately 1 min after treatment with saturating toxin concentrations) and for loss of intracellular ²⁺K⁺ (approximately 20 min) under these conditions is similar.

The loss of K⁺ represents a potential source of error in experiments in which cells are incubated with combinations of scorpion toxin plus an alkaloid toxin. This effect is exaggerated, however, in the experiments of Fig. 9 since supersaturating concentrations of both veratridine (200 µM) and scorpion toxin (256 ng/ml) were used. Under these conditions, a large permeability is generated almost immediately and substantial loss of K⁺ occurs. At the lower concentrations of scorpion toxin and veratridine employed in the titration experiments presented here, these effects are minimal. This conclusion is supported by experiments like the one illustrated in Fig. 8 in which incubations with toxin alone or with toxin plus veratridine give similar results.

The action of scorpion toxin is slowly reversible (Fig. 10) over a 1-h time course in the absence of veratridine. Veratridine does not affect the reversibility of the scorpion toxin effect. Thus veratridine has little effect on the rate of scorpion toxin action, the rate of reversal of scorpion toxin action, or the apparent dissociation constant of scorpion toxin action. Aconitine and batrachotoxin have little effect on the apparent dissociation constant for scorpion toxin and presumably have no effect on rates of action and reversal, although direct experiments with these slower acting agents have not been carried out.

Scorpion venom causes a decrease in the apparent dissociation constant of veratridine, aconitine, and batrachotoxin (25) in addition to increasing Vₐ. The purified scorpion toxin acts similarly. Data from titrations of veratridine at different scorpion toxin concentrations conform closely to theoretical curves drawn assuming interaction with a single class of binding sites (Fig. 11). In this experiment, Kₐₜ (veratridine) was reduced from 24 to 7 µM and Vₐ was increased from 17.5 to 111 nmol/min/mg by treatment with scorpion toxin. Similar results are obtained in experiments in which aconitine titrations are carried out in the presence of varying scorpion toxin concentrations.

Data from experiments in which batrachotoxin titrations were conducted in the presence of scorpion toxin did not follow the theoretical curves closely. Further analysis revealed that data at a low concentration of batrachotoxin fit the theoretical curves adequately whereas data from concentrations greater than Kₐₜ gave a smaller rate of uptake than expected. These results did not reflect homotropic cooperativity because log-log plots of the data had slopes of 1.0 throughout the linear range. Since incubation with scorpion toxin plus a high concentration of batrachotoxin causes substantial loss of K⁺, it is probable that the high concentration data are in error due to reduction of the driving force for Na⁺ uptake. In order to obtain a concentration-response curve for batrachotoxin under these conditions, complete time courses for batrachotoxin action in the presence and absence of scorpion toxin were carried out essentially as in Fig. 7A for 12 different concentrations of batrachotoxin. Data from each time course which reached a stable plateau level of ²⁺Na⁺ uptake were used to construct a concentration-response curve illustrated as a double reciprocal

**Fig. 10.** Reversibility of scorpion toxin action. N18 cells were incubated for the indicated times in Na⁺-free medium containing 50.5 ng/ml of scorpion toxin and then ²⁺Na⁺ uptake was determined in assay medium containing a concentration of veratridine (200 µM) which has an immediate effect (O). Other cells were incubated 10 min as above, rinsed to remove scorpion toxin, and incubated for the indicated times in Na⁺-free medium without scorpion toxin (G). Initial rates of ²⁺Na⁺ uptake were then determined as above.

**Fig. 11.** Effect of scorpion toxin on increase of ²⁺Na⁺ uptake by veratridine. N18 cells were incubated for 30 min at 36°C in Na⁺-free medium containing the indicated concentrations of veratridine and no scorpion toxin (D), 4 µg/ml of scorpion toxin (G), 12.8 µg/ml (CL), or 128 µg/ml (△) of scorpion toxin. Initial rates of ²⁺Na⁺ uptake were determined as described under "Experimental Procedure." Best fit curves are drawn as described under "Experimental Procedure" for Kₐₜ = 24 µM and Vₐ = 17.5 nmol/min/mg with no scorpion toxin, Kₐₜ = 20 and Vₐ = 45.5 with 4 ng/ml, Kₐₜ = 10.5 and Vₐ = 61 with 12.8 ng/ml, and Kₐₜ = 6.9 and Vₐ = 111 with 128 ng/ml of scorpion toxin.
Divalent cations inhibit activation of the action potential Na⁺ ionophore by veratridine and batrachotoxin competitively (26). In order to test whether divalent cations inhibit the action of scorpion toxin, cells were incubated with scorpion toxin in the presence of divalent cations, washed free of divalent cations, and then assayed in the presence of a concentration of veratridine (200 μM) that causes complete activation of the ionophore without prolonged incubation (26). Divalent cations at a concentration of 5 mM caused an increase in $K_{a}$, for scorpion toxin (Fig. 13) and, in some experiments, a small decrease in $V_{m}$. The variable decrease in $V_{m}$ can probably be ascribed to inability to wash out all divalent cations from the scorpion toxin incubation with resultant partial inhibition of the action of veratridine. The effect on $K_{a}$ appears to reflect competitive inhibition of scorpion toxin action by divalent cations. The divalent cation specificity is $\text{Mn}^{2+} > \text{Ca}^{2+} > \text{Ni}^{2+} > \text{Mg}^{2+} > \text{Co}^{2+} > \text{Sr}^{2+}$ which is somewhat different from the specificity observed for competitive inhibition of the action of veratridine and batrachotoxin (26). Divalent cations are approximately $\alpha$-fold less effective in inhibiting scorpion toxin action.

Tetrodotoxin is a noncompetitive inhibitor of the increase in $^{22}\text{Na}^{+}$ uptake caused by veratridine or batrachotoxin (26). It also inhibits the action of scorpion toxin noncompetitively (Fig. 14) with a $K_{i}$ of approximately 4 mM. Thus tetrodotoxin inhibits the action potential Na⁺ ionophore by interaction with a site that is separate from the sites of action of scorpion toxin and the alkaloid toxins. This site may be a coordination site for transported monovalent cations (36).

**DISCUSSION**

Scorpion venoms have diverse effects on the regulation of activity of the action potential Na⁺ ionophore (7-11). The venom of the North African scorpion *Leiurus quinguestriatus* acts cooperatively with the alkaloid neurotoxins veratridine, batrachotoxin, and aconitine to cause activation of the action potential Na⁺ ionophore (25). In this report, we describe the purification of a toxic protein from this venom using its ability to activate the action potential Na⁺ ionophore as a specific assay. The purified protein contains all of the activity of the venom in this assay system and interacts cooperatively with all three alkaloid toxins.

![Fig. 12](image1.png)

**Fig. 12.** Effect of scorpion toxin on increase of $^{22}\text{Na}^{+}$ uptake by batrachotoxin. N18 cells were incubated for 2, 5, 10, 15, or 20 min at 36°C in Na⁺-free medium containing from 0.01 to 3 μM batrachotoxin and no scorpion toxin (O) or 107 ng/ml of scorpion toxin (■). Initial rates of $^{22}\text{Na}^{+}$ uptake were determined as described under "Experimental Procedure." The value of $K_{m}$ during the plateau in each time course is plotted as a function of batrachotoxin concentration in a double reciprocal plot. Data from time curves which did not maintain a plateau level of $^{22}\text{Na}^{+}$ uptake were discarded. The best fit curves chosen as described under "Experimental Procedure" correspond to $K_{a} = 2$ and $V_{m} = 125$ in the absence of scorpion toxin and $K_{a} = 0.2$ and $V_{m} = 125$ in 107 ng/ml of scorpion toxin.

![Fig. 13](image2.png)

**Fig. 13.** Effect of divalent cations on scorpion toxin action. N18 cells were incubated for 30 min at 36°C in Na⁺-free medium containing the indicated concentrations of scorpion toxin and no added divalent cation (O), 5 mM SrCl₂ (△), 5 mM CaCl₂ (●), or 5 mM MnCl₂ (■). Initial rates of $^{22}\text{Na}^{+}$ uptake were determined in medium containing 200 μM veratridine as described under "Experimental Procedure." Curves are drawn for a fit to a common $V_{m} = 64$ nmol/min/mg and $K_{i} = 19.2$ nmol/min/culture at 2 nM tetrodotoxin (A), 5 nM tetrodotoxin (A), 5 nM tetrodotoxin (O), or 10 nM tetrodotoxin (O). Initial rates of $^{22}\text{Na}^{+}$ uptake were determined as described under "Experimental Procedure" in assay medium containing 200 μM veratridine and the same tetrodotoxin concentrations. Best fit curves are drawn as described under "Experimental Procedure." The best fit curves correspond to $K_{a} = 11$ ng/ml and $V_{m} = 26$ nmol/min/culture at 0 nM tetrodotoxin, 19.3 nmol/min/culture at 2 nM, 11.6 nmol/min/culture at 5 nM, and 8.4 nmol/min/culture at 10 nM.

![Fig. 14](image3.png)

**Fig. 14.** Inhibition of $^{22}\text{Na}^{+}$ uptake by tetrodotoxin. N18 cells were incubated for 30 min at 36°C in Na⁺-free medium containing the indicated concentrations of scorpion toxin with no tetrodotoxin (O), 2 nM tetrodotoxin (△), 5 nM tetrodotoxin (●), or 10 nM tetrodotoxin (■). Initial rates of $^{22}\text{Na}^{+}$ uptake were determined as described under "Experimental Procedure" in assay medium containing 200 μM veratridine and the same tetrodotoxin concentrations. Best fit curves are drawn as described under "Experimental Procedure." The best fit curves correspond to $K_{a} = 11$ ng/ml and $V_{m} = 26$ nmol/min/culture at 0 nM tetrodotoxin, 19.3 nmol/min/culture at 2 nM, 11.6 nmol/min/culture at 5 nM, and 8.4 nmol/min/culture at 10 nM.
Analysis of the preparation by gel electrophoresis and isoelectric focusing indicates that the protein is highly purified. Sequence studies of toxic proteins from scorpion venoms suggest considerable homology among different toxins (37, 38) and in one case indicate that an apparently homogeneous toxin preparation contained two toxins differing in only 1 amino acid residue (39). The results presented here, while providing strong evidence for purity, do not exclude the presence of such closely related proteins in the preparation.

In analyzing the results, we have assumed (see “Experimental Procedure”) that scorpion toxin modifies the properties of the Na+ ionophore by reversible, noncovalent interaction rather than by causing conformational changes in the ionophore or the surrounding membrane by enzymatic action. The data presented support this conclusion. Thus, the toxin acts without a detectable lag time (Fig. 7); the rate and extent of toxin action are concentration-dependent (Fig. 7); its action is reversible (Fig. 10); and the concentration dependence is consistent with a simple binding reaction. Other experiments not described in this report also support this interpretation. Assays for protease activity using denatured casein as substrate and for phospholipase activity using egg lecithin as substrate were negative, even when toxin concentrations greatly exceeding those required for activation of the Na+ ionophore were tested. Binding experiments using radioactively labeled toxin detect a small number of specific binding sites in electrically excitable neuroblastoma cells, but not in inexcitable cells. While these results do not rigorously exclude involvement of enzymatic activity in toxin action, they favor the conclusion that only reversible, noncovalent interaction with the Na+ ionophore is required.

The purified scorpion toxin reduces the apparent dissociation constant for batrachotoxin, veratridine, and aconitine and veratridine and aconitine. Thus, there is an apparent heterotrophic cooperative interaction between the sites of action of the alkaloid toxins and scorpion toxin. Homotropic cooperative interactions were not observed in studies of activation of the Na+ ionophore by veratridine and batrachotoxin (20). The concentration-response data for scorpion toxin under various conditions (Figs. 6, 7, 12, 13) are fit closely by theoretical curves drawn for reversible interaction with a single class of independent binding sites having a dissociation constant of 9 to 15 nM (1.3 to 2.3 nM). Thus, these experiments do not detect homotropic cooperative interactions. In our early experiments with purified scorpion toxin, we consistently observed apparent dissociation constants of 100 to 1000 nM and cooperative concentration-response curves corresponding to Hill coefficients from 2 to 5. Subsequent experiments showed this behavior to be an artifact caused by inactivation of the toxin and/or irreversible adsorption of the toxin to labware at very low concentrations. These effects were eliminated by inclusion of a high concentration of a “carrier” protein (bovine serum albumin or gelatin) in solutions containing toxin.

Despite the large effect of scorpion toxin on apparent Kd for alkaloid toxins, careful analysis did not reveal an effect of alkaloid toxins on the affinity of cells for scorpion toxin or on the rate of action of the toxin. Thus, the heterotropic cooperativity observed is not reciprocal. The mechanism of this interaction remains uncertain.

Tetrodotoxin inhibits the increase in 22Na+ uptake caused by scorpion toxin, but has no effect on the apparent dissociation constant for scorpion toxin. Thus, the results with purified scorpion toxin support a hypothesis presented previously (28) which suggests that the action potential Na+ ionophore complex includes three functionally separable components: two regulatory components which specifically bind either alkaloid toxins or scorpion toxin and interact allosterically in controlling the activity of a third ion transport component which binds tetrodotoxin. The relationship between these hypothetical regulatory sites and the processes of activation and inactivation of the Na+ ionophore as described in the Hodgkin-Huxley model of ionophore function (40) is uncertain. Batrachotoxin, veratridine, and aconitine appear to affect both activation and inactivation (2, 4, 41, 42). Leiurus quinques-tratus venom affects inactivation specifically in the frog node of Ranvier (7). Since the purified toxin from Leiurus quinques-tratus venom has little capacity to activate the Na+ ionophore when tested alone, it may exert its effects by specifically inhibiting the process of inactivation.

Purification of the action potential Na+ ionophore and examination of its molecular properties requires use of ligands which bind specifically to the Na+ ionophore. The purified scorpion toxin described in this report should prove useful in studies of activation and inactivation of the Na+ ionophore and, in experiments to be described subsequently, bind specifically to cells containing the Na+ ionophore.

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