ISOLATION OF A SCORPION TOXIN FOR USE AS A PROBE OF THE ELECTRICALLY EXCITABLE SODIUM CHANNEL

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SUMMARY. Fractionation of the venom of the scorpion Tityus serrulatus yielded two toxic components that caused an increase in the uptake of $^{22}Na^+$ by neuroblastoma cells. Scorpion toxins and veratridine stimulated $^{22}Na^+$ uptake by cells in a synergistic manner while tetrodotoxin blocked this effect. A radiolabeled derivative of scorpion toxin retained biological activity similar to unmodified material.

The electrically excitable sodium channel (1), is defined as a component of nerve and muscle membranes that mediates transient permeability of the membrane to sodium ions in response to a small local depolarization (2,3). This transient change in sodium permeability is the first in a series of steps that result in the propagated nerve impulse or action potential (4,5). Tetrodotoxin (TTX)[1] is a potent neurotoxin that binds specifically to the sodium channel and blocks the inward passage of sodium ions (6,7). To date, radiolabeled TTX has been the only readily available probe for use in biochemical characterization of the sodium channel (8-12). Various alkaloid neurotoxins, such as veratridine, grayanotoxin, aconitine and batrachotoxin appear to interact with the sodium channel in a regulatory manner (1). With the exception of batrachotoxin, the low binding affinities of the alkaloid neurotoxins limit their utility as specific probes of sodium channels.

Several reports suggest that components of scorpion venoms also interact with the sodium channel (13-15). A number of toxins have been isolated from these venoms and characterized; all are small (mol. wt. 6,000-8,000) basic proteins (16-18). They affect nerve preparations by causing a prolonged depolarization ("delayed sodium inactivation" (14,19,20)) and occassionally stimu-

[1] Abbreviations: tetrodotoxin, TTX; ammonium acetate, NH_4Ac.

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lating spontaneous depolarizations (13,21). Catterall (1) demonstrated that a toxic fraction from venom of the scorpion Leirus quinquestriatus caused a TTX sensitive stimulation of $^{22}Na^+$ uptake by neuroblastoma cells similar to that caused by alkaloid neurotoxins. The combined effects of alkaloid neurotoxins and scorpion toxin appeared to be synergistic. From this and the work of Romey et al. (20) and Balerna et al. (22), it appears that scorpion toxins interact with a site on the sodium channel that is nonidentical with the TTX and alkaloid binding sites.

Since these toxins are small proteins, they are likely to be more amenable to chemical modification than TTX or the alkaloid neurotoxins and thus might be useful probes for the sodium channel not only in situ but in preparations isolated from sources such as garfish nerve (9,12,23) and the electroplax of electric eel (24). We present here studies of the isolation and characterization of two toxins from the venom of the scorpion Tityus serrulatus that stimulate $^{22}Na^+$ uptake in electrically excitable neuroblastoma cells.

EXPERIMENTAL

MATERIALS. Lyophilized Tityus serrulatus venom was obtained from Sigma Chemical Co. Veratridine was purchased from Aldrich Co., ouabain and trypsin from Sigma Chemical Co., and tetrodotoxin was obtained from Sankyo, Ltd., Japan. Schwarz Mann Ultra-Pure urea was freshly prepared and deionized before use. All other chemicals were reagent grade and obtained from commerical sources. Chromatographic resins were obtained from Bio-Rad Labs. Fetal calf serum and Dulbecco's modified Eagle's medium were obtained from Gibco. Falcon plastic tissue culture dishes and flasks were used for growth of neuroblastoma cells. Aqueous $^{22}NaCl$ was obtained from Amersham Radiochemical Centre and carrier-free $Na^{125}I$ was from International Chemical and Nuclear Co.

METHODS

Stock cultures of the clonal neuroblastoma cell line N18 were the kind gifts of Drs. Harvey Herschman and William C. Catterall. Cultures were maintained in 60 mm petri dishes containing 5 ml growth medium (10% fetal calf serum, 90% Dulbecco's modified Eagle's medium) in a water saturated atmosphere of 10% CO_2/90% air at 37°C. Cells were subcultured as described by Catterall (1).

Assays of the effect of neurotoxins on $^{22}Na^+$ uptake by cultured N18 cells were performed as described by Catterall (1).

Fractionation of scorpion venom. Crude venom (250 mg) was extracted for 30 min with 5 mM NH_4Ac containing 6 M urea at 4°C. Insoluble material was removed by centrifugation for 10 min at 12,000 g. The yellow supernatant was removed and the residue re-extracted. Pooled supernatant fractions were applied to a 500 ml column of Bio-Gel P-30 and eluted with 5 mM NH_4Ac/6 M urea. Fractions were pooled as indicated and processed to remove urea as follows: the pooled material was diluted to give a concentration of 2 mM NH_4Ac and passed over a 5 ml column of BioRex 70 (NH_4^+ form). The resin was then washed extensively with 1 mM NH_4Ac, and the protein eluted with 0.5 M NH_4Ac. This material was repeatedly lyophilized. Samples of each peak from the P-30 column were assayed for ability to stimulate $^{22}Na^+$ uptake in neuroblastoma cells, and fractions stimulating $^{22}Na^+$ uptake in the cell assay were subjected to further purification by ion-exchange chromatography.

For ion-exchange a sample of the lyophilized protein was suspended in buffer (5 mM NH_4Ac in 6 M urea), applied to a 100 ml bed of BioRex 70 (NH_4^+ form, preequilibrated in the same buffer) and eluted with a gradient of 5 mM to 300 mM NH_4Ac in 6 M urea. Pooled fractions were treated as described above to remove urea and NH_4Ac. Following lyophilization, the fractions were assayed for biological activity.

Analytical methods. Column eluates were monitored for protein content by absorbance at 280 nm. Parallel assays of protein content by the method of Lowry (26) gave essentially identical profiles. Homogeneity of pooled fractions was assessed by polyacrylamide gel electrophoresis as described by Swank and Munkres (27).

Iodination of scorpion toxin: One milligram of lyophilized protein was exposed to 2 mCi of $Na^{125}I$ in the presence of chloramine T and KI (28). The reaction mixture was chromatographed on a 45 ml bed of Bio-Gel P-2 in 0.25 M NH_4Ac to separate the iodinated protein from unreacted $Na^{125}I$. The fractions where radioactivity and protein were coincident were pooled and lyophilized several times. The product yielded an initial specific activity of 9.7×10^7 cpm/mg.

RESULTS

The elution profile of crude venom fractionated on a gel filtration column is shown in Fig. 1. The four protein peaks were pooled as shown and assayed for ability to stimulate $^{22}Na^+$ uptake in neuroblastoma cells. In Table 1 the activities of the four fractions are summarized. Peaks 3 and 4 appeared to contain comparable activities and these materials were subjected to further fractionation by ion-exchange chromatography.

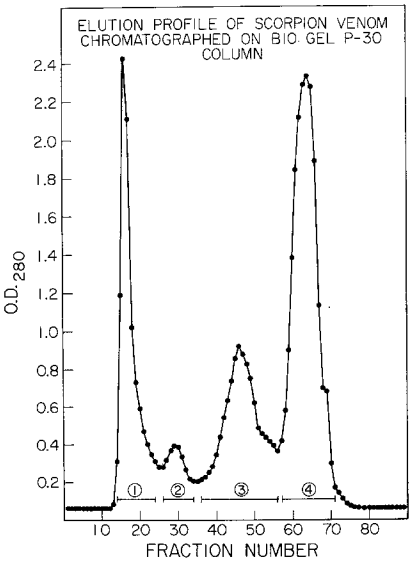

Figure 1. Gel-filtration of T. serrulatus venom on Bio-Gel P-30 in the presence of 6 M urea. The protocol is described in the experimental section. Fraction size was 8 ml.

TABLE 1

STIMULATION OF $^{22}Na^{+}$ UPTAKE BY POOLED FRACTIONS FROM BIO-GEL P-30 COLUMN

Fraction (10μg/ml)	$^{22}Na^{+}$ Uptake nmoles $min^{-1}mg^{-1}$ cell protein[a]
1	8.0
2	3.6
3	24.9
4	14.1

[a]Assays performed as described in text.

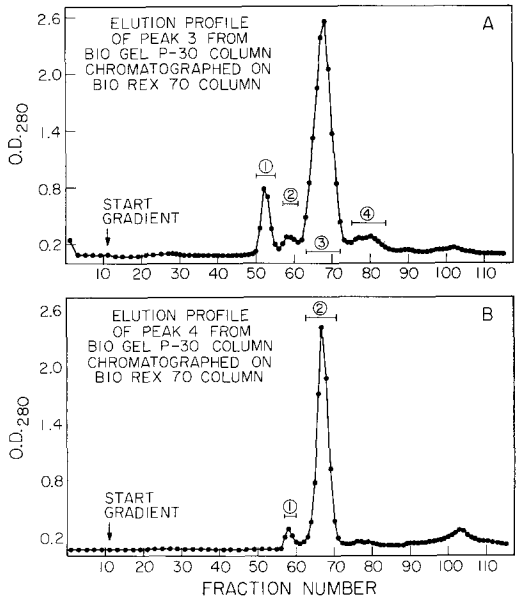

Figure 2. Ion-exchange chromatography on BioRex-70 in the presence of 6 M urea. Protocol is described in the experimental section. Fraction size was 2.8 ml. A. Ion exchange of pooled material from peak 3 of Fig. 1. B. Ion exchange of pooled material from peak 4 of Fig. 1.

Figs. 2A and 2B show the elution profiles from BioRex 70 columns of materials from Bio-Gel P-30 peaks 3 and 4 respectively. The activities of the fractions obtained from the BioRex 70 columns are shown in Table 2; fractions A3 and B2 contained the bulk of the activity in stimulating $^{22}Na^{+}$ uptake by neuroblastoma cells. Although these two active fractions were resolved

TABLE 2

STIMULATION OF $^{22}Na^+$ UPTAKE BY POOLED FRACTIONS FROM BIOREX 70 COLUMNS

nmoles $^{22}Na^+$ uptake $min^{-1} mg^{-1}$ cell protein[a]

FRACTION (5 g/ml)	NO ADDITIONS	100 nM TTX	200 M VERATRIDINE
A1	3.3	2.0	87.2
A2	21.6	3.2	167.2
A3	27.6	0	164.4
A4	4.7	0	123.6
-	-	-	38.3
B2	27.2	0	178.3

[a]Assays performed as described in text.

TABLE 3

REVERSIBILITY OF TOXIN STIMULATION OF $^{22}Na^+$ UPTAKE[a]

nmoles $^{22}Na^+$ uptake min^{-1} mg^{-1} cell protein

EFFECTOR PRESENT DURING PREINCUBATION	WASH CELLS (+) EFFECTOR	WASH CELLS (-) EFFECTOR
10µg/ml A3	23.1	13.3
10µg/ml B2	20.4	9.8
200 µM veratridine	23.5	0

[a]Experimental procedure described in text.

from different materials from the Bio-Gel P-30 column, no differences were observed in their properties upon ion-exchange chromatography, gel electrophoresis or assay of biological activity. A minimum molecular weight of 7,200 daltons was estimated from an amino acid analysis of toxin samples.

Effects of scorpion toxin on $^{22}Na^+$ uptake by neuroblastoma cells. The reversibility of the effects of toxin fractions A3 and B2 was compared with reversibility of stimulation of $^{22}Na^+$ uptake by veratridine, a compound known to bind to the sodium channel with low affinity (1,29). Cell cultures were incubated as usual for 30 min in the presence of test compounds, the preincubation buffer was removed and the cells incubated for 3 consecutive 10 min periods with buffer plus or minus test compounds. Radioactive $^{22}Na^+$ uptake was then assayed for 2 min. As shown in Table 3, the effects of veratridine were completely reversed

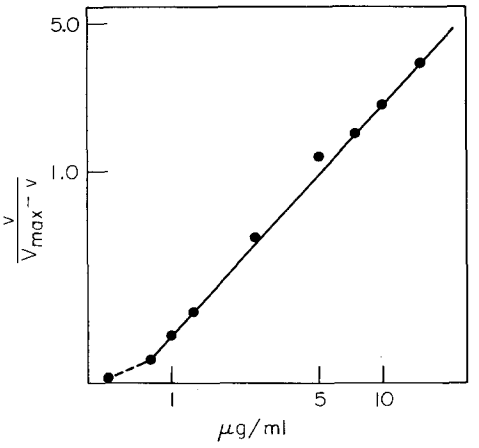

Figure 3. Hill plot of data obtained from dose-response experiment of scorpion toxin stimulated $^{22}Na^{+}$ uptake by neuroblastoma cells.

TABLE 4

STIMULATION OF $^{22}Na^{+}$ UPTAKE BY IODINATED TOXIN[a]

nmoles $^{22}Na^{+}$ uptake min^{-1} mg^{-1} cell protein

FRACTION	NO ADDITIONS	100nM TTX	200μM VERATRIDINE
^{125}I-A3 (4.8μg/ml)	26.6	9.3	204.4
A3 (5 μg/ml)	29.2	6.9	182.9
veratridine (200 μm)	39.8	0	–

[a]Assays performed as described in text.

by the 30 min wash. The effects of the two active scorpion toxin fractions were reduced to approximately one-half during this period.

Dose-response experiments with fractions A3 and B2 gave essentially identical results. Fig. 3 shows a Hill plot of these data. The apparent K_d was estimated at 690 nM and the Hill coefficient, n, was 1, suggesting that the toxin affected a single class of non-interacting sites.

Toxin fraction A3 was iodinated as described, and the data presented in

Table 4 shows that the iodinated toxin retained biological activity comparable to that of the unmodified material.

DISCUSSION

Published methods for the fractionation of T. serrulatus venom (30-32) proved unsatisfactory in our hands. Using the procedures described here, we have obtained two protein components from scorpion venom which differ in their gel filtration properties but appear to be identical by other physical chemical criteria and in their ability to interact with the electrically excitable sodium channel of neuroblastoma cells. As expected on the basis of earlier results with Leirus venom (1), the increased $^{22}Na^{+}$ uptake stimulated by T. serrulatus toxin is blocked by TTX and enhanced by veratridine. Under the conditions of our assays, the toxin appeared to bind to the sodium channel and its effects were slowly reversible. A radiolabeled derivative of the toxin retained the biological activity of the unmodified material. However, it was observed that the iodinated toxin was adsorbed to plastic and glassware and therefore the estimated K_d for the effect of toxin on stimulation of $^{22}Na^{+}$ uptake by neuroblastoma cells may be a low estimate of the true dissociation constant.

It now appears that the sodium channel contains three non-identical binding sites for different families of neurotoxins. TTX and a similar compound, saxitoxin, are thought to bind to a site of monovalent cation ligation (24,33) which could well be the actual channel. The alkaloid neurotoxins bind to a site separate from the TTX binding site (34), and scorpion toxins bind to yet a third site (1,20,22). Catterall has discussed in detail the possible interactions between the alkaloid and scorpion toxin binding sites (1). A radiolabeled scorpion toxin should be a useful probe for the sodium channel in situ and in isolated form. Preliminary experiments revealed that there appeared to be a component of specific binding of the scorpion toxin described here to membranes isolated from the eel electroplax.

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