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INHIBITORY EFFECTS OF SCORPION VENOM ON THE UPTAKE OF AMINO ACIDS BY SYNAPTOSOMES AND SYNAPTOSOMAL MEMBRANE VESICLES

D. E. RHOADS, N. A. PETERSON, H. SANKARAN and E. RAGHUPATHY*
University of California, Langley Porter Psychiatric Institute, Brain-Behavior Research Center, Sonoma Developmental Center, Eldridge, CA 95431, U.S.A.

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Abstract—Scorpion (*Tityus serrulatus*) venom strongly inhibited the Na^+-dependent uptake of [^{14}C]proline by rat brain synaptosomal preparations. In addition, the efflux of proline was enhanced markedly by scorpion venom. The inhibitory effects of the venom were also demonstrated in synaptosomal vesicle preparations where proline uptake was energized by an artificially imposed Na^+ gradient. In both preparations, the effect of scorpion venom was additive with the inhibitory effect of veratridine on Na^+-dependent amino acid uptake. The inhibitory effects of both compounds were abolished by tetrodotoxin. The Na^+-dependent uptakes of amino acids (e.g. proline, glutamic acid, and γ-aminobutyric acid) were much more sensitive to inhibition by the toxin than the Na^+-independent uptakes (e.g. leucine and phenylalanine). The results of the present study indicate that the scorpion venom may exert its inhibitory effect on Na^+-dependent transport by decreasing the transmembrane Na^+ gradient. Efflux of accumulated proline, which is presumably controlled by maintenance of this Na^+ gradient, was stimulated 3- to 4-fold by the scorpion venom.

The venoms from numerous species of scorpions contain neurotoxins which interact with the action potential Na^+-ionophore (voltage-dependent Na^+ channel and/or its gating mechanism). Electrophysiological studies of several nerve or neuromuscular preparations under voltage-clamp conditions have shown that the principal mechanism of action of scorpion toxins is inhibition of Na^+-current inactivation [1–3]. Further, there was delayed rectification under these conditions due to suppression of the K^+ current [1, 3]. Characterization of these effects of scorpion toxins has been extended to cultured neuroblastoma cells where the cooperativity of the action of scorpion toxin with that of the alkaloid veratridine in the activation of the action potential Na^+-ionophore has been demonstrated by $^{22}Na^+$ flux studies [4, 5].

A number of the physiological effects of scorpion venoms appear to be mediated by the stimulation of neurotransmitter release by the toxins [6–8]. High affinity, sodium-dependent uptake systems for a variety of established and putative neurotransmitters have been described in rat brain synaptosomal preparations [9–11]. These transport systems may function as reuptake mechanisms in the termination of transmitter action [9]. In this paper, we describe the inhibitory effects of the venom from the Brazilian scorpion *Tityus serrulatus* on the Na^+-dependent uptake of amino acids by rat brain synaptosomes. We have extended these results to a synaptosomal membrane vesicle preparation in which the energetics of transport can be defined more precisely. In addition to inhibiting the uptake mechanism, the venom also stimulates efflux (release) of previously accumulated amino acids.

* To whom all correspondence should be addressed: E. Raghupathy, University of California, Brain-Behavior Research Center, Sonoma Developmental Center, Eldridge, CA 95431, U.S.A.

MATERIALS AND METHODS

Amino acid uptake into synaptosomal fractions [12] or crude synaptosomal–mitochondrial fractions (P_2 fraction in Ref. 13), prepared from freshly excised cortices of Sprague–Dawley rats, was measured as described previously [10]. Results obtained with either of the fractions were essentially the same. Unless otherwise stated, 0.1 mg of synaptosomal fraction protein was incubated with ^{14}C-amino acids (0.1 μCi; 0.3 to 0.5 nmole) at 25° in 1 ml of a medium (TMNaK) consisting of 10 mM Tris–HCl (pH 7.4), 15 mM $MgCl_2$, 150 mM NaCl, and 1 mM KCl. Protein was quantified by the method of Lowry *et al.* [14]. *T. serrulatus* venom (TsV) was dissolved in TMNaK and added to the incubation mixtures in final concentrations of 0–20 μg/ml. Incubations were terminated by the addition of 5 ml of cold TMNaK. Synaptosomal particles were then isolated for radioactivity determination by filtration as described previously [10].

Efflux of proline from synaptosomal fractions was measured after incubation for sufficient time (15 min) to allow maximal accumulation of radiolabeled proline. Efflux was initiated by 5-fold dilution of the incubation mixture with TMNaK, with or without TsV (5 μg/ml). Aliquots of the diluted incubation mixture were removed at appropriate time intervals. The synaptosomal particles were harvested, and radioactivity was determined as described above.

Membrane vesicles were obtained from synaptosomal fractions by methods previously described [15, 16]. Synaptosomal preparations were homogenized (three strokes) in 10 mM Tris–HCl buffer (pH 8.1) and incubated for 30 min on ice with frequent mixing. The suspension was centrifuged for 20 min at 27,000 *g* and the pellet (membrane preparation) was resuspended and frozen at a protein concentration of 10–20 mg/ml in 0.32 M sucrose containing 5 mM Tris–HCl (pH 7.4), 1 mM $MgSO_4$, and 0.5 mM EGTA.* These vesicles were loaded at 37° with 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM $MgSO_4$ by rapid thawing and diluting with loading solution to a protein concentration of 1–3 mg/ml [17]. Of this latter suspension, 0.1 ml was added to an external solution containing 10 mM Tris–HCl, 1 mM $MgSO_4$, 150 mM NaCl, and 0.1 μCi (0.35 nmole) [^{14}C]proline in a final volume of 1 ml. Incubations were terminated and the uptake of radiolabeled proline was determined as described above for synaptosomal incubations. Proline transport under these conditions is essentially driven by ΔNa^+, the artificially created Na^+ gradient (Na^+-extravesicular > Na^+-intravesicular) (manuscript in preparation).

Uniformly labeled ^{14}C-amino acids were purchased from the New England Nuclear Corp. (Boston, MA) at the following specific activities (mCi/mmole): proline, 260; γ-aminobutyric acid, 203; glutamic acid, 254; leucine, 298; and phenylalanine, 536. *T. serrulatus* venom was obtained from the Sigma Chemical Co. (St. Louis, MO). Veratrine mixture was obtained from the New York Quinine Chemical Works, Inc. (New York, NY). An average molecular weight of 591 and a 25% (by weight) veratridine content were assumed for this preparation. Tetrodotoxin (TTX) was purchased from CalBiochem (San Diego, CA).

RESULTS

T. serrulatus venom (TsV) inhibited synaptosomal proline uptake in a dose-dependent manner (Fig. 1). Inhibition was seen at TsV concentrations as low as 0.1 μg/ml and was maximal at 5 μg TsV/ml. At this maximum level of inhibition, proline accumulation was reduced to approximately 25% of the control uptake in the absence of TsV. Proline has been shown previously to be transported by synaptosomes by a strictly Na^+-dependent mechanism [10]. The uptake of leucine, which has been shown previously to be Na^+ independent [10], was also inhibited by TsV (Fig. 1). However, even at the maximum level of inhibition, leucine uptake was at 75% of the control value and was therefore much less sensitive to the inhibitory effect of TsV than was proline uptake. Other amino acids whose uptakes are Na^+ independent (e.g. phenylalanine and histidine) followed the pattern of leucine, and these results further illustrate the greater sensitivity of the Na^+-dependent transport systems (e.g. proline, glutamic acid, and γ-aminobutyric acid) to TsV inhibition in these synaptosomal preparations (data not shown).

* EGTA, ethyleneglycol-bis(β-amino-ethylether) *N,N'*-tetra-acetic acid.

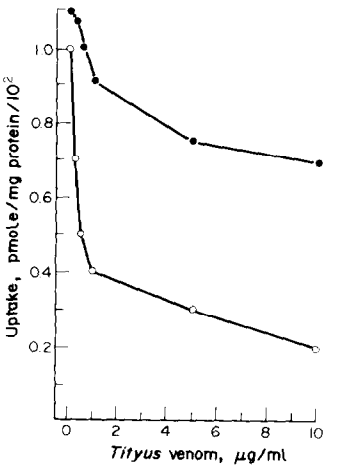

Fig. 1. Inhibition of synaptosomal leucine and proline uptake as a function of *Tityus* venom concentration. Synaptosomal fractions were prepared by the method of Kurokawa *et al.* [12] and incubated for 10 min with [^{14}C]proline and [^{14}C]leucine in medium TMNaK under conditions described in text. Key: (●—●) [^{14}C]leucine uptake; and (○—○) [^{14}C]proline uptake. Each value is the mean of six closely agreeing determinations. The extent of inhibition of leucine uptake in a Na^+-free medium was essentially the same as that observed in TMNaK.

The dose-response in Fig. 1 was based on the maximum accumulation of amino acid, which occurred after 10–15 min in the case of proline uptake (Fig. 2, open circles). The time course presented in Fig. 2 also indicates that the initial rate of proline uptake was reduced significantly by TsV (5 μg/ml) just as was the overall capacity to accumulate proline. If synaptosomes were allowed to accumulate proline in the absence of TsV and TsV was added after a steady-state level of uptake had been reached (indicated by the arrow in Fig. 2), efflux of proline

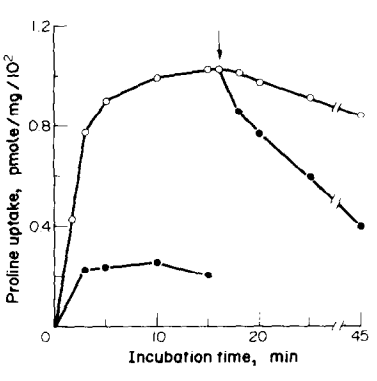

Fig. 2. Effect of *Tityus* venom on the influx and efflux of [^{14}C]proline as a function of time. Experimental conditions were as in Fig. 1, with the exception that incubation times were varied. Venom was used at a concentration of 5 μg/ml. Initial uptake rates were: (a) control, 26 pmoles·min^{-1}·mg^{-1}; (b) venom, 8 pmoles·min^{-1}·mg^{-1}; and (c) venom + TTX, 17 pmoles·min^{-1}·mg^{-1}. Each value is the mean of five closely agreeing determinations. Key: control (○—○); and venom (●—●).

Table 1. Interactions of scorpion venom with tetrodotoxin and veratridine in the inhibition of synaptosomal amino acid uptake*

Addition	Amino acid uptake† [pmoles·(mg protein)$^{-1}$·(10 min)$^{-1}$]					
	Proline		γ-Aminobutyric acid		Leucine	
	Uptake	% of Control	Uptake	% of Control	Uptake	% of Control
None (control)	91	100	1000	100	104	100
TsV‡ (1 μg/ml)	35	38	701	70	78	75
Veratridine (25 μM)	48	53	614	61	93	90
TsV + veratridine	15	16	307	31	81	78
TTX§	93	102	991	99	95	91
TTX + TsV	89	98	980	98	96	92
TTX + veratridine	93	102	1007	101	110	106
TTX + TsV + veratridine	79	87	921	92	100	97

* Synaptosomal amino acid uptake was measured as described in the text with appropriate additions to the incubation mixture as indicated. Results for phenylalanine and glutamic acid were similar to those of leucine and γ-aminobutyric acid respectively.

† Each value is the mean of at least four independent determinations and the standard errors did not exceed 15% of the mean.

‡ *Tityus serrulatus* venom.

§ Synaptosomes were preincubated for 10 min at 4° with 5 μg/ml of tetrodotoxin.

was enhanced markedly. The average rate of efflux [pmoles proline·min^{-1}·(mg protein)$^{-1}$ was 0.6 in the absence of TsV and 2.1 in the presence of TsV. This 3- to 4-fold stimulation of the rate of efflux was comparable to that initiated under depolarizing conditions such as elevated extrasynaptosomal KCl or the addition of 50 μM veratridine (data not shown).

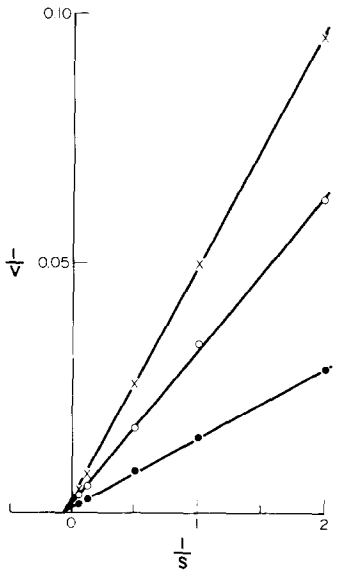

Fig. 3. Double-reciprocal plot showing the effect of *Tityus* venom and veratridine on synaptosomal proline uptake. Experimental conditions were as in Fig. 1 except that the incubation time was 3 min and the proline concentration(s) was varied between 0.5 and 20.0 μM. The initial velocity (V) was expressed as pmoles proline·(mg protein)$^{-1}$·min^{-1} and is the average of three independent determinations. *Tityus* venom was used at a concentration of 5 μg/ml and veratridine at 50 μM. Key: control (●—●); venom (○—○); and veratridine (×—×).

The inhibitory effect of the combined actions of veratridine (25 μM) and TsV (1 μg/ml) on proline uptake was greater than that which was obtained with either compound alone (Table 1). Uptake of proline in the presence of veratridine was 53% of that occurring in its absence, and TsV alone reduced the uptake to 38% of the control value. Both compounds together further decreased the uptake to a level which was only 16% of control value. This result was obtained at several different concentrations of the two compounds. Data on inhibition of synaptosomal proline uptake by veratridine and TsV are expressed as double-reciprocal plots in Fig. 3. This analysis indicates that the inhibition was of the noncompetitive type. Table 1 shows that prior incubation of the synaptosomal preparation with tetrodotoxin (TTX) completely abolished the individual inhibitory effects of veratridine and TsV on proline uptake. Their combined inhibitory effects were reduced by TTX to a level which was 87% of the control proline uptake. The results for the Na^+-dependent uptakes of γ-aminobutyric acid and glutamic acid (not shown) were essentially the same as that of proline. At the TsV concentrations studied, γ-aminobutyric acid transport was less affected than was proline transport. However, the inhibitory effects of veratridine and TsV were additive. The uptake of leucine (and phenylalanine, not shown) was not substantially inhibited by veratridine, and the combined action of veratridine and TsV was not greater than the effect of TsV alone. This lack of additive action of TsV and veratridine, together with the lesser sensitivity to inhibition by TsV, sets the Na^+-independent transport systems apart from the strictly Na^+-dependent systems.

A major consequence of the activation of the action potential Na^+-ionophore would be a rapid increase in the intrasynaptosomal Na^+ concentration and, thus, a reduction in the electrochemical potential of the Na^+ gradient (ΔNa^+), which is thought to energize Na^+-dependent amino acid uptake. The

Table 2. Na^+-dependent uptake by synaptosomal membrane vesicles: Effect of neurotoxins and reduction of ΔNa^+*

	Initial rate of proline uptake (pmoles·mg^{-1}·min^{-1})	% of Control	Total accumulation of proline (pmoles/mg)	% of Control
Control (Na^+ = 150 mM)	13.1	100	9.8	100
Control + TTX†			10.0	102
TsV‡ (5 μg/ml)	8.6	65	6.4	66
TsV + TTX			8.8	90
Veratridine (50 μM)	8.3	63	6.3	65
Veratridine + TTX			9.1	93
Veratridine + TsV	4.3	33	2.8	28
ΔNa^+ = 100 mM§	9.7	77	6.9	70
ΔNa^+ = 75 mM§	7.5	62	5.6	57
ΔNa^+ = 50 mM§	5.4	38	4.2	43

* Membrane vesicles were prepared from hypoosmotically-lysed synaptosomes and loaded with 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM $MgSO_4$. Uptake of proline was measured in an external solution of 150 mM NaCl, 1 mM $MgSO_4$, 10 mM Tris–HCl (pH 7.4), and 0.35 μM [^{14}C]proline. The initial rate of uptake was calculated from the radioactivity accumulated within 20 sec. Values for total accumulation were calculated from the maximum radioactivity accumulated. The time necessary for maximal accumulation was 2 min under these conditions. Each value is the mean of at least four independent determinations and the standard errors did not exceed 15% of the mean.

† Tetrodotoxin (5 μg/ml).

‡ *Tityus serrulatus* venom.

§ Uptake of proline was measured under conditions where the initial Na^+ gradient was reduced by replacing a fraction of the NaCl in the external solution with Tris–HCl to achieve the Na^+ concentration indicated.

effects of TsV were therefore examined in a membrane vesicle preparation in which the energy for proline uptake was supplied by an artificially imposed ΔNa^+ (Na^+-extravesicular > Na^+-intravesicular). Both the initial rate of proline uptake and the overall extent of proline accumulation in the vesicle preparation were reduced by TsV to 65 and 66% of the control uptake respectively (Table 2). As in studies with intact synaptosomal preparations, the effect of TsV was decreased by TTX and was additive with the inhibitory effect of veratridine. Also shown in Table 2 is the reduced proline uptake which occurred upon reduction of the magnitude of the initial ΔNa^+ from 150 to 50 mM by replacement of a fraction of the extravesicular NaCl with either KCl or Tris–HCl. Such a reduction in ΔNa^+ decreased the uptake of proline to a degree comparable to the inhibition of the uptake by TsV. However, other mechanisms of decreased proline transport resulting from reduced external Na^+ cannot be ruled out and may contribute to the observed level of inhibition under these conditions.

DISCUSSION

Several features of the inhibition of synaptosomal Na^+-dependent amino acid uptake by TsV are consistent with the proposed action of TsV on the action potential Na^+-ionophore. First, tetrodotoxin, which is a specific inhibitor of the action potential Na^+ionophore [18, 19], blocked the inhibitory effects of TsV. Second, the combined actions of TsV and veratridine, which has been shown to activate the Na^+-ionophore [20, 21], were greater (nearly additive) compared to the inhibitory effect of either compound alone. Third, *T. serrulatus* venom resulted in a stimulation in the efflux of previously accumulated proline, and the magnitude of this stimulation was comparable to that occurring under other depolarizing conditions. Fourth, the characteristics of the reduction in proline uptake into membrane vesicles prepared from synaptosomes further indicated that the inhibition of proline uptake by TsV may have been a direct consequence of a reduction in ΔNa^+. The dual action of TsV on both aspects of proline transport, influx and efflux, can be accounted for by such an action of the transmembrane Na^+ gradient. This gradient is bifunctional in that it is required not only to drive the uptake of proline, but also to maintain the proline inside the synaptosomal compartment against its own concentration gradient. Fifth, on the other hand, Na^+-independent transport systems (e.g. for leucine and phenylalanine) were much less sensitive to inhibition by TsV than were the Na^+-dependent systems. Further, veratridine did not enhance the inhibition of Na^+-independent transport caused by TsV alone.

The venom of *T. serrulatus* has been shown to contain a small molecular weight (7000 daltons) protein, tityustoxin (TsTX), which when purified has all of the electrophysiological characteristics of the native venom [6]. Neurotoxins from a number of scorpion species are similar in that they are low molecular weight proteins which bind specifically to sites associated with voltage-dependent Na^+ channels [22]. These sites of binding are different from those for either tetrodotoxin or veratridine binding [23]. Biochemical studies of $^{22}Na^+$ influx into neuroblastoma cells lead to a firm distinction between the action of veratridine on the activation of the action potential Na^+-ionophore (opening of the channel) and the action of scorpion toxin to prevent its closing [4]. The purified toxin from *Leiurus quinquestriatus* had little capacity to activate the Na^+-ionophore

when tested alone in the absence of veratridine. In the presence of veratridine, $^{22}Na^+$ uptake was initiated and was further stimulated by the presence of toxin.

Abita *et al.* [24] have studied the effects of sea anemone toxin on γ-aminobutyric acid accumulation and release by a synaptosomal preparation and conclude that this system offers an easy *in vitro* assay for the interaction of toxins with their receptors. Our studies with *T. serrulatus* venom appear to support this conclusion. Neurotoxins such as the *Tityus* venom studied in the present paper should also prove to be useful probes in defining the mechanisms involved in certain Na^+-dependent processes.

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