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TITYUS SERRULATUS TOXIN VII BEARS PHARMACOLOGICAL PROPERTIES OF BOTH β-TOXIN AND INSECT TOXIN FROM SCORPION VENOMS

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SUMMARY : Some β-toxins from the South American scorpion Tityus serrulatus (e.g. Ts VII) are highly toxic both for mouse and fly larva. Radioiodinated Ts VII and the insect toxin from the North African scorpion Androctonus australis Hector (AaH IT) bind to the same site on a house fly head synaptosomal fraction. These results reinforce the hypothesis about the existence of a correlated series of scorpion toxins as previously defined by amino acid compositions and sequences, and immunological and circular dichroism studies, in suggesting that Ts VII constitutes a link which may fill the pharmacological gap existing between β -toxins and insect toxins such as AaH IT.

Scorpion toxins form a family of polypeptides made of one single chain of 60 to 70 amino acid residues cross-linked by four disulfide bridges (1). They have been called mammal, insect and crustacean toxins according to the test animal (mouse, blowfly larva or isopod) used to follow their purification. Scorpion toxins active on mammals have been divided into two types, i.e. α and β , according to different pharmacological effects and specific binding sites related to the voltage dependant sodium channel of excitable membranes (2, 3). However insect toxins, e.g. Androctonus australis Hector (AaH IT) show unique selectivity for insect nervous tissues. Indeed, AaH IT binds specifically to a single class of non-interacting high affinity (K_D = 1.2-2 nM) binding sites most likely related to the insect voltage dependant sodium channel (4). A comparative study on ten scorpion toxins and their relative conformations in solution using circular dichroism (5) showed that these toxins differ from each other, despite a high degree of amino acid sequence homology. They form a series of related conformational variants with similarities among the individual spectra that can be correlated with sequence resemblance and pharmacological specificities. This study also suggests that there are two limiting conformational variants being approximated, on the one hand, by toxin II of Androctonus australis

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Hector (AaH II), an α-toxin, and, on the other hand, by toxins like toxin II of Centruroides suffusus suffusus (Css II), a β-toxin, and AaH IT. These findings agree with a previous theoretical classification made according to amino acid compositions, insertion/deletion events and sequences which also segregates α-toxins, β-toxins and AaH IT and shows some structural affinity between AaH IT and the β-toxins group as a whole. As toxins active on the mouse were recently purified (6, 7) i.e. toxin VI from Centruroides suffusus suffusus (Css VI) and toxins I to VIII from Tityus serrulatus (Ts I to Ts VIII), we decided to test their toxicity on blowfly larva and their binding characteristics to a house fly head synaptosomal fraction in a search for scorpion toxins able to recognize and act on both mammal and insect nervous systems. Such a finding would bring new evidence that scorpion toxins constitute a continuous conformational series pharmacologically active on ion channels.

MATERIALS AND METHODS

Toxins : AaH II (1) and AaH IT (8), Css II and Css VI (6), Ts I to VIII (7) were purified in the laboratory. The sequence of Ts VII has been recently determined (9). **Assays of toxicity** : Larvae of the blowfly Sarcophaga argyrostoma and C57/BL6 mice were used. Injection procedure into larvae and definition of contraction paralysis unit (CPU) have been previously described (10).Toxicity to mice was determined both by subcutaneous (s.c) and intracerebroventricular injections (i.c.v), LD 50 values being calculated as already stated (11). **Iodination of the toxins** : For Ts VII iodogen was used as the oxidizing reagent (0.5 mCi of iodide 125-carrier free from Amersham-England was reacted for 60 min, at 40°C, in 20 µl of 10 mM Tris-HCl buffer, pH 8.6 with 0.5 nmole toxin and 3 nmoles dry iodogen). Purification of [^{125}I]Ts VII and determination of radiolabelling yield were done as previously described (4). For AaH IT the lactoperoxidase method was used and [^{125}I]AaH IT was purified by immunoprecipitation (12). Specific radioactivities of 500 to 1000 Ci/mmole were routinely obtained. **Preparation of synaptosomal fractions** : Heads of house flies (Musca domestica) were provided by Procida (Marseille, France) and stored frozen (-80°C) until use. They were homogeneized with a Potter-Elvejhem apparatus (3000 rpm, 30 strokes, with a 2 min interval each 10 strokes in order to avoid heating), at 10 % (w/v), in an ice cold buffer containing 0.2 M sucrose, 20 mM HEPES-Tris, pH 7.0 (13), and protease inhibitors (14). The homogenate was centrifuged at 2000g for 10 min at 4°C and the supernatant was saved. The pellet was homogeneized and centrifuged in the same conditions as above. The pooled supernatants were centrifuged at 33000 g for 30 min at 4°C. The pellet was resuspended (1 ml/g heads) in an assay buffer made of 10 mM glucose, 140 mM choline chloride, 5.4 mM KCl, 0.8 mM Mg SO_4, 1.8 mM $CaCl_2$, 25 mM HEPES-Tris, pH 7.4. The protein content was determined according to Lowry et al. (15) with bovine serum-albumin (BSA) as a standard . The protein yield was 1.5 to 2 % from the starting material. Fractions (200 µl) were frozen in liquid nitrogen and stored at -80°C. No loss of [^{125}I]Ts VII and [^{125}I]AaH IT binding activities was detectable after two months. A synaptosomal fraction (P2) was prepared from rat brains as already described (16). **Binding experiments** : They were performed at 25°C. Glass microfibre filters GF/B 2.5 cm (Whatman Ltd. Maidstone. England) pre-wetted 2 hrs with the assay buffer containing 2 % of BSA were used. Incubations were made in the assay buffer supplemented with 0.2 % of BSA. The washing buffer was the assay buffer in which HEPES was 5mM and BSA 0.5 %.

Table I

Comparison between the activities of different scorpion venoms and toxins on fly larva and mouse

Sample	Fly larva : CPU[a] µg/100 mg	Mouse : LD 50 (s.c.) µg/20 g	(i.c.v.) ng/20 g
AaH venom	0.300 (b)	8.40 (b)	n.d.
Ts venom	0.260	17.50	n.d.
AaH IT	0.001 (b, c)	> 1000.00	> 50 x 10^3
I	n.t (b, c)	0.34 (b)	10.0
II	n.t (b, c)	0.18 (b)	0.5
III	n.t (b, c)	0.45 (b)	7.0
Css II	> 2.000	0.5	5
Css VI	> 1.000	0.05	1.7
Ts I	> 1.780	3.50	24.0
II	> 1.780	3.70	6.0
III	> 2.800	2.70	80.0
IV	> 2.800	0.40	24.0
VI	0.091	8.50	2.5
VII	0.046	4.70	0.6
VIII	0.051	7.20	11.0

AaH : Androctonus australis Hector; Css : Centruroïdes suffusus suffusus; Ts: Tityus serrulatus; n.d. not determined; nt : non-toxic ; s.c. : subcutaneous; i.c.v. : intracerebroventricular; [a]CPU: contraction paralysis unit as defined in (10), (b, c) according to Zlotkin et al. (8, 17).

RESULTS AND DISCUSSION

From a comparison of the activities of different scorpion venoms and toxins on fly larva and mice (Table I) one can make several remarks . Firstly, the tested venoms are both active on the mouse, regardless of the injection method used (s.c. or i.c.v.), and fly larva. Secondly, contrary to Ts toxins, AaH toxins show a clear cut specificity : AaH I, II and III are mammal toxins and AaH IT is an insect toxin. A few Ts toxins only, characterized as mammal toxins β-type (7) are also highly active on fly larva : Ts VI, VII and VIII. All α-toxins, i.e. AaH I, II, III and Ts III, IV, are inactive at the maximum injected doses. These results prompted us to compare AaH IT and Ts VII in specific binding and competition experiments on the insect nervous system.

Fig. 1 shows that, in the concentration ranges used, both [^{125}I]AaH IT and [^{125}I]Ts VII bind in a specific and saturable manner on a synaptosomal fraction from house fly heads. The Scatchard plots (inserts) are linear which indicate that each toxin binds to a single class of receptor sites. Binding site capacities (Bmax) and equilibrium dissociation constants (Kd) were calculated from four independant experiments : 70 ± 20 (± s.d.) fmol/mg of protein and 0.20 ± 0.02 (± s.d.) nM for [^{125}I]AaH IT and 100 ± 20 (± s.d.) fmol/mg of protein and 0.07 ± 0.02 (± s.d.) nM for [^{125}I]Ts VII. These experiments on the same preparation indicate that the number of AaH IT

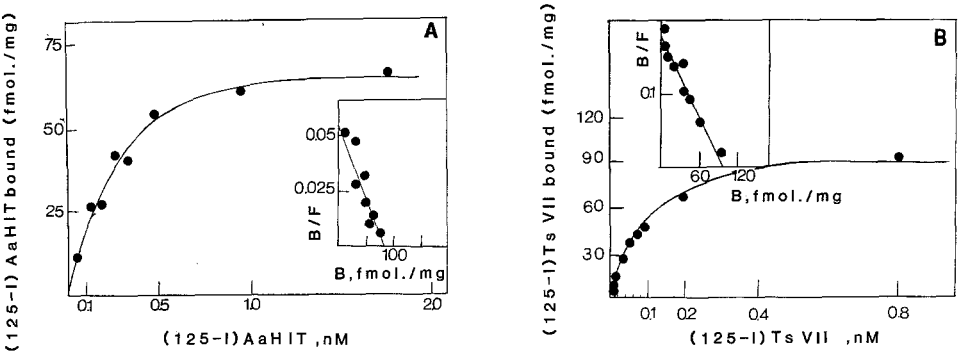

Fig. 1 : Specific binding of [^{125}I] toxins to house fly head synaptosomal fraction. Fly head synaptosomal fraction (0.133 mg and 0.053 mg protein per ml in A and B) was incubated 40 min (A) or 120 min (B) at 25°C, in the assay buffer (see methods) in the presence of the indicated concentrations of [^{125}I] AaH IT (A) or [^{125}I] Ts VII (B). The reaction was stopped by rapid filtration of 1 ml samples followed by 2 x 5 ml of washing medium through glass microfibre filters. Non specific binding was measured in the presence of AaH IT 1 µM (A) or Ts VII 0.1 µM (B). It represented 60-80 % (A) and 46-70 % (B) of the total radioactivity bound to the membranes in the range of concentrations used. Grafic specific binding is the difference between total and non specific binding. Each value is the mean of assays in triplicate. Inset : Scatchard plots.

binding sites represents roughly 70 % of that of Ts VII. The binding data are in good agreement with previous results obtained, on the one hand, with AaH IT on cricket central nervous system and house fly heads (4, 18) and, on the other hand, with Ts γ (19), a β-toxin purified from Ts venom (20), using a preparation of house fly head purified membranes very similar to the preparation we used.

Results of competition experiments (Fig. 2A) on the insect neuronal membranes show that the binding of [^{125}I]AaH IT is fully prevented by AaH IT ($K_{0.5}$ = 0.3 nM), Ts VII ($K_{0.5}$ = 0.08 nM) and by Css VI (but for a far greater concentration : $K_{0.5}$ = 50 nM). Concentrations of AaH II and Css II up to 10 µM were ineffective. The binding of [^{125}I]Ts VII to the same preparation (Fig. 2B) is fully prevented by Ts VII ($K_{0.5}$ = 0,08 nM) and Css VI (but with a far greater concentration, $K_{0.5}$ = 200 nM) and partially (70 % only) by AaH IT ($K_{0.5}$ = 3 nM). Here again, AaH II and Css II are ineffective in competing with [^{125}I] Ts VII even at concentrations up to 10 µM . The data point out that, on the same preparation, if Ts VII can prevent 100 % of [^{125}I]AaH IT binding (Fig. 2A), AaH IT can only prevent 70 % of [^{125}I]Ts VII binding (Fig. 2B). This can be correlated with the difference in the number of binding sites for the two toxins (see above). From these results, which have been repeatedly obtained, one can say that the experiment with AaH IT suggests heterogeneity in the Ts VII binding sites although the Scatchard plot of the specific binding of [^{125}I] Ts VII (Fig. 1B) is linear. Experiments are in progress in order to try and clarify this point, as a non competitive displacement of one toxin by the other cannot be completely

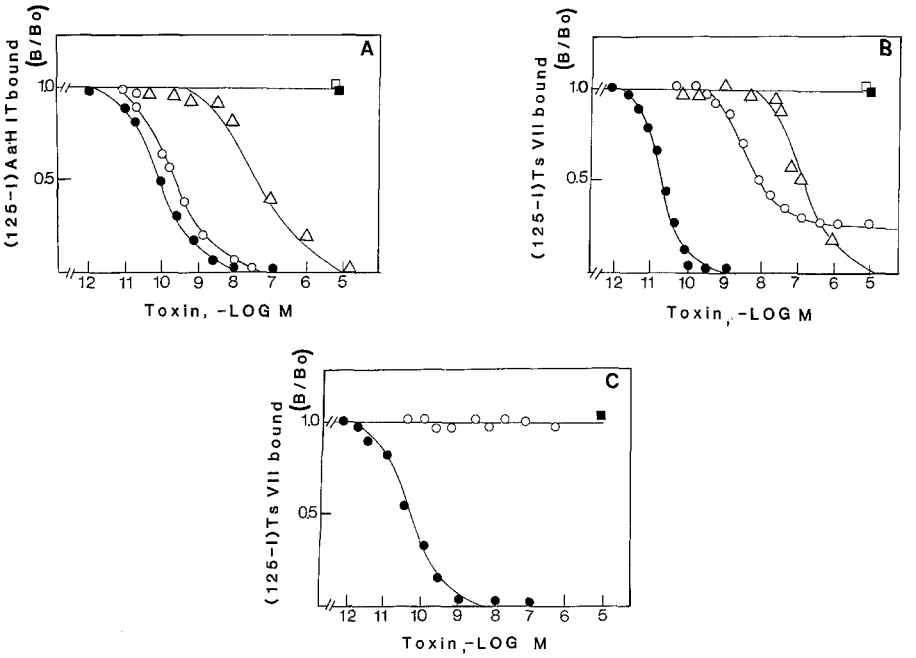

Fig. 2 : Competition between radiolabelled and unlabelled toxins for mammalian and insect synaptosomal fractions. Fly head synaptosomal fraction (0.133 mg and 0.053 mg per ml in A and B and 0.013 mg per ml in C) was incubated at 25°C, for 120 min, with either 0.1 nM [^{125}I]AaH IT (A) or 0.04 nM [^{125}I]Ts VII (B and C), in the assay buffer, and increasing concentrations of unlabelled toxins, i.e. AaH II (■), AaH IT (○), Css II (□), Css VI (△), Ts VII (●). The reaction is stopped as in experiments in Fig. 1. Bo and B are the respective bindings of the radiolabelled toxins in the absence and in the presence of the indicated concentrations of the unlabelled toxins. The non specific binding has been substracted. House fly head synaptosomal fraction (A and B), rat brain synaptosomal fraction (C).

excluded. Ts VII is not the only β-toxin able to prevent [^{125}I]AaH IT binding : Css VI is active but for concentrations 10^3 higher. As regards Css II, another β-toxin, it is inactive on larva and it cannot compete with [^{125}I]Ts VII. All the α-toxins tested are non toxic for fly larva, and do not compete with AaH IT. When AaH IT prevents [^{125}I]Ts VII binding (Fig. 2B), another question arises regarding the discrepancy between its K_D (0.2 nM) obtained by Scatchard plot (Fig. 1A) and its K_D (1.9 nM); calculated using the relationship :

$K_{0.5} = K_D (1 + ([[^{125}I]Ts\ VII])/K_D\ of\ [^{125}I]Ts\ VII))$. On explanation could be major differences relative to binding and dissociation kinetics for the [^{125}I]-toxins studied. Experiments are in progress to provide new elements. Competition experiments on rat brain synaptosomes (Fig. 2C) show that Ts VII, but not AaH IT and AaH II, competes with [^{125}I]Ts VII ($K_{0.5}$ = 0.08 nM). So, AaH IT can prevent [^{125}I]Ts VII binding on the insect nervous system preparation, but not on rat brain synaptosomes. These results show that Ts VII recognizes different binding sites in mammal and insect nervous systems,

and suggest that scorpion toxins may be interesting tools to search for and identify sodium channel sub-types.

Our results reinforce the hypothesis which considers the scorpion toxin family as consisting of a series of conformational variants related to the target recognition. It is now well established that α -, β- and insect scorpion toxins are able to bind to specific sites related to the voltage dependent sodium channel (3, 18, 21, 22). Ts VII, which is toxic both for mammals and insects, has been proved to bind to the same site as β-toxins on the mammalian sodium channel and to the same site as AaH IT on the insect sodium channel. Thus Ts VII appears to constitute a link between mammalian β-toxins and insect toxins. Its conformation will therefore be interesting to elucidate and compare with those of AaH II and AaH IT. Moreover, this work is also in agreement with the hypothesis that the toxin targets may themselves form a related series (5). As a conclusion, a better knowledge of scorpion toxins structure-toxicity relationships may prove to be useful in the insecticide field given that scorpion toxins specificity for mammals or insects appears to be the result of large differences in affinities of the various toxins for the different sodium channels.

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