Bot IT_2: a new scorpion toxin to study receptor site on insect sodium channels

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Abstract The insect-specific *Bothus occitanus tunetanus* IT_2 toxin is distinguishable from other scorpion toxins by its amino acid sequence and effects on sodium conductance. The present study reveals that Bot IT_2 possesses in cockroach neuronal membranes a single class of high affinity ($K_d = 0.3 \pm 0.1$ nM) and low capacity ($B_{max} = 2.4 \pm 0.5$ pmol/mg) binding sites. Competitive binding experiments with several known sodium channel neurotoxins reveal that the Bot IT_2 binding site is in close proximity to the other toxins.

Key words: Insect sodium channel; Scorpion toxin

1. Introduction

Scorpion venoms contain toxins which interact with the voltage-dependent sodium channels that are responsible for the increase in sodium permeability during the initial phase of the action potential in most excitable cells [1]. Scorpion neurotoxins modify the function of sodium channels in mammals or other vertebrates [2] as well as molluscs [3] and insects [4,5]. Insect selective scorpion neurotoxins have revealed four different receptor sites on insect sodium channels. Among them, two groups, namely the excitatory and depressant toxins, are only active on insects as demonstrated by toxicity, electro-physiology and binding assays [6]. The excitatory toxins cause rapid excitatory paralysis and induce repetitive firing in insect nerves. The depressant toxins cause a slow depressory flaccid paralysis due to the depolarization of nerve membranes and blockage of sodium conductance in axons [4,6,7]. Some α-scorpion toxins demonstrate high activity in insects and low activity in mammals [8]. These toxins recognize the receptor site 3 on insect sodium channels which is also shared by sea anemone toxin ATX II [9,10]. These toxins induce prolongation of the action potential due to the slowing of inactivation [5]. Receptor binding site 4 on insect sodium channels is recognized by the β-scorpion toxin Ts VII, isolated from the venom of *Tityus serrulatus*, which is highly toxic for both insects and mammals [11]. β-scorpion toxins affect sodium activation and cause channels to remain open or reopen at the resting potential, resulting in repetitive trains of action potentials [12,13].

Recently, a new scorpion toxin Bot IT_2 was isolated from the venom of *Buthus occitanus* which has high activity in insects and less activity in mammals [14]. Bot IT_2 is distinguishable from other scorpion toxins by (a) its new action on the activation kinetics of the insect sodium channels [15] and (b) its primary structure [14]. The present study examines the interaction of Bot IT_2 with insect neuronal membranes and its relationships with known receptor sites on insect sodium channels.

2. Materials and methods

2.1. Materials

The insect selective neurotoxins AaH IT, Bot IT_2, Bot IT_1, Bot IT_4 were purified according to [16] and [14,17], respectively. The β-toxin Ts VII and the α-toxin AaH II were purified according to [18,19], respectively. ATX II was purchased from Sigma (USA).

2.2. Radioiodination and purification of the derivatives

Bot IT_2 was radioiodinated as follows: 1 nmol Bot IT_2, 1 mCi of carrier free $Na^{125}I$, 5 μg lactoperoxidase and 20 μl H_2O_2 diluted 1/50 000 were incubated for 2×2 min in 20 mM phosphate buffer, pH 7.4 in a final volume of 85 μl. The solution was then injected into an HPLC RP8 column. Elution was performed at 20°C with a linear gradient from 5 to 35% B in A in 10 min (A = 0.1% TFA, B = acetonitrile, 0.1% TFA), followed by another linear gradient from 35 to 75% B in A for 70 min. The flow rate was 1 ml/min. The monoiodotoxin was eluted as the first peak of radioactive protein. The purification procedure gave the same results as described in [20], which describes the first peak as monoiodotoxin and the second as diiodotoxin. The top fraction of the monoiodinated peak was used in binding studies. The concentration of radiolabeled toxin was estimated according to the specific activity of ^{125}I and was 2000 Ci/mmol of monoiodotoxin.

2.3. Neuronal membrane preparation

Insect synaptosomes (P_2L preparation) were prepared from the VNS of adult cockroach (*Periplaneta americana*) according to established methods [21]. All buffers contained a cocktail of proteinase inhibitors composed of phenylmethylsulfonyl fluoride (50 μg/ml), pepstatin A (1 mM), iodoacetamide (1 mM) and 1 mM 1,10-phenanthroline. Membrane protein concentrations were determined using a Bio-Rad Protein assay, with BSA as standard.

2.4. Binding assays

Equilibrium saturation assays were performed using a series of concentrations of the unlabeled toxin in the presence of fixed concentration of the radioactive toxin. To obtain saturation curves, the specific radioactivity and amount of bound toxin were calculated for each toxin concentration. The binding medium was (in mM): choline Cl (140), $CaCl_2$ (1.8), KCl (5.4), $MgSO_4$ (0.8), HEPES (25), pH 7.4;

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Abbreviations: AaH IT and AaH II, an excitatory insect selective toxin and an alpha mammal toxin, respectively, from the venom of the scorpion *Androctonus australis Hector*; ATX II, toxin II from the sea anemone *Anemonia sulcata*; Ba IT_2 and Bot IT_4, depressant toxins from the venom of the scorpion *Buthacus arenicola* and *Buthus occitanus tunetanus*, respectively; Bot IT_1 and LqhαIT, alpha insect selective toxins from the venom of the scorpion *Buthus occitanus tunetanus* and *Leiurus quinquestriatus quinquestriatus*, respectively; Ts VII, a beta toxin from the venom of the scorpion *Tityus serrulatus*

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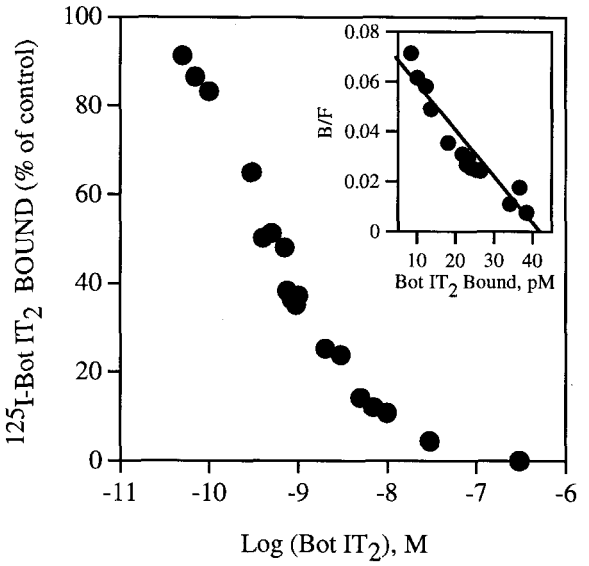

Fig. 1. Binding of Bot IT_2 to cockroach neuronal membranes. Cockroach neuronal membranes (2.5–3 μg protein) were incubated in the presence of 84–89 pM ^{125}I-Bot IT_2 and increasing concentration of Bot IT_2. Non-specific binding, determined in the presence of 1 μM Bot IT_2, was subtracted from all data points. Data are shown as percent inhibition of specific Bot IT_2 binding. Binding was analysed using the computer program LIGAND. The IC_{50} for Bot IT_2 is 0.7 ± 0.3 nM (mean ± S.E.M. of three experiments). (Inset) Scatchard transformation of the displacement curve indicating Bot IT_2 affinity of 0.3 nM.

glucose (10) and BSA (2 mg/ml). The reaction mixture (0.3 ml) was incubated for 60 min at 22°C and then diluted with 2 ml of ice-cold binding medium and filtered over GF/C filters (Whatman, UK) under vacuum. The filters were then washed twice in binding medium. Non-specific binding was determined in the presence of 1 μM unlabeled toxin and was 10–20% of total binding. Equilibrium saturation or competition experiments were analyzed using the iterative computer program LIGAND (Elsevier Biosoft, UK). Each experiment was performed at least three times.

3. Results

3.1. Pharmacological characterization of Bot IT_2

Using the monoiodo derivative of Bot IT_2 and *P. americana* neuronal membranes, we have studied the specific binding of Bot IT_2 on insect sodium channels. Scatchard transformation of the displacement curve of Bot IT_2 (Fig. 1) reveals that Bot IT_2 binds to a single class of binding sites of high affinity (K_d = 0.3 ± 0.1 nM) and low capacity (B_{max} = 2.4 ± 0.5 pmol/mg). This affinity is comparable to those observed for other insect scorpion toxins in the same preparation [11,21].

3.2. Inhibition of Bot IT_2 binding by sodium channel toxins

In order to classify the receptor site of Bot IT_2, we examined the inhibition of its specific binding by several known sodium channel neurotoxins.

The data presented in Fig. 2 reveal that the specific binding of ^{125}I-Bot IT_2 to cockroach neuronal membranes is completely inhibited by three distinct neurotoxins. (A) The excitatory insect selective toxin AaH IT, shown to bind to a single class of binding sites of high affinity in cockroach neuronal membranes [13]. Moreover, we have shown that the binding of the radiolabeled AaH IT is completely inhibited by Bot IT_2 [14]. (B) The depressant insect selective toxin Bot IT_4; Bot IT_4 possesses the same primary structure as Ba IT_2, previously shown to possess the pharmacological properties of a depressant toxin [20]. (C) The β-toxin Ts VII, shown to compete with other β-toxins on binding to receptor site 4 in vertebrate

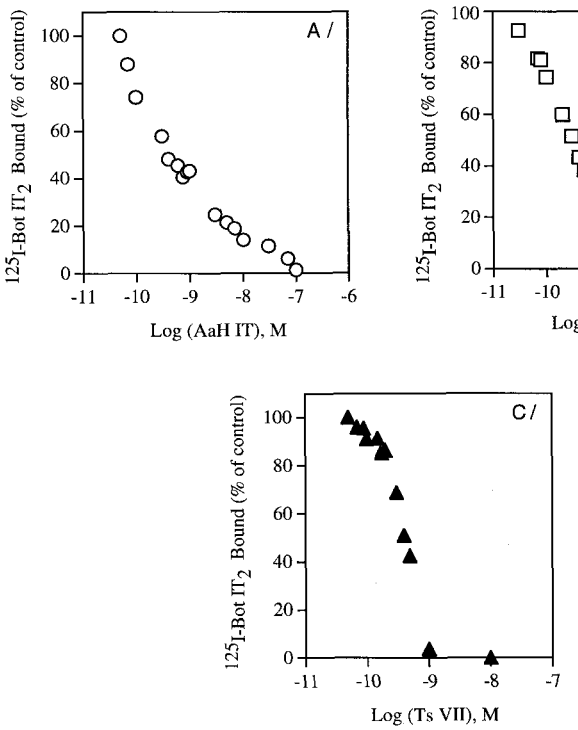

Fig. 2. Competition for ^{125}I-Bot IT_2 binding by sodium channel neurotoxins. Neuronal membranes of *Periplaneta americana* (2.4–3 μg protein) were incubated in the presence of 73–84 pM ^{125}I-Bot IT_2 and increasing concentration of (A) the excitatory toxin AaH IT (IC_{50} = 0.6 ± 0.2 nM), (B) the depressant toxin Bot IT_4 (IC_{50} = 0.25 ± 0.08 nM) and (C) the β-toxin Ts VII (IC_{50} = 0.3 ± 0.1 nM).

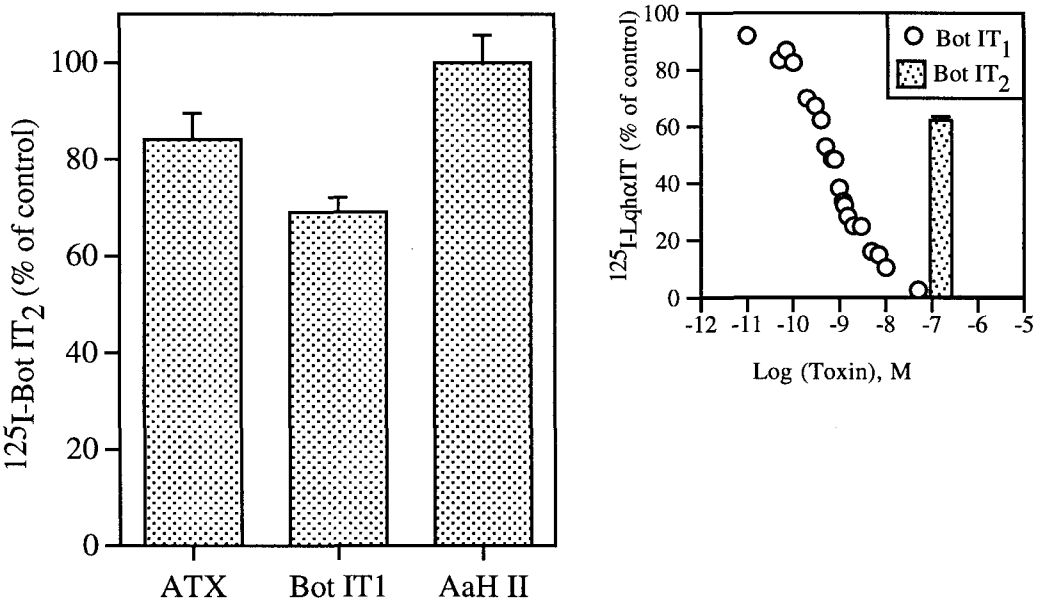

Fig. 3. Competition for ^{125}I-Bot IT_2 binding by α-toxins. Neuronal membranes of *Periplaneta americana* (2.4–3 μg protein) were incubated in the presence of 73–84 pM ^{125}I-Bot IT_2 and 1 μM ATX II or Bot IT_1 or AaH II. The data are presented as percentage of inhibition of ^{125}I-Bot IT_2 specific binding. The percent inhibition of Bot IT_2 specific binding is 84.1 ± 5.4% for ATX II, 68.9 ± 3.1% for Bot IT_1 and 103 ± 5.6% for AaH II (mean ± S.E.M. of three experiments). (Inset) Competition for ^{125}I-LqhαIT binding by Bot IT_1 and Bot IT_2. Cockroach neuronal membranes (2.1 μg protein) were incubated for 60 min in the presence of 80–95 pM ^{125}I-LqhαIT and increasing concentrations of Bot IT_1 or 1 μM Bot IT_2. Data are shown as percent inhibition of specific ^{125}I-LqhαIT binding. The IC_{50} for Bot IT_1 is 0.6 ± 0.04 nM and percent inhibition of specific LqhαIT binding is 73.6 ± 1.8% for Bot IT_2.

sodium channels [22] as well as with the insect-selective toxins on binding to insect sodium channels [13,23].

On the other hand, sea anemone toxin ATX II [9], and α-scorpion toxin Bot IT_1, shown to inhibit completely the binding of α-scorpion toxin to receptor site 3 in the insect sodium channel (Fig. 3, inset), partially inhibit at high concentration (1 μM) ^{125}I-Bot IT_2 binding to insect neuronal membranes (Fig. 3). At high concentration (1 μM) Bot IT_2 also partially inhibits the binding of the radiolabeled α-scorpion toxin LqhαIT (Fig. 3, inset). Moreover, the α-scorpion toxin AaH II that binds to receptor site 3 in vertebrate sodium channels [24,25] as well as in insect sodium channels [10] does not affect ^{125}I-Bot IT_2 binding (Fig. 3).

4. Discussion

When comparing the binding properties of Bot IT_2 in cockroach neuronal membranes to those of the other insect selective scorpion toxins in the same preparation it may be concluded that:

(1) Bot IT_2 shows the typical pharmacological properties of an excitatory toxin as suggested by the inhibition of its specific binding by excitatory [11] and depressant [21] insect toxins, the β-toxin Ts VII [11] and by the partial inhibition of its binding by high concentration of ATX II (Fig. 3). This means that the different insect selective scorpion toxins and sea-anemone toxin ATX II share the same receptor site on insect sodium channel.

(2) Bot IT_2, however, differs from the excitatory toxin by the reciprocal partial competition with α-scorpion insect selective neurotoxin which is also characteristic of a depressant toxin (Cestèle et al., unpublished results). These results emphasize the notion that ATX II and α-scorpion toxin do not share exactly the same receptor site on sodium channels [10,26]. Moreover, these two groups of toxins allow discrimination of the binding properties of Bot IT_2 and excitatory toxins. According to these observations, it may be concluded that Bot IT_2 binds to a receptor site localized in close proximity to but not identical to one of the excitatory toxins in spite of the competitive interaction between these two toxins.

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BoT IT2     --DGYIKGYKGCKIT-CVI----NDDYCDTEC--K---AE-GGTYGYC-WKWGLACWC-EDL-PED--KRWKPE-T-NT----C------
Bot IT4     --D**IRRRD*C*VS-*LF---GNEG-*DKE*--*---AY-GGSY***-WIWGLA*W*-E-*GPDD--*TWKSE-T-NT----*G-----
T.s. VII    -KE**LMDHE*C*LS-*FIRPSG---Y*GRE*GI*-K----GSS-***AWP---A*Y*Y-G*-P-NWV*VWDRA-T-N--K--*------*
A.a.H.IT1   KKN**AVDSS*-*APE*LL--S-N--Y*NNQ*T-*VHYADK----***CL---LS*Y*F-G*-NDD-K*VL--EISD-TRKSY*DTTIIN
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	Bot IT2	Bot IT4	Ts VII	AaH IT
Bot IT2	100 %	67%	42 %	31 %

Fig. 4. Comparison of Bot IT_2, Bot IT_4, AaH IT and Ts VII amino acid sequences. The table represents the percentage of aligned positions with identical residues.

Results obtained by binding experiments are in accordance with those from electrophysiological experiments suggesting that the global effect of Bot IT_2, studied under current-clamp conditions, are comparable to those of excitatory insect toxins such as AaH IT [4,7,27]. Moreover, electrophysiological data obtained under voltage-clamp conditions revealed that sodium channel activation is the main target of Bot IT_2 action as is the case with β-toxins [28].

On the other hand, the pharmacological properties of Bot IT_2 are surprising when comparing its primary structure to those of the other toxins used in this study. Bot IT_2 shares the highest amino acid sequence similarity with the depressant toxin Bot IT_4 (67%, Fig. 4) which is known to possess two binding sites on sodium channels [20] and to display a typical depressant electrophysiological activity [17]. However, Bot IT_2 and Bot IT_4 possess similar binding characteristics (see above); it may be suggested that the Bot IT_2 receptor site overlaps the high-affinity binding site of depressant toxin. Moreover, Bot IT_2 represents an interesting tool to discriminate the amino acid residues of the depressant toxin implicated in the high and low affinity binding sites. Future binding experiments and structural studies of these two toxins will clarify this point.

There is no evident correlation between the amino sequence similarities (Fig. 4) and the interaction with sodium channel of the scorpion insect selective neurotoxins used. However, it may be pointed out that the two amino acid residues (Lys-28 and Lys-51) of AaH IT, previously shown to be important for the toxicity and binding activity of this toxin [29], are conserved in the other toxins used. This can explain why, in spite of their sequence divergence, these toxins acts on a common receptor site in insect sodium channels and emphasize the notion that binding competition between the various toxins is due to the site being composed of or requiring several segments of the sodium channel. A partial overlap, even at one of the several attachment points of the various toxins to the receptor, may be sufficient to inhibit binding of another toxin. The common features of the tertiary structure of these toxins may allow them to interact with the same or overlapping receptor site on sodium channel.

Since Bot IT_2 reveals particular electrophysiological and binding properties and particular features in its primary amino acid sequence, this toxin represents a new interesting tool to study the structure-function relationships of scorpion toxins with insect sodium channels. Future studies of Bot IT_2 on the molecular and structural level will be important to understand its interaction with insect sodium channels.

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