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Comparative pharmacology and cloning of two novel arachnid sodium channels: Exploring the adaptive insensitivity of scorpion to its toxins

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Abstract **Scorpion toxins have been found lacking effect on Na^+ current of its own sodium channel, whereas the molecular mechanism remains mystery. In this study, the binding affinity of pharmacologically distinct scorpion toxins was found much weaker to scorpion (*Buthus martensii*) nerve synaptosomes than to spider (*Ornithoctonus huwena*) ones. The sodium channel cDNA from these two species were further cloned. The deduced proteins contain 1871 and 1987 amino acids respectively. Several key amino acid substitutions, i.e., A1610V, I1611L and S1617K, are found in IVS3–S4 constituting receptor site-3, and for receptor site-4, two residues (Leu-Pro) are inserted near IIS4 of scorpion sodium channel.**

Keywords: Sodium channel; Adaptive insensitivity; Scorpion toxins; Receptor site; Binding properties

1. Introduction

Voltage-gated Na^+ channel (Nach) is a key transmembrane protein responsible for the initiation and propagation of action potentials in excitable cells [1]. The available Nach proteins identified from different phyla all contain four homologous domains (DI-DIV), each having six putative transmembrane segments (S1–S6) [2]. Due to high sequence similarity, the large family of Nachs provides great challenge in attempts to characterize or target specific members. Fortunately, the venomous scorpions accommodate toxins capable of selectively targeting different subtypes of Nachs, i.e., α-toxins and β-toxins. α-Toxins could slow down the inactivation of Nach by interacting with receptor site-3, whereas β-toxins enhance the activation process of Nach upon binding to receptor site-4 [3]. Either class could be further divided into different groups according to the phyletic- or isoform-selectivity, i.e., α-mammal toxins, α-insect toxins, α-like toxins; and β-mammal toxins, depressant or excitatory insect-specific β-toxins, β-like toxins likewise [4].

Due to the high affinity and selectivity of scorpion toxins, localization of their receptor sites would provide unique information on the structure–function relationship of Nachs. Till now, extracellular loop S3–S4 of D4 has been inferred to constitute receptor site-3 for all three groups of α-toxins, though involving different residues in this region [5,6]. Receptor site-4 has been mapped to extracellular loop of D2 and D3 on mammal Nachs [7–9]. D2 has also been implicated in the binding of excitatory toxins on insect Nach [10].

To further elucidate the molecular basis of the binding properties of Nachs, we chose to study the non-conventional Nach from scorpion species which evolved resistance to the toxins it secretes [11]. In the present study, we investigated the binding properties of Nach from scorpion and its close relative spider species, and isolated the two full-length Nach cDNAs encoding them. By evolutionary analysis, we may gain insight into the molecular origin of the specific pharmacological properties of the scorpion Nach and a general knowledge of the structure–function relationship of the receptor site for scorpion toxins.

2. Materials and methods

2.1. Materials

Scorpion *Buthus martensii* karsch, a widely distributed scorpion species in Asia [12], and spider *Ornithoctonus huwena* Wang, one of the most venomous spiders in China [13], were used to isolate nerve tissues. The three scorpion toxins, BmK AS, BmK IT_2 and BmK I, were purified as previously published [14–16]. Sea anemone toxin ATX-II was purchased from Alomone Labs (Jerusalem, Israel). All other reagents used were of analytical grade.

2.2. Binding assays

The binding experiments were performed employing surface plasmon resonance-based technology (BIAcore, Pharmacia). Firstly, both scorpion and spider nerve cord synaptosomes were prepared and quantified according to the method previously described [17]. Secondly, each scorpion toxin was dissolved in a buffer of 10 mM sodium acetate at pH lower than the corresponding pI to give a positive charge and immobilized onto research grade sensor chip CM5 (Uppsala, Sweden) with standard amine coupling at 22 °C. Thirdly, scorpion and spider synaptosomes diluted to different concentrations were injected over immobilized neurotoxins. The dissociation rate constant of each concentration was determined by fitting the dissociation phase of each curve in a manner of 1:1 interaction between sodium channel and

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immobilized toxins and the generated dissociation rate constants were finally averaged and presented as means ± S.D. (Table S1). The association kinetic parameters were not calculated since the molarity of injected mixture of nerve synaptosomes could not be determined precisely.

For competitive binding assay, native toxins were preincubated with scorpion and spider synaptosomes at 22 °C for 30 min before the complex being injected over the sensor chip. The same concentration of nerve synaptosomes in the absence of competitors was used as control. All reaction procedures were carried out in running buffer (140 mM choline chloride, 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 25 mM HEPES and 10 mM glucose) at a flow rate of 5 μl/min for 6 min at 22 °C. Disruption of any complex that remained bound after dissociation was achieved using 0.05 N NaOH, which allows almost full retention of the original binding activity of the immobilized neurotoxins. Data were processed using Biacore 3000 Control software Version 3.1.1 and analyzed using Biaevaluation Version 3.1 (Biacore AB) and a simple 1:1 (Langmuir) dissociation model.

2.3. RNA extraction, RT-PCR, RACE

Total RNA was respectively extracted from the nerve cord tissues of scorpion and spider using Trizol reagent (Promega, USA)according to manufacturer's instructions. About 10 μg total RNA sample was primed with an Oligo (dT) $_{15}$ primer and reverse-transcribed into single-stranded cDNA using Superscript II reverse transcriptase (Gibco/BRL, USA). Degenerate primers derived from conserved amino acid residues of Para channels of fly and cockroach, as well as vertebrate Nachs, were used for amplification of the homologous sequence from the scorpion and spider cDNA. Sequence of the primers and their corresponding position are listed (Table S2). PCR amplification was carried out with Advantage high-fidelity polymerase (Clontech, USA) based on the PE-2400 (Perkin–Elmer, USA). The PCR products were analyzed on agarose gels and purified for direct sequencing or cloned into the pGEM-T easy T/A cloning vector (Promega). Finally, they were sequenced on an ABI PRISM 377 DNA sequencer (Perkin–Elmer). After the partial homologous Nach cDNA sequence generated by a series of degenerate PCR, 5′ and 3′ RACE (rapid amplification of cDNA ends) were subsequently performed to in both species (see Fig. S1). For both species, the 3′ end was achieved according to BD SMART™ RACE cDNA Amplification Kit (Clontech). The gene-specific primers (GSPs) used for scorpion was S5158 (5′-TAAACCAGGTGTAGCAGTGGCATAT-3′), and for spider, were S4393 (5′-TTGGTTCAAGCCATCCCAGCCATTTTCA-3′) and S5883 (5′-AGGAACCAACGAGGCGGGAGAAGAG-3′).

In 5′RACE, the 5′ end of spider-derived cDNA was also achieved with the same RACE Kit. Two anti-sense GSPs were A1966 (5′-ACTGGCTTCCTTTGCTATGGCG-3′) and A1156 (5′-GCAAACCCAATAAGGCAAACA-3′). As to scorpion, briefly, cDNA was initially transcribed using GSP A1967 (5′-ATAACCAGCAGTTACAACAGTC-3′) and purified on a Glassmax column (GIBCO/BRL). Homopolymeric dC tails were subsequently added to their 3′-ends by terminal deoxynucleotidyl transferase. Gene-specific primers A1351 (5′-ACAGCAGGTCCTTGGCAT-3′) and A1275 (5′-GCAAGAAATGCCCAATAAAA-3′) and cDNA adaptor primers (Gibco/BRL: AAP and AUAP) were then employed to obtain the 5′-end.

3. Results and discussion

3.1. Binding properties of scorpion and spider nerve synaptosomes

Scorpion toxins have been found lacking effect on sodium current of its own channel [11,18]. One possible suggestion is that the binding affinity of toxins to scorpion sodium channel is diminished. In this study, both synaptosomes could be rapidly bound to the immobilized BmK AS or BmK IT_2 in a concentration-dependent manner, but scorpion synaptosomes was much weaker in affinity than for spider ones (Figs. 1A and B). The estimated dissociation rate constant regarding scorpion synaptosomes is $(1.08 \pm 0.47) \times 10^{-4}\,S^{-1}$ for BmK AS and even higher for BmK IT_2, about $(2.98 \pm 0.34) \times 10^{-4}\,S^{-1}$, contrasting with the "quasi-irreversibility" of the binding to spider synaptosomes. The binding of immobilized BmK AS or BmK IT2 could be competed by native counterpart (Fig. 1C). The detected decrease in binding potency is most pronounced for the spider synaptosomes treated with native BmK AS (81.8% at 1.0 μM), less for that with native BmK IT_2 (60.4% at 1.0 μM and 81.1% at 10 μM), and least for the scorpion synaptosomes with native BmK AS (48.7% at 1.0 μM and 64.8% at 10 μM).

It is of note neither synaptosomes was detected binding to the immobilized BmK I (Figs. 1A and B) as well as the blank sensor surface (data not shown). In accordance, native BmK I could not inhibit the binding of BmK AS to scorpion synaptosomes even at the high concentration of 10 μM (Fig. 1D). Unexpectedly, BmK I was capable of inhibiting either BmK AS or BmK IT_2 binding to spider synaptosomes in a concentration-dependent manner (Fig. 1D). The potency of both BmK AS and BmK IT_2 binding was almost decreased to half in the presence of 10 μM BmK I. The most possible explanation for the discrepancy of BmK I on spider synaptosomes is the disruption of appropriate conformation required for BmK I binding to sodium channel during the covalent immobilization by amine coupling. This hypothesis derives support from the critical role of Asn11 and Arg58 in the binding of BmK I [19]. On the other hand, though the functional surface of BmK AS and BmK IT2 remains unknown, the critical residues are predicted significantly different from BmK I as these two toxins belong to β-toxins, which seem to have the conserved interactive surface composed of a number of non-polar and negative charged amino acids (e.g., Tyr26 and Val34 for Bj-xtrIT and Trp 58 and Glu15 for Css4) [20,21]. The non-polar and negatively charged amino acids are not responsible for the amine-coupling immobilization and thereby the immobilized β-toxin retains the binding capacity of its native counterpart. Besides BmK AS and BmK IT2 in the present study, immobilized BmK AS1 [22] and BmK abT [17], both are also β-toxins, exhibit the binding manner similar to their native counterparts.

To further investigate the binding properties of scorpion and spider synaptosomes, reciprocal competitions between neurotoxins were carried out. BmK AS and BmK IT_2 accommodated strong mutual competition in binding to spider synaptosomes (Fig. 1E). About 77.3% BmK IT_2 was inhibited by 1.0 μM BmK AS while 54.7% BmK AS was inhibited by 1.0 μM BmK IT2. As to scorpion synaptosomes, BmK IT_2 at a concentration of ten times (10 μM) could also compete for BmK AS binding, but to a less extent (<30%), which corresponds well with the detected low binding affinity of immobilized BmK IT_2 to scorpion synaptosomes (Fig. 1A). ATX-II, a well identified site-3 toxin, competes in a similar way of BmK I against the binding of BmK AS and BmK IT_2 (Fig. 1F). Combined, the weak affinity of applied toxins to the scorpion synaptosomes should account for the adaptive insensitivity occurred in scorpion.

3.2. Natural mutation in receptor site-3 and site-4 of BmNav1 and OhNav1

The yielded full-length cDNAs comprise 8065 and 8844 bp, respectively (Genebank™ accession number AY322171 and DQ839489). The open reading frame starts with an initiation codon ATG at nucleotide 194 in scorpion and 310 in spider one, both conforming to the Kozak rule with a G base at position -3 relative to the start codon [23], and terminates with a