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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

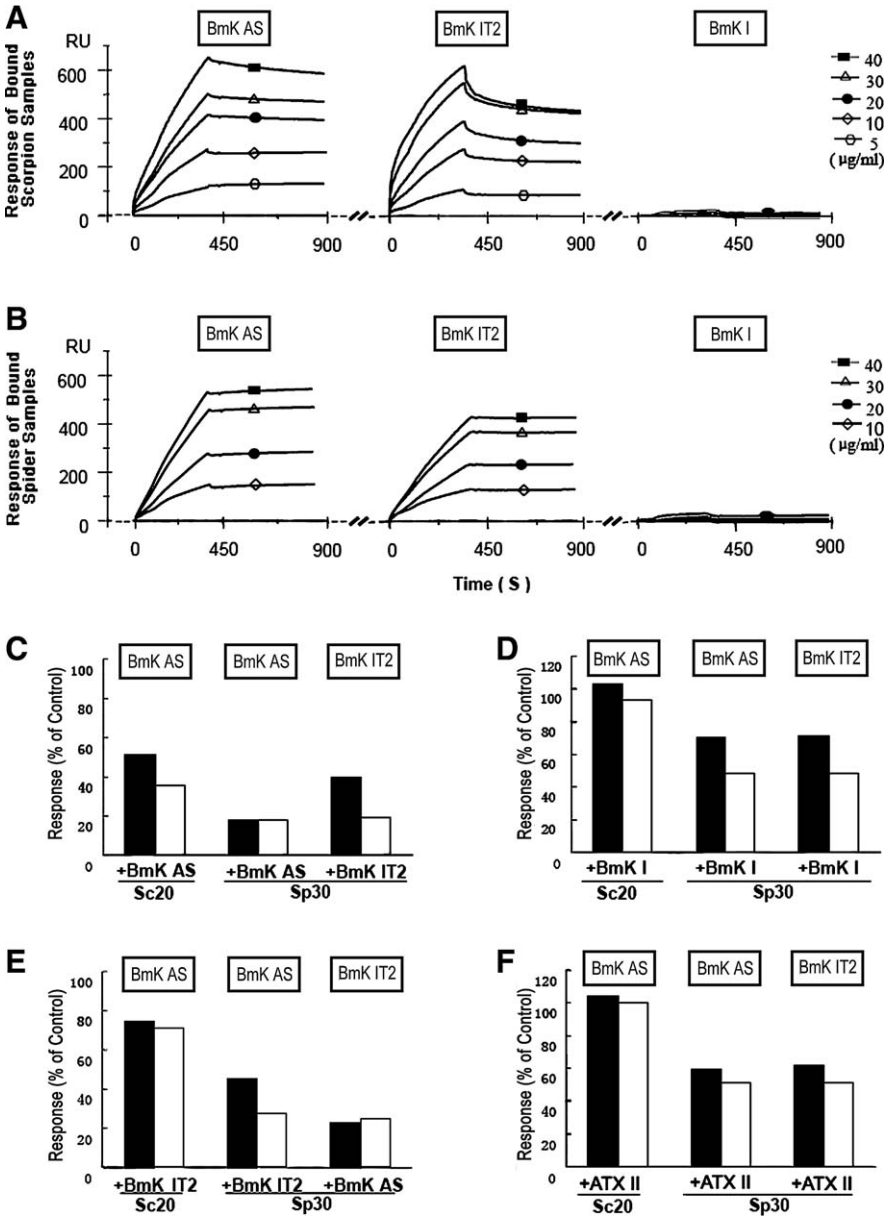

Fig. 1. Binding properties of toxins to scorpion and spider synaptosomes. (Panels A and B): BIAcore analysis of immobilized toxins binding to various concentrations of synaptosomes samples of scorpion (A) or spider (B). BmK AS, BmK IT2 and BmK I were immobilized (shown as Boxed) as described in Section 2. Their immobilization amounted to 2500, 1075, 1060 resonance units (RU) and were at pH value of 5.0, 4.4 and 4.5, respectively. (Panels C–F): competition for immobilized toxin binding to scorpion synaptosomes (20 μg/ml, Sc20) or spider synaptosomes (30 μg/ml, Sp30) by different native toxins at 1.0 μM (open symbols) or 10 μM (solid symbols). Data were analyzed as the percent binding compared with control. See Section 3 for detail.

TAA stop codon at nucleotide 5807–5809 in the former and TGA at nucleotide 6271–6273 the latter. The open reading frame accordingly encodes 1871 amino acids and 1987 amino acids. The deduced sequence and size of both novel proteins are similar to known Nach proteins (Fig. 2). The essential functional motifs for voltage-dependent gating, Na^+ selective permeation and TTX binding are absolutely conserved there (Fig. 2). Furthermore, the constructed phylogeny of BmNav1 and OhNav1 among Nach family (Fig. 3) agrees with the phylogeny of scorpion or spider organisms [24]. According to the nomenclature [2], these two deduced proteins are named $BmNa_v1$ and $OhNa_v1$. To our knowledge they are the first two full-length Nach cDNAs from arachnid order.

Though BmNav1 and OhNav1 form a closely related group sitting on the sister clade of arachnid channels, there are some difference in receptor site-3 and site-4. For clear reference, all amino residues are numbered corresponding to their position in rNav1.2 (Fig. 3).

In IVS3-S4, three amino acid substitutions between OhNav1 and BmNav1, V1610A, L1611I and K1617S, are found in a short stretch of 18 amino acids (residues 1605–1622) encompassing the negative residue Asp (position 1613) critical for α-toxin binding [5]. These three amino acids are evolutionarily conserved as $F_{1610}L_{1611}K_{1617}$ among most mammals and absolutely identical as $V_{1610}L_{1611}K_{1617}$ among insects. Hence, the substitutions mentioned above might result from conservation

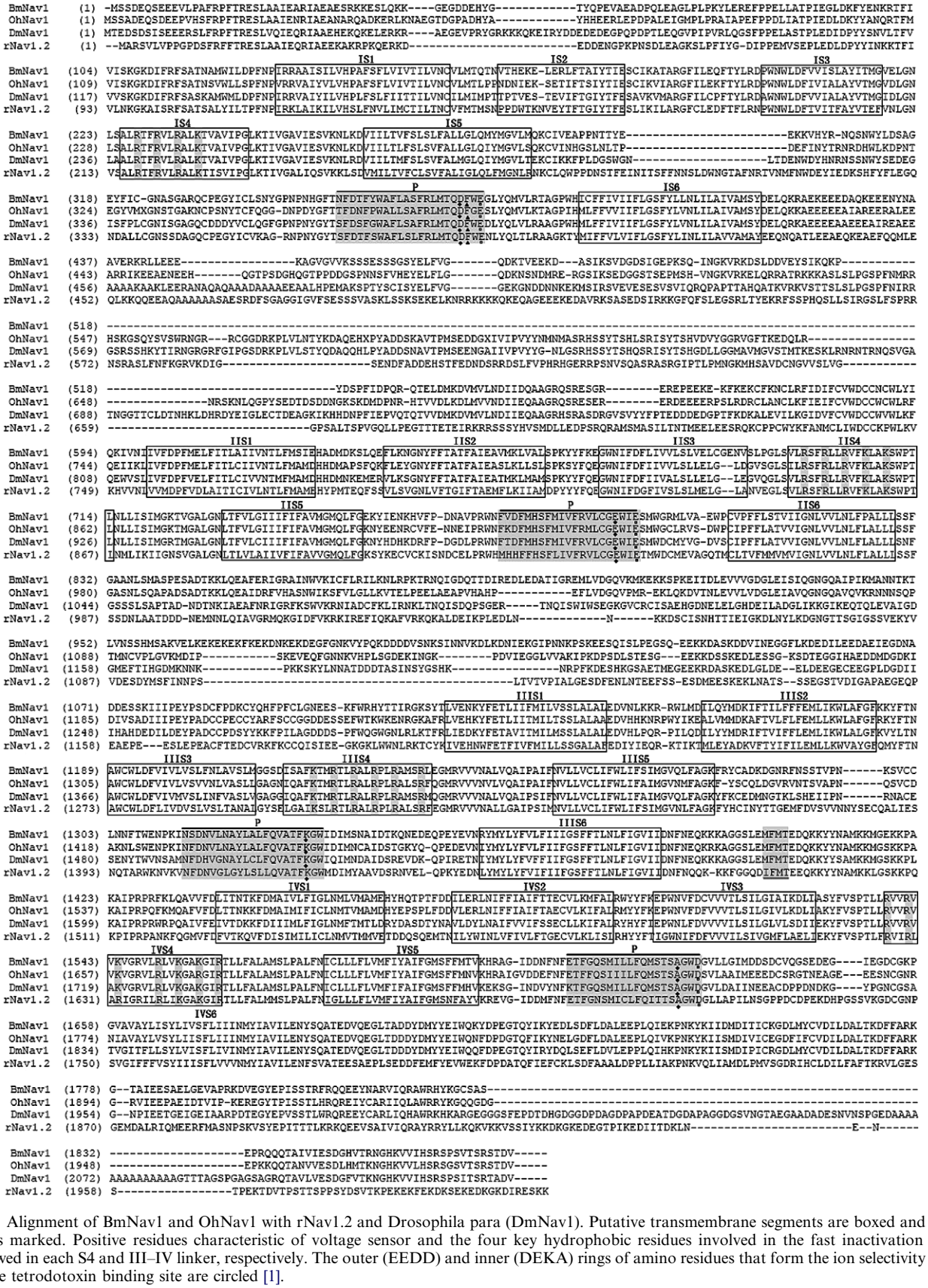

Fig. 2. Alignment of BmNav1 and OhNav1 with rNav1.2 and Drosophila para (DmNav1). Putative transmembrane segments are boxed and pore regions marked. Positive residues characteristic of voltage sensor and the four key hydrophobic residues involved in the fast inactivation were shadowed in each S4 and III–IV linker, respectively. The outer (EEDD) and inner (DEKA) rings of amino residues that form the ion selectivity filter and the tetrodotoxin binding site are circled [1].

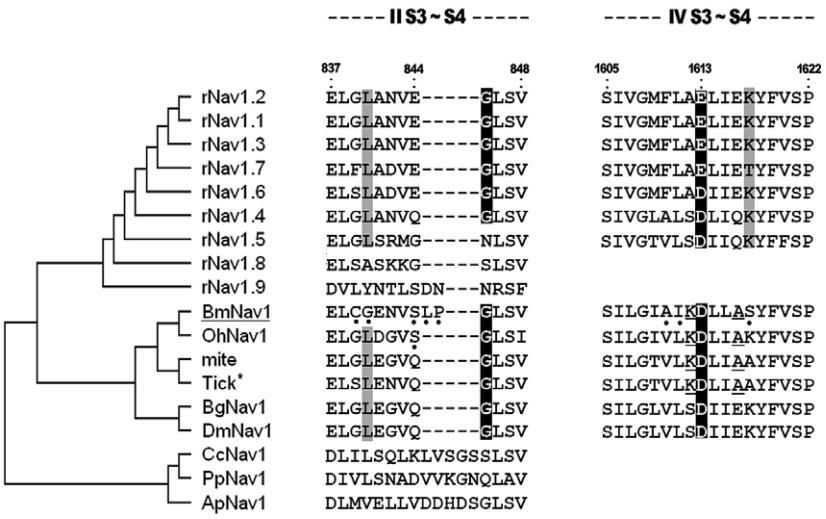

Fig. 3. Sequence comparison of BmNav1 and OhNav1 with phylogenetically distinct sodium channels in receptor site-3 (IIS3-S4) and site-4 (IVS3-S4). The channels used are mammal channels (rNav1.1-rNav1.9) and channels from invertebrate species, including the jellyfish *Polyorchis penicillatus* (PpNav1, AF047380) and *Cyanea capillata* (CcNav1, L15445), sea anemone *Aiptasia pallida* (ApNav1, AF041851), fruitfly *Drosophila melanogaster* Para (DmNav1, M32078), German cockroach *Blattella germanica* Para (BgNav1, U73583), mite *Varroa destructor* (VdNav1, AY259834), and tick *Boophilus microplus* partial sequence (AF134216). Sequences were aligned and numbered corresponding to their position in rNav1.2. Dashes indicate gaps introduced to maximize similarities. The most important residues demonstrated for toxin-binding are shadowed by dark gray and less important residues in gray, and the natural mutations occurred in BmNav1 are dotted.

in OhNav1 and natural mutation developed in BmNav1. It holds a clue to scorpion's adaptive insensitivity. Coincidentally, the K1617T replacement was found decreasing the affinity of scorpion toxins significantly in rNav1.7 [6], strongly supporting the involvement of natural mutation K1617S in the scorpion's adaptive insensitivity. Furthermore, residues at 1610 and 1611 are near the extracellular end of the IVS3 and this region has also been demonstrated participating in the binding of α-toxin by mutagenesis research on rNav1.2 previously [5].

Interspecies comparison further reveals that Lys_{1612} and Ala_{1616} are conserved in all four available arachnid channels, but different in insect and vertebrate counterparts. Position 1612, is Ser in insects and Ser/Ala in vertebrates; position 1616, is Glu in insects and Glu/Gln in vertebrates. As both residues are adjacent to the critical negative residue Glu/Asp at position 1613, these two substitutions are easily supposed to interfere with the electrostatics in this region for site-3 toxin binding, which otherwise might be favored by the corresponding uncharged residues and negative residues in sensitive Nachs. Based on the features, two substitutions, S1612K and E1616A, might have occurred in primitive arachnids before the divergence into different orders but seem not to alter the binding ability of scorpion toxins to spider sodium channel. It still deserves investigation whether they function in harmony with three substitutions mentioned above in scorpion's adaptive insensitivity and play a role in the insensitivity to other site-3 toxins, such as those from spider [25].

It is worth noting that besides IVS3-S4, IS5-S6 and IVS5-SS1 have also been suggested to be the constituents of receptor site-3 by site-directed antibody experiment [26]. But the mutation of negatively charged residues in IS5-S6 and IVS5-SS1 appeared not to alter the binding of α-toxin. Further mutagensis studies would be required to identify the individual amino acid residues in these regions that may participate in α-toxin binding. The obvious difference between BmNav1 and others is the residues at position 355 and 356, which are highly conserved as Lys-Ala in mammal channels rNav1.1–1.7 or Gln-Gly in arthropod channels, only except for BmNav1 (Ser-Asn) (Fig. S2). Analyzing the sequence conservation and substitution in these regions between diverse channels may hold clue to this question.

As to receptor site-4, IIS3-S4 constitutes the major part [8]. OhNav1 is highly similar to those toxin binders (Insect Nachs and rNav1.1–1.7 except rNav1.5) in this loop, especially to insect ones as only 2 out of 12 amino acids (residues 837–848) are different. One is a conservative change E841D and the other is Q844S (Fig. 3), which seems not to affect the binding of β-toxin suggested by Q844E mutation [9]. On the other hand, despite the overall similarity to toxin binders in this stretch, BmNav1 displays several substitutions: G839C, L840G, Q844S and the most amazing insertion of two amino acids (Leu-Pro) adjacent to the key residue Gly_{845} [8,9]. The sequence and size of the region is found evolutionary conserved among all known Nachs with the exception of rNav1.9 and channels from the ancestral cnidarians, indicating that the di-peptide insertion in BmNav1 should result from some evolutionary pressure unique to scorpion. Considering that this region is right the β-toxin binding site, the insertion accounts for the adaptive insensitivity to β-toxins in all likelihood. The different binding properties of β-toxins (BmK AS and BmK IT2) to scorpion and spider synaptosomes (Fig. 1) possibly suggest a causal relationship between the unique di-peptide insertion and scorpion's adaptive insensitivity to β-toxins. Furthermore, L840 is inferred to make productive binding interaction with amino acid residues in β-toxin as L840C significant reduced the binding affinity [27]. Hence, L840G, the renewed mutation in scorpion after the separation of scorpion and spider order, is expected to work together with the di-peptide insertion for the adaptive insensitivity towards scorpion toxins.

Besides IIS3-S4, IIS1-S2 and III SS2 have also been found constituting the receptor site 4 [8,9]. Sequence alignment in

these two regions reveals some residues that have been known important for the binding of β-toxins, such as $N_{1466}V_{1467}$ in rNav1.2 [8,9], are changed into QNE in BmNav1, and some residues uniquely occur in the scorpion channel whereas conserved among other channels, for example, Ala at position 781 and Arg at position 1498 (Fig. S3). These natural substitutions may be hint to the involvement in the receptor site-4.

3.3. BmNav1 and OhNav1: insight into the localization of toxin binding sites

The overlapping binding site of β-like toxin BmK AS and depressant toxin BmK IT_2 as well as their partially overlapping with that of α-like toxin BmK I, revealed by the competitive binding on spider synaptosomes, are consistent with the previous reciprocal competition observed on insect synaptosomes [16]. ATX-II inhibits in a similar manner of BmK I, strongly suggesting that though BmK AS and BmK IT_2 are deemed to bind to receptor site-4, the structural elements forming receptor site-3 might be involved as well. This idea derives support from previous findings that another two β-like group toxins, AaH IT_4 and Lqhβ1, were capable of inhibiting the binding of α-mammal toxins on rat brain synaptosomes [28,29]. It is of note that depressant toxins (by extension of BmK IT_2) and β-like group toxins constitute a sister clade separated from other β-toxins [4]. Thereby it is not surprising that these two groups share a binding character distinct from classical β-toxins. Furthermore, BmNav1 and OhNav1 may be developed to construct chimera and study the structural elements of Nachs that are involved in the interaction with toxins from natural venomous animals, especially site-3 and site-4 toxins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.07.024.

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