Localization of Receptor Site on Insect Sodium Channel for Depressant β-toxin BmK IT2

Huiqiong He1,2, Zhirui Liu1,2, Bangqian Dong1, Jianwei Zhang1, Xueqin Shu1, Jingjing Zhou1, Yonghua Ji1

1 Lab of Neuropharmacology and Neurotoxicology, Shanghai University, Shanghai, People's Republic of China, 2 Graduate School of Chinese Academy of Sciences, Shanghai Institute of Physiology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, Shanghai, People's Republic of China

Abstract

Background: BmK IT2 is regarded as a receptor site-4 modulator of sodium channels with depressant insect toxicity. It also displays anti-nociceptive and anti-convulsant activities in rat models. In this study, the potency and efficacy of BmK IT2 were for the first time assessed and compared among four sodium channel isoforms expressed in Xenopus oocytes. Combined with molecular approach, the receptor site of BmK IT2 was further localized.

Principal Findings: 2 μM BmK IT2 strongly shifted the activation of DmNav1, the sodium channel from Drosophila, to more hyperpolarized potentials; whereas it hardly affected the gating properties of rNav1,2, rNav1,3 and mNav1,6, three mammalian central neuronal sodium channel subtypes. (1) Mutations of Glu896, Leu899, Gly904 in extracellular loop Domain II S3–S4 of DmNa1, abolished the functional action of BmK IT2. (2) BmK IT2-preference for DmNa1,1 could be conferred by Domain III. Analysis of subsequent DmNa1,1 mutants highlighted the residues in Domain III pore loop, esp. Ile1529 was critical for recognition and binding of BmK IT2.

Conclusions/Significance: In this study, BmK IT2 displayed total insect-selectivity. Two binding regions, comprising domains II and III of DmNa1,1 play separate but indispensable roles in the interaction with BmK IT2. The insensitivity of Na1,2, Na1,3 and Na1,6 to BmK IT2 suggests other isoforms or mechanism might be involved in the suppressive activity of BmK IT2 in rat pathological models.

Introduction

Voltage-gated sodium channels (VGSC) are key membrane proteins responsible for neuron excitability, consisting of an ion-conducting α-subunit accompanied by one or more auxiliary subunits [1]. Generally, the α-subunit comprising four repeated domains (DI–DIV), each containing six transmembrane α-helixes (S1–S6) and a hairpin-like pore loop between S5 and S6 [2], split into an N-terminal part (SS1) and a C-terminal part (SS2). Despite the high structure similarity, various VGSC subtypes display distinct distribution, gating properties and function activities. Some neurotoxins can differentiate among them with preference for certain subtype(s) [3], thus providing clues about the structure-function relationship of VGSCs and a potential molecule library for novel drug design or insecticide development.

Amongst the neurotoxins purified from scorpions, β-toxins shift the voltage dependence of VGSC activation to cause subthreshold channel opening, which can be enhanced when channels are prracticed by a depolarizing prepulse [4]. According to the phyletic-bioactivity, the β-toxins may be further divided into β-mammal toxins, depressant or excitatory insect-specific β-toxins and TsVII-like toxins acting on both mammals and insects [3]. The group of β-toxins is deemed to bind to a common receptor site-4 on VGSC α-subunits, which, however, shows a rather complex picture. The binding sites for β-mammal toxin CssIV (from Centruroides suffusus suffusus) and TsVII-like toxin Tz1 (from Tityus zulianus) have been mapped to DII S1–S2, DII S3–S4 and DIII SS2-S6 on mammalian VGSCs [4,5,6,7]. The effect of TsVII (i.e. Tzγ from Tityus serrulatus) on reducing peak currents are also conferred by the S4 segments of DIII and DIV [8,9]. The excitatory and depressant β-toxins act distinctly, though they both target insect VGSCs [10,11]. DII of DmNa1,1 is implicated in the selective recognition of excitatory toxin AahIT (from Androctonus australis Hector) [12], while several channel regions (DI S5-SS1, DI S8-S6, DII SS2-S6, and DIV SS2-S6) may be involved in the interaction with depressant toxin LqhIT2 (from Leiurus quinquesstratus helvus) [11]. Based on the results of mutation experiments applied on rat VGSCs and information provided by structure analysis of LqhIT2 [13,14], some possible interaction spots in DIII S3–S4 of DmNa1,1 were deduced. However, no site-directed mutagenesis has been performed on insect VGSC yet as to dissect the receptor site for depressant β-toxins.

BmK IT2, a depressant β-toxin from the scorpion Buthus martensi Karsch, can induce strong insect toxicity [15]. Like other...
depressant toxins, such as LqhIT2 [11,16], BmK IT2 possesses a series of mutations into DmNav1 and the resulting mutants were accomplished with PCR-based mutagenesis, giving rise to two non-interacting binding sites (the high/low-affinity binding sites) on insect nerve membranes [17,18]. Despite typical anti-insect features of depressant β-toxins, BmK IT2 displayed antinociceptive and anticonvulsant activities in rat models [19], which were attributed to the specific modulation on brain VGSCs [20]. Such effects against mammals have also been observed in other depressant β-toxins [21,22,23] and explained as a consequence of adaptive evolution of these toxins. However, the binding affinity of BmK IT2 to rat brain synaptosomes was quite low [17,18] and the specific target is still unidentified.

To forward the understanding for the binding features of depressant β-toxins and their intriguing functional diversity, in the present study, we attempted to address the following issues: 1) Can BmK IT2 modulate the mammalian VGSC subtypes from central neuronal system (i.e. Na\(_{1.2}\), Na\(_{1.3}\) and Na\(_{1.6}\))? 2) What is the selectivity of BmK IT2 between these mammalian subtypes and insect VGSC DmNav1? 3) What is the binding/recognition site on insect VGSC for BmK IT2?

Materials and Methods

Materials

BmK IT2 was purified by column chromatography from the crude venom of the Asian scorpion _Buthus martensi_ Karsch as described previously [15]. The purity of the toxin was confirmed by mass spectrometry.

The genes encoding the sodium channel α-subunit DmNa\(_{1.1}\) (P35500.3) from _Drosophila_ paralytic temperature-sensitive and the auxiliary TipE subunit were kindly provided by J. Warmke (Merck, New Jersey, USA) and M. S. Williamson (IACR-Rothamsted, UK), respectively. Plasmids in combination with cDNAs of rat/mouse VGSC α-isoforms i.e. rNa\(_{1.2}\) (CAA27287), rNa\(_{1.3}\) (CAAA68735) and mNa\(_{1.6}\) (Q9WTU3.1), as well as β1 subunit were originally from Dr. Alan L. Goldin (University of California, USA).

Construction of channel chimeras and mutants

Five endogenous restriction sites in rNa\(_{1.2}\), rNa\(_{1.3}\) ORF were used to excise DNA fragments coding for the four channel domains (DI: XhoI/XmaI, DII: XmaI/BglII, DIII: BglII/BstEII, DIV: BstEII/PacI). The parallel DNA fragments of DmNav1 corresponding to XhoI/XmaI, DII: XmaI/BglII, DIII: BglII/BstEII, DIV: BstEII/PacI were replaced by those of DmNav1 channel. The resulting mutants were introduced into the mammalian sodium channel (rNav1.2) to examine their binding properties and pharmacology.
Figure 1. Effect of BmK IT2 on wild-type VGSCs expressed in *Xenopus* oocytes. A. Current responses of rNa_1.2, rNa_1.3, mNa_1.6, and DmNa_1 channels to a test voltage of −50 mV, where channels were closed under control conditions (gray traces). Black traces represented currents
in the presence of 2 μM BmK IT2 without a prepulse (−PP, upper panel) and with a prepulse (+PP, lower panel). The scale bar in figure 1A covered all four embodied currents. B. Normalized conductance plotted as a function of voltage for the indicated channel subtypes. C. Current-voltage curves for the indicated channel types.

Results

Efficacy of BmK IT2 on VGSC isoforms from insect and mammalian central neuronal system

Using the two-electrode voltage clamp recording, BmK IT2 was subjected to a comparative study for the effects on four VGSC subtypes, rNav1.2/β1, rNav1.3/β1, mNav1.6/β1 and DmNav1/TipE expressed in Xenopus oocytes (Fig. S1). The voltage-dependent channel activation was investigated by a three-step protocol (see Materials and Methods). 2 μM BmK IT2 induced significant subthreshold currents (at −50 mV) in DmNav1/TipE channels with a depolarizing prepulse (PP) of 25 ms (Fig. 1A). The half-maximal activation voltage (V_{1/2}) of DmNav1/TipE was shifted by about −11 mV and the slope factor (k_{n}) was increased from 3.72 to 7.97 mV (p<0.001, n = 10) by 2 μM BmK IT2 (EC_{50} = 2.9±0.36 μM, Table 1). This shift was also observed only in the presence of a prepulse (Fig. 1B–C). In contrast, rNav1.1/β1, rNav1.3/β1, mNav1.6/β1 and DmNav1/TipE were totally insensitive to BmK IT2 at concentrations of 2 μM (Fig. 1A and B) and even up to 20 μM (Fig. S2). Prolonging the PP duration to 50 ms was unable to enhance the efficacy of BmK IT2 (data not shown). Though the activation of rNav1.3/β1 eventually responded to BmK IT2 at a rather high concentration (50 μM; ΔV_{1/2} = −4.84 mV, data not shown), rNav1.2/β1 and mNav1.6/β1 still remained insensitive. Whether or not β1 subunit was coexpressed with these mammalian VGSC subtypes did not influence the action of BmK IT2 (not shown).

On all investigated VGSC subtypes, a small depression of current amplitude was observed (<10% for mammalian VGSCs and ~20% for DmNav1/TipE, Fig. 1C) after application of BmK IT2. There were no significant BmK IT2-induced changes in inactivation property of channels (data not shown). The results suggest that BmK IT2 exhibited distinguished subtype selectivity on sodium channels, preferring the insect target rather than mammalian central neuronal isoforms.

Mutations in DII S3–S4 impacted BmK IT2 function on insect VGSC

Previous reports demonstrated that substitutions introduced to DII (e.g. E{779}Q in DII S1–S2, and E{837}Q, L{840}C, G{845}N in DII S3–S4 of rNav1.2; G{850}N in DII S3–S4 of rNav1.4) reduced the effects of the β-toxins Css4 and Tz1 [4,5,6,7]. As for the case of mammalian channels, structural bioinformatics analysis deduced three analogous residues in DmNav1 (E{896}, L{899} and G{904}) might also be crucial in the interaction with LqhIT2 [15,14]. Based on these studies and considering the high homology between LqhIT2 and BmK IT2, mutations of D{838}, E{896} and G{904} (corresponding to E{779}, E{837}, L{840} and G{845} in rNav1.2, Fig. 2A), were individually introduced into DmNav1.

The mutants were co-expressed with TipE subunit ensuring the functional expression and currents were recorded in the same condition as that of wild type DmNav1. The gating property of all mutants was not altered with respect to those of wild-type channels, thus the subsequent electrophysiological analysis was not “contaminated” by mutagenesis. The normalized conductance-voltage relationship of mutants were assessed in the absence and presence of 2 μM BmK IT2 with a 25 ms-PP. Mutant D{838}C showed the similar response to BmK IT2 as wild type DmNav1 (Fig. 2B), whereas the mutations of GhG{896}, LcG{899} and GhG{904} totally abolished negative shift of voltage-dependent activation induced by 2 μM BmK IT2 (ΔV_{1/2}≤2.0 mV, ΔV_{0.36}<1.0 mV, n = 7 or 8, Fig. 2C–E, Table 2). Besides, the mutants DmE{896}C, DmL{899}C and DmG{904}N were also resistant to BmK IT2 at higher concentrations (Table 1). This result verified that residues E{896}, L{899} and G{904} in DII S3–S4 of DmNav1 play critical roles in responding to BmK IT2.

DII from DmNav1 conferred BmK IT2 sensitivity to rNav1.2

Although E{896}, L{899} and G{904} positively support the action of BmK IT2, sequence alignments (Fig. 2A) indicate these residues are also conserved in corresponding positions of all BmK IT2-insensitive mammalian VGSCs investigated. It appears that they are necessary, but not sufficient to fulfill the interaction with BmK IT2, suggesting additional channel region(s) might be involved.

To find out the region(s) responsible for BmK IT2 recognition, four chimeras (ChD1, ChD2, ChD3 and ChD4; Fig. 3A) were thus constructed by replacing each domain of rNav1.1/β1 with that of DmNav1 respectively. Current recordings demonstrated that

| Channels          | EC_{50} (μM) | n |
|-------------------|-------------|---|
| rNav1.2           | >50         | 3 |
| rNav1.3           | >20         | 4 |
| mNav1.6           | >50         | 3 |
| DmNav1            | 2.9±0.36    | 5 |
| ChD1              | >50         | 3 |
| ChD2              | >50         | 3 |
| ChD3              | 22.5±6.65   | 3 |
| ChD4              | >50         | 3 |
| DmD{838}C         | 3.6±0.90    | 3 |
| DmE{896}C         | >35         | 3 |
| DmL{899}C         | >35         | 3 |
| DmG{904}N         | >50         | 3 |
| L/DmNav1.2        | ND          | / |
| L1/D2/DmNav1.1    | ND          | / |
| DmE{1523}KR{1524}Y | ND          | / |
| DmMS              | ND          | / |
| DmE{1523}N        | 2.4±0.46    | 3 |
| DmD{1525}E        | 3.3±0.73    | 3 |
| DmK{1526}L        | ND          | / |
| DmI{1527}K        | 15.6±3.60   | 3 |
| DmR{1528}Y        | ND          | / |
| DmT{1529}D        | ND          | / |
| DmI{1530}L        | ND          | / |

EC_{50} values (μM) were determined as described in Methods. The data were represented as the mean ± SEM and n is the number of independent experiments. ND, not determined; /, null.

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Table 1. EC_{50} values (μM) of BmK IT2 on wild-type and chimeric/mutated VGSCs.
the channel activities were not impaired by cross-species domain substitution. Like rNav1.2, most chimeric channels were regulated by mammalian β1 subunit but not TipE from insect (data not shown). The only exception was ChD4 that seemed insensitive to either β1 or TipE.

The activation of chimeras ChD1, ChD2 and ChD4 were hardly modified by 2 mM BmK IT2 (Fig. 3B), like wild type Nav1.2. In contrast, ChD3 gained the response to 2 mM BmK IT2, which caused a statistically significant shift of channel activation (DV1/2 = 25.64 mV, p < 0.005, n = 10) (Fig. 3B, Table 2). The increased sensitivity in ChD3 (EC50 = 22.5 ± 6.65 nM, Table 1) also suggested DIII seemed to play a necessary role in the interaction between insect sodium channel and BmK IT2.

Residues in DIII SS2-S6 critical for the sensitivity of DmNav1 to BmK IT2

To further clarify the possible interaction site in DIII, a series of mutations have been performed in DIII SS2-S6 pore loops of rNav1.2 and DmNav1.1. The mutagenesis design was based on the previous report that suggested DIII SS2-S6 might be involved in the binding of LqhIT2 [11]. First, to verify whether this region accounted for BmK IT2 binding, the DIII SS2 loops were compared (Fig. 4A) and exchanged between DmNav1 and rNav1.2 (Fig. 4B), giving rise to two loop chimeras: L(Dm)Nav1.2 and L(1.2)DmNav1. Unexpectedly, the whole loop replacement in DmNav1 (I1512 to I1534) by that of rNav1.2 (M1425 to L1447) resulted in channels hardly expressed in Xenopus oocytes even accompanied by TipE subunit. Thus for generating robust Na+ currents, two residues in rNav1.2-type loop had to be restored as present in DmNav1(I1529/R1530) (See Material and Methods). Double mutant DmI1529K/R1530Y was then produced as the compensation of the incomplete loop substitution.

Similar to the case of chimera ChD3, in the presence of 2 mM BmK IT2 and a 25 ms prepulse, the voltage-dependent activation of L(Dm)Nav1.2 displayed a mild but significant shift with ΔV1/2 of about −3 mV (p < 0.05, n = 8, Fig. 4C and Table 2). As for L(1.2)DmNav1 (Fig. 4D), the substitution by most part of the DIII SS2 loop from rNav1.2 could not prevent BmK IT2-induced shift in the voltage of half-maximal activation (ΔV1/2 = −12.48 mV).
Table 2. Parameters for the voltage dependent activation of wild-type and chimeric/mutated VGSCs.

| Channels         | \( V_{1/2} \) (mV) | \( \Delta V_{1/2} \) (mV) | \( k_m \) | \( k_m(\text{+BmK IT2}) \) | \( n \) |
|------------------|---------------------|--------------------------|----------|--------------------------|------|
| rNa,1.2          | -25.45 ± 0.67       | 1.14 ± 0.01              | 4.78 ± 0.54 | 4.88 ± 0.55             | 8    |
| rNa,1.3          | -20.06 ± 0.32       | 0.90 ± 0.04              | 3.03 ± 0.54 | 3.14 ± 0.50             | 8    |
| mNa,1.6          | -19.14 ± 0.72       | 0.32 ± 0.08              | 3.93 ± 0.76 | 4.48 ± 0.76             | 8    |
| DmNa1             | -18.52 ± 0.34       | -11.92 ± 0.36            | 3.72 ± 0.40 | 7.97 ± 0.65             | 10   |
| ChD1             | -20.60 ± 0.32       | -0.05 ± 0.04             | 2.13 ± 0.24 | 2.33 ± 0.33             | 10   |
| ChD2             | -17.41 ± 0.22       | -0.87 ± 0.01             | 4.82 ± 0.60 | 4.92 ± 0.49             | 10   |
| ChD3             | -23.40 ± 0.29       | -5.64 ± 0.00             | 3.85 ± 0.25 | 3.59 ± 0.26             | 10   |
| ChD4             | -20.22 ± 0.39       | -21.66 ± 0.41            | 4.11 ± 0.40 | 4.40 ± 0.38             | 10   |
| DmD452E/C        | -18.95 ± 0.62       | -24.90 ± 0.98            | 3.33 ± 0.76 | 7.68 ± 0.88             | 7    |
| DmL696E/C        | -20.68 ± 0.38       | -22.34 ± 0.52            | 3.10 ± 0.57 | 4.05 ± 0.45             | 6    |
| DmL699E/C        | -22.89 ± 0.38       | -24.28 ± 0.39            | 2.76 ± 0.28 | 2.90 ± 0.22             | 7    |
| DmG904N          | -25.55 ± 0.57       | 0.66 ± 0.00              | 4.80 ± 0.46 | 4.79 ± 0.45             | 8    |
| L(Dm)Nav1,1.2    | -21.01 ± 0.31       | -26.06 ± 0.39            | 3.71 ± 0.34 | 4.79 ± 0.32             | 7    |
| Li(1/2)DmNav1    | -21.87 ± 0.64       | -34.35 ± 0.72            | 4.76 ± 0.58 | 5.48 ± 0.62             | 9    |
| Dm1529Y/R1350Y   | -24.82 ± 0.48       | -29.88 ± 0.48            | 4.85 ± 0.39 | 5.05 ± 0.46             | 8    |
| DmM5             | -21.68 ± 0.35       | -28.24 ± 0.55            | 2.85 ± 0.40 | 5.73 ± 0.49             | 6    |
| Dm1522N          | -25.32 ± 0.56       | -37.42 ± 0.84            | 6.89 ± 0.75 | 7                     | 7    |
| Dm1522E          | -21.32 ± 0.34       | -31.05 ± 0.58            | 6.50 ± 0.52 | 6                     | 6    |
| Dm1526L          | -21.54 ± 0.39       | -33.83 ± 0.59            | 5.71 ± 0.51 | 8                     | 6    |
| Dm1522K          | -23.10 ± 0.17       | -22.98 ± 0.18            | 4.02 ± 0.14 | 7                     | 7    |
| DmR1350Y         | -20.35 ± 0.51       | -25.46 ± 0.98            | 7.69 ± 0.85 | 8                     | 8    |
| Dm1522D          | -20.57 ± 0.36       | -30.01 ± 0.70            | 7.41 ± 0.64 | 6                     | 6    |
| Dm1526L          | -24.64 ± 0.42       | -32.63 ± 0.55            | 5.90 ± 0.45 | 7                     | 7    |

The values of half-maximum activation voltage \( V_{1/2} \) and corresponding slope factor \( k_m \) were determined in the absence and presence of 2 μM BmK IT2. Application of BmK IT2 shifted channel activation by \( \Delta V_{1/2} \). The data were represented as the mean ± SEM and \( n \) is the number of independent experiments.

The values of \( V_{1/2} \) and \( \Delta V_{1/2} \) were determined in the absence and presence of 2 μM BmK IT2. Application of BmK IT2 shifted channel activation by \( \Delta V_{1/2} \). The data were represented as the mean ± SEM and \( n \) is the number of independent experiments.

Interestingly, however, unlike wild type DmNa1, the slope factor of its activation curve was barely affected by BmK IT2 (L(1/2)DmNa1: \( \Delta k_m < 1 \text{ mV} \), \( n = 8 \); DmNa1: \( \Delta k_m = +4.25 \text{ mV} \), \( n = 10 \); Table 2). It was noticeable that double-mutant DmI1529K/R1350Y exhibited largely attenuated sensitivity to 2 μM BmK IT2 as the toxin-induced \( \Delta V_{1/2} \) decreased to -5.06 mV with the slope factor \( k_m \) unchanged (Table 2). The results indicate that the DIII SS2-S6 pore-loop of DmNa1 plays a major role in BmK IT2 interaction and it was the main contributor in conferring BmK IT2 sensitivity to rNa1,2.

To determine the residue(s) in this region critical for the interaction with BmK IT2, a series of site-directed mutations of DmNa1 were produced (see Materials and Methods). All mutants displayed gating parameters (Table 2) similar to those of wild type DmNa1, ruling out the possibility that the alteration of gating behavior was involved in variation of BmK IT2 sensitivity. Subsequent analysis demonstrated that among all the mutants (Fig. 5), the potency of 2 μM BmK IT2 was obviously decreased on DmM5, DmI1522K, DmR1350Y and DmI1526K/R1350Y, with respect to wild-type DmNa1. The alterations in voltage-dependent activation induced by 2 μM BmK IT2 were in the order that \( \Delta V_{1/2} \) of DmNa1 (-11.92 mV, 4.25 mV) > DmR1350Y (-5.11 mV, 3.66 mV), DmM5 (-6.36 mV, 2.88 mV) > DmI1526K/R1350Y (-5.06 mV, 0.20 mV) > DmI1526K (+0.12 mV, 0.60 mV). Notably, mutant DmI1526K was less sensitive to BmK IT2 (\( E_{50} = 15.6 \pm 2.6 \text{ nM}, \) Table 1), indicating an especially critical role of residue I1526 in the interaction with BmK IT2.

Discussion

VGSC subtype-selectivity of BmK IT2

BmK IT2 was classified into the group of β-depressant insect toxin because: 1) it shares high sequence similarity with other well-defined depressant anti-insect toxins, such as LqhIT2, LqqtIT2 and BjIT2 [26]; 2) BmK IT2 is toxic to insect but not mammals [27,28]. This insect-selectivity was also observed in binding experiments tested on cockroach nerve cords which displayed a 200–300 fold higher affinity with BmK IT2 than rat brain synaptosomes [17]. However, like some other depressant β-toxins [21,22,23], BmK IT2 also evolves function against mammals, e.g. antiinsective and anticonvulsant activities in rat models [19]. As recent studies have mostly focused on the pharmacological phenotype of BmK IT2, the underlying mechanism and molecular target in rat brain remain unidentifiable. In this study, the efficacy and selectivity of BmK IT2 was assayed for the first time among independently cloned VGSCs from insect (DmNa1) and mammalian central nervous system (i.e. rNa1,2, rNa1.3 and mNa1,6) expressed in Xenopus oocytes.

Results showed that the main effects of BmK IT2 on DmNa1 included a decrease of peak Na⁺ current (by ~20%) and a significant hyperpolarizing shift of the activation. These are typical effects for scorpion depressant β-toxins. The increase of the slope value of activation curve, reflecting the decreased voltage dependence of activation process and a larger subthreshold channel open probability, is also observed in previous reports
Figure 3. Schematic composition of DmNav1-Nav1.2 domain chimeras and effect of BmK IT2 on four chimeric channels. A. Cartoons illustrating the construction of channel chimeras. The channel domains of rNav1.2 were shown in grey, while the domains from DmNav1 were shown in black. B. Normalized conductance-voltage plotted for chimeras ChD1-ChD4 before (■) and after (○) application of 2 μM BmK IT2, with a prepulse (PP).
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characterizing the function of depressant β-toxins LqqIT2 and LqhIT2 [29,30].

In contrast, three mammalian VGSCs were totally insensitive to BmK IT2. The low affinity of BmK IT2 to rat brain synaptosomes can be explained by the insensitivities of Nav1.2 and Nav1.6, which are dominant VGSC subtypes spreading throughout CNS [31,32], to BmK IT2. It is noteworthy that BmK IT2 was capable of inhibiting the total Na\(^{+}\) currents in rat dorsal root ganglion (DRG) neurons [20]. According to our results that Nav1.2, Nav1.3 and Nav1.6 are BmK IT2-insensitive, the action of BmK IT2 on Na\(^{+}\) currents of DRG neurons may be a result of selective modulation on other neuronal VGSC subtypes, most likely Nav1.7, Nav1.8 and/or Nav1.9 channels. Thus, it may allow us to speculate that peripheral nerve VGSC subtypes might be the major targets responsible for BmK IT2-induced anti-nociception and anti-convulsant effects in rat models, though the subtypes or other membrane proteins that are possibly involved in the working mechanism of BmK IT2 still need to be further characterized.

Construction of insect-mammalian chimeric channels

Since the insect and mammalian VGSCs are highly similar in both structural and functional properties, insect-mammalian chimeras could be constructed to determine the regions responsible for the toxin recognition and interaction. Previously for localizing the insect VGSC domain that binds β-excitatory toxin AahIT, a chimeric channel was constructed from rat brain rNav1.2 in which DII was replaced by that of Drosophila [12]. Here we also chose rNa\(_{1.2}\) as backbone of chimeric channels that accepted insect VGSC domains considering that: 1) rNa\(_{1.2}\) channel is insensitive to BmK IT2 at very high concentration (e.g. 20 μM); 2) as a typical VGSC subtype from mammalian nervous system, rNa\(_{1.2}\) channel has been well characterized in Xenopus oocytes and displays an excellent performance in expression level.

The binding feature of BmK IT2 on DmNa\(_{1}\)

The classical voltage-sensor trapping model indicates that β-toxins function as a stabilizer of activated state of VGSCs by trapping the outward DII S4 and hereby shift the activation threshold to more hyperpolarized potentials [4].

To directly reveal the BmK IT2 binding region(s) in DmNa\(_{1}\) channel and confirm the results obtained in Na\(_{1.2}\) backbone chimeras, we also attempted to generate the mammalian-insect chimeras in which the independent domains of DmNav1 were replaced by those of rNav1.2. Unfortunately, due to the rather low expression level, these chimeras failed to serve as satisfying candidates for the subsequent pharmacological analysis.

**Figure 4. Analysis of DmNa\(_{1}\)-Nav1.2 DIII SS2 loop chimeras.** A. Sequences of SS2 loop in DIII of wild-type VGSCs. B. Diagram illustrating the composition of the SS2 loop chimeras L(Dm)Na\(_{1.2}\) and L(1.2)DmNa\(_{1}\) (Na\(_{1.2}\) SS2 loop: grey; DmNa\(_{1}\) SS2 loop: black). C–D. Effect of BmK IT2 on voltage-dependent activation of L(Dm)Na\(_{1.2}\) and L(1.2)DmNa\(_{1}\) with a prepulse (PP) of -10 mV for 25 ms. ■, control conditions; ○, 2 μM BmK IT2.

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sensitivity as they are well conserved in the BmK IT2-insensitive mammalian channels like rNav1.2, rNav1.3 and mNav1.6. The subsequent study revealed that DIII rather than DII could confer BmK IT2 insect-preference to mammalian sodium channel. The channel epitope that interacts with BmK IT2 was further narrowed down to residues around the N-part of DIII SS2-S6 loop (I1512/Q1513/N1516/D1517/I1519) as well as the hydrophobic I1529 and the positive R 1530, implying the hydrophobic and electrostatic interactions may both be decisive for toxin binding. Although the residue alterations at positions 1512–1526 and at position 1530 had minor impact on toxin efficacy, the exchange of hydrophobic Ile at position 1529 in DmNav1 to the Lys present in rNav1.2 largely impaired the toxin-channel interaction. Thus the central role of I1529 seemed to support the hydrophobic interaction in toxin-channel inter-recognition (Fig. 6).

Interestingly, despite targeting VGSCs from different phyla, the binding features of BmK IT2 and Tz1, a β-like toxin that can strongly affect the activation of muscular Na\textsubscript{v} channel but was incapable of affecting the activation of cardiac and peripheral nerve Na\textsubscript{v} channels [5], appear very similar: toxins recognize and bind to the pore loop of DIII and then are capable of trapping the outward movement of voltage-sensor in DII, thus lowering the threshold for channel activation.

**Figure 5. Site-directed mutations introduced in DIII SS2-S6 loop of DmNa\textsubscript{v}1.** A–I. Normalized conductance-voltage curves for the indicated mutant channels before (■) and after (○) application of 2 μM BmK IT2, with a prepulse (+PP) in all cases. doi:10.1371/journal.pone.0014510.g005
Figure S1 Sequences of the DmNav1 mutants indicating the mutated residues in DII and DIII. The loop chimera L(Dm)Nav1,2 was produced by replacing the diverged residues within DIII SS2-S6 loop of rNa1,2, by those from DmNav1 (underlined residues) correspondingly. In addition, single- or multiple-mutations were also employed on DmNav1, giving rise to the loop-chimera or mutants listed below. Black dots in loop-chimera/mutants indicated the unchanged residues compared to the sequence of L(Dm)Nav1,2 (or DmNav1). Found at: doi:10.1371/journal.pone.0014510.s001 (1.80 MB TIF)

Figure S2 Effect of BmK IT2 on mammalian wild-type VGSCs. Normalized conductance-voltage (G-V) curves of rNa1,2, rNa1,3, rNav1.6 and mNa1.6 in absence (■) and presence of 20 μM (△) and 50 μM (△) BmK IT2, with a 25 ms prepulse. Found at: doi:10.1371/journal.pone.0014510.s002 (1.34 MB TIF)

Figure S3 Dose-response curves for effects of BmK IT2 at DmNav1 and indicated mutants. The EC50 values were determined by measuring the currents induced by the toxin at a test pulse of ~40 mV (Table 1). The protocol used are shown in the inset. Found at: doi:10.1371/journal.pone.0014510.s003 (5.03 MB TIF)

Table S1 The localizations of mutated bases are underlined in nucleotide sequence of all the primers. For loop chimeras, the deduced amino acid residues of mutated positions are indicated beneath. Found at: doi:10.1371/journal.pone.0014510.s004 (0.07 MB DOC)

Supporting Information

Figure 6. Schematic presentation of domain arrangement and key residues involved in BmK IT2-DmNav1 interaction. Schematic BmK IT2 structural model (in amino residue) was constructed by Swiss-model Workspace (http://swissmodel.expasy.org) based on the known structure of the depressant β-toxin LqhIT2 (>80% similarity in sequence) (PDB accession 2i61A). Key residues involved in BmK IT2 structural model (in amino residue) was constructed by

Conclusion

The insect-selectivity of BmK IT2 was highlighted in this study when differentiating between heterologously expressed VGSC subtypes from insect and mammalian central nervous system. The results suggested Na1,2, Na1,3, and Na1,6 channels were not involved in mediating the BmK IT2-induced antinociceptive and anticonvulsant effect in rat models. The study revealed the receptor site on insect VGSC DmNav1 for depressant β-toxin BmK IT2 consisted of at least two regions, i.e. DII and DIII. The recognition epitope for insect-preference were localized to the hydrophobic residues within DIII pore-loop SS2-S6. Finally, the inter-species chimeric channels employed here may provide a promising opportunity for identifying putative binding site(s) in VGSCs targeted by other specific modulators.

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Author Contributions

Conceived and designed the experiments: HH YJ. Performed the experiments: HH ZL. Analyzed the data: HH ZL. Contributed reagents/materials/analysis tools: JZ XS JZ. Wrote the paper: HH ZL.
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