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Molecular game theory for a toxin-dominant food chain model

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ABSTRACT
Animal toxins that are used to subdue prey and deter predators act as the key drivers in natural food chains and ecosystems. However, the predators of venomous animals may exploit feeding adaptation strategies to overcome toxins their prey produce. Much remains unknown about the genetic and molecular game process in the toxin-dominant food chain model. Here, we show an evolutionary strategy in different trophic levels of scorpion-feeding amphibians, scorpions and insects, representing each predation relationship in habitats dominated by the paralytic toxins of scorpions. For scorpions preying on insects, we found that the scorpion α-toxins irreversibly activate the skeletal muscle sodium channel of their prey (insect, BgNaV1) through a membrane delivery mechanism and an efficient binding with the Asp/Lys-Tyr motif of BgNaV1. However, in the predatory game between frogs and scorpions, with a single point mutation (Lys to Glu) in this motif of the frog’s skeletal muscle sodium channel (fNaV1.4), fNaV1.4 breaks this interaction and diminishes muscular toxicity to the frog; thus, frogs can regularly prey on scorpions without showing paralysis. Interestingly, this molecular strategy also has been employed by some other scorpion-feeding amphibians, especially anurans. In contrast to these amphibians, the Asp/Lys-Tyr motifs are structurally and functionally conserved in other animals that do not prey on scorpions. Together, our findings elucidate the protein–protein interacting mechanism of a toxin-dominant predator–prey system, implying the evolutionary game theory at a molecular level.

Keywords: toxin, receptor, molecular game, amphibian, scorpion

INTRODUCTION
Evolution has fine-tuned the ability of venoms in many venomous animals, such as snakes, spiders, centipedes and scorpions, to rapidly incapacitate both prey and predators—especially for fast-moving targets—as a mechanism for hunting prey or deterring predators [1–4]. To achieve a paralytic envenomation, targeting skeletal muscle sodium channel NaV1.4 in mammals or its counterpart receptor BgNaV1 in insects is an efficient strategy, because this channel is crucial for skeletal muscle contraction as it regulates the generation and propagation of action potentials [5–8]. During the long evolutionary game process of natural selection, the formation of food chains containing venomous animals was likely dominated by venom components that elicited muscular toxicity [9, 10].

Scorpions (Mesobuthus martensi Karsch) inflict potentially paralytic and lethal stings mainly through their α-toxins acting on the skeletal muscle sodium channel [11–13]. Scorpion α-toxins slow or inhibit the inactivation process of NaV channels and thus induce prolongation of action potentials [14–16]. For scorpions, causing the dysfunction of the muscular system by α-toxins is a unique evolutionary and molecular mechanism that rapidly renders prey incapable of retaliation or escape. Although toxins have been underlined by their powerful bioactivities and were thought to be crucial for predation, venomous animals are not located at the top of the food chain in...
most ecosystems [17–19]. This raises questions regarding how the higher players maintain their dominance in the food chain and how they invalidate their preys’ toxins.

Interestingly, as predators of scorpions, some amphibians might have evolved a tolerance to the assault of scorpion toxins on the muscular system [20]. Anuran species, such as *Leptodactylus pentadactylus*, *plethodontohyla inguinais* [21,22], have been recorded to prey on scorpions and this evolutionary phenomenon is supported by several physiological mechanisms at a molecular level. Based on these observations, we questioned whether the frog or toad employs certain resistance mechanisms to the paralytic toxins of scorpion and whether these molecular strategies are crucial for this predator-prey relationship. Our observations showed that the frog (*Pyxicephalus adspersus*) exhibited resistance to stings of the scorpion (*Mesobuthus martensii*), preyed and consumed the scorpion without a paralytic response (Supplementary Movie S1, available as Supplementary Data at NSR online). This suggests that the frog evolved to reduce sensitivity to the paralytic toxins. By contrast, these toxic stings play an extremely successful defensive or lethal role in other vertebrates and insects [23–25]. Are scorpion α-toxins invalidated by encountering a detoxification mechanism in frogs? In the present study, we unraveled the molecular strategies and the evolutionary game theory in a food cycle composed of anuran species, scorpions and insects through integrating the results from animal tests, electrophysiology, mutagenesis, fluorescent dynamics and computational modeling.

RESULTS

**Significant tolerance of frog-to-scorpion stings and α-toxin**

As shown in Fig. 1A and Supplementary Movie S1 (available as Supplementary Data at NSR online), although the scorpion displayed a powerful chemical defense via several toxic stings, these stings had no impact on the frog during the predatory process. By contrast, scorpion stings exhibited a successful defensive role in laboratory mice (Supplementary Movie S2, available as Supplementary Data at NSR online). Therefore, frogs may possess some molecular strategies to diminish the physiological effects induced by these toxins. To obtain a representative muscle-paralytic α-toxin, considered as a major component for subduing prey and deterring predators, we purified an α-toxin (BmK-M9) in abundance (Fig. 1B and Supplementary Fig. S1A, available as Supplementary Data at NSR online), we used this toxin as a prototypic α-toxin and established the homologous model of BmK-M9 (Fig. 1E). As expected,
Figure 2. DIV of fNaV1.4 possesses the resistance property to α-toxins. (A) Representative whole-cell currents of rNaV1.4 and fNaV1.4 before and after 10 μM BmK-M9 application. The cells were perfused by 10 μM BmK-M9 for 30 seconds to ensure a saturated concentration of the toxin on the channel. (B) The I5ms/Ipeak value of each sodium channel following application of 10 μM BmK-M9. The statistical values are given as mean ± SEM (n = 3 cells). (C) Comparison of binding affinity of BmK-M9 on rNaV1.4 and fNaV1.4. The association traces were plotted by application of 10 μM BmK-M9 and dissociation traces were plotted by washing with bath solution within 40 seconds. (D) The toxin dissociation rate was recorded following a series of images from rNaV1.4-expressing (top row) and fNaV1.4-expressing (bottom row) HEK293T cells incubated with F-BmK-M9 (10 μM). (E) Schematic representation of the chimeras between rNaV1.4 (blue) and fNaV1.4 (red). (F) Representative whole-cell currents of DIV chimeric channel before and after 10 μM BmK-M9 application. (G) Schematic representation of the chimeras between BgNaV1 (blue) and Shaker (grey). (H) Representative whole-cell currents of Shaker˙D4 chimeric channel were recorded before and after 10 μM BmK-M9 application. (I) Diagram of BgNaV1 channels indicating the location of mutated cysteine labeled with TAMRA-MASt (up); Fluorescence signals evoked at −180 mV, −80 mV, −20 mV, 40 mV were recorded from channel mutant S1678C before (left panel) and after (right panel) 10 μM BmK-M9 application (middle); The changes in fluorescence signals (at 40 mV) for the four fluorescence-labeled channel mutants were analysed in the presence of 10 μM BmK-M9. The statistical values are given as mean ± SEM (n = 3 cells) (down). (J) The voltage-dependent fluorescence (circle) and conductance-voltage relationship (dash line) of channel mutant S1678C were analysed before and after 10 μM BmK-M9 application. The statistical values are given as mean ± SEM (n = 3 cells).

by calculating the lethal dosage, we found that cockroaches exhibited more than 100,000-fold sensitivity to BmK-M9 compared to frogs (Fig. 1F). Given that the skeletal muscle sodium channels are the general main target of scorpion α-toxins (Fig. 1G) [12,13,27–29], we hypothesized that the counterpart receptor (frog NaV1.4, fNaV1.4) of the BgNaV1 channel may be the molecular basis of frog’s detoxification mechanism, thus making the frog a higher-level predator in this food chain.

Domain IV of fNaV1.4 and BgNaV1 determines the sensitivity to α-toxin

To test our hypothesis, we first cloned fNaV1.4 from a frog (Pyxicephalus adspersus) and expressed it in HEK293 cells. As shown in Supplementary Fig. S2A (available as Supplementary Data at NSR online), the steady activation of fNaV1.4 (Va1/2 = −28.1 mV) is similar to that of the BgNaV1 channel (Va1/2 = −26.5 mV). By comparing the fraction of remaining current at 5 milliseconds after the peak versus the peak current amplitude (Table S2, available as Supplementary Data at NSR online), fNaV1.4 possesses unique molecular mechanisms that significantly resist BmK-M9, unlike BgNaV1 (Fig. 2A–C). In agreement with our electrophysiological results, fluorophore-linked BmK-M9 (F-BmK-M9) revealed the fast dissociation of BmK-M9 on toxin-insensitive fNaV1.4-expressing cells (Fig. 2D). To focus on the structural basis of fNaV1.4 containing the resistance, a series of chimeric channels were made between fNaV1.4 and mammalian NaV1.4 (Fig. 2E and F). We found that only the homologous domain IV (DIV) of fNaV1.4 retained its resistance properties to BmK-M9 (Fig. 2F and Supplementary Fig. S2B, available as Supplementary Data at NSR online). A previously reported approach [30] helped us to further confirm the interaction between BmK-M9 and the voltage-sensing domains (VSDs), in which specific VSD paddles from each homologous domain of BgNaV1 channel were transplanted into a Shaker channel (Fig. 2G). Consistently, 1 μM BmK-M9 exclusively interacted with the DIV-VSD construct (Shaker˙D4), whereas domain I, II, III constructs and WT Shaker were unaffected (Fig. 2H, Supplementary Fig. S2C and D, available as Supplementary Data at NSR online). Saturated BmK-M9 partially inhibited the gating current of BgNaV1 and completely suppressed that of Shaker˙D4 (Supplementary Fig. S3A and B, available as Supplementary Data at NSR online). We also labeled a fluorophore (TAMRA-MTS) onto four VSDs (L224C, S870C, I1361C and S1678C, Fig. 2I, Supplementary Fig. S3C and D, available as Supplementary Data at NSR online) of BgNaV1 to track...
A glutamic acid mutation in the Asp/Lys-Tyr motif provides the species selectivity for α-toxins. (A) Screening $\tau_{\text{off}}$ values of 17 single-point toxin mutants on rNaV1.4. (B) Dissociation of BmK-M9 on rNaV1.4 and three single-point channel mutants. (C) Comparison of the dissociation traces of BmK-M9 on single-point mutant K1432A and double-point mutant K1432A/Y1433A (left); single point-mutant D1428A and double-point mutant D1428A/Y1433A channel (right). (D) Sequence alignment of the Asp/Lys-Tyr motif of rNaV1.4 (r1.4), BgNaV1 (B1), fNaV1.4 (f1.4) and hNaV1.7 (h1.7). The corresponding $\tau_{\text{off}}$ values of BmK-M9 on these channels are shown. (E,F) The dissociation traces of toxin (T in red) on mutants on channel (C in red) mutants. (G) Comparison of the $I_{\text{res}}/I_{\text{peak}}$ values on BmK-M9 treated BgNaV1 and fNaV1.4 with a single amino acid change.

A point mutation bestows fNaV1.4 with resistance to paralytic α-toxin

Additionally, glycine/alanine screening revealed the key residues in the toxin-channel interaction. Two residues (42Y and 62K) were identified as the key sites of the toxin by the washing-out time-course analysis, yielding $\tau_{\text{off}}$ values of 25.82 and 15.07 seconds, respectively (Fig. 3A). Asp/Lys-Tyr motif was identified as the binding pocket of BmK-M9, given that the three residues located in this motif of mammalian DIV-VSD were found to be important for the toxin-channel interaction (Fig. 3B–D). To experimentally test the site-to-site interaction, we employed an analysis based on thermodynamic mutant cycling [34,35]. Briefly, if one of these two residues specifically interacts with one residue in the Asp/Lys-Tyr motif, then the $\tau_{\text{off}}$ value of double mutation should be nonadditive compared with that of a single mutation. Otherwise, the effects of accelerating the decrease in $I_{\text{res}}/I_{\text{max}}$ value by mutating these residues would be additive. Except for fNaV1.4, the tyrosine (42Y) of BmK-M9 directly interacts with the lysine located in Asp/Lys-Tyr motif (Fig. 3E and F), which likely provides the species selectivity for BmK-M9 (Supplementary Fig. S4A, available as Supplementary Data at NSR online). Based on these understandings of the site-to-site interaction, it is hardly surprising that a single-point mutation could largely alter the bioactivity of BmK-M9 on both fNaV1.4 and mammalian NaV1.4 (Fig. 3G). Compared to the skeletal muscle sodium channels of other animals, a glutamic acid in the Asp/Lys-Tyr motif of fNaV1.4 acts as the molecular determinant and reverses the charge by replacing lysine in this motif, which may bestow the frog with biological resistance to the paralytic α-toxin.

Structural and molecular strategies of the predatory game process

We used Rosetta to simulate the resting state of DIV-VSD by aligning the first arginine (R1448) resolved in the activated state (PDB: 6AGF) to the fourth arginine (R1457) and rebuild the loop between transmembrane segment 3 and 4. As shown in Supplementary Fig. S4B (available as Supplementary Data at NSR online), the Asp/Lys-Tyr motif is embedded in the lipid membrane in the resting state model, suggesting a lipid-dependent interaction between toxin and DIV-VSD. BmK-M9 was incorporated into the cell membrane in our partition experiments (Fig. 4A and B). In agreement with the toxin-membrane interaction, BmK-M9 showed effective bioactivity when applied from the intracellular side (Fig. 4C). When BmK-M9 was docked into this resting state model, the toxin largely resided within the membrane (Fig. 4D). The docking model is fully consistent with our results from point-mutation screening, toxin-lipid partition experiments and patch-clamp recordings. In the
Figure 4. The detoxification receptor rNaV1.4 repels the binding of α-toxins. (A) The RP-HPLC detection of BmK-M9 in DMEM medium supernatant with HEK293T cells (red) and without (grey) HEK293T cells. BmK-M9 was detected after 24 hours of toxin application. (B) The interaction between BmK-M9 and lipid membranes. The bands represent the toxin extracted from liposome and lipid-bilayer membranes of HEK293T cells, which were incubated with 10 μM BmK-M9, respectively. The 10 μM BmK-M9 in DMEM medium without lipid membranes was used as control. (C) Representative whole-cell currents of rNaV1.4 at the beginning of whole-cell formation and 30 seconds after whole-cell patch constructed whereby 10 μM BmK-M9 was added in the pipette solution. (D) The docking model of BmK-M9 with domain IV of rNaV1.4 at resting state. (E) A zoomed-in view of the interaction between BmK-M9 and rNaV1.4. (F) A cartoon showing the molecular game theory for a toxin-dominant food chain model among frogs, scorpions and insects.

DISCUSSION

Venomous animals are consistently excellent predators due to possession of formidable venom biochemical armaments and thereby often occupy dominant positions in food chains [36,37]. To be higher-level predators of these venomous animals, evolutionary game processes at the molecular level are necessary to equip several crucial detoxification mechanisms that circumvent the risk of poisoning [38,39]. The present study highlights a representative example of this type of predatory game theory among scorpion-eating amphibians, scorpions and insects.

Scorpions evolved a gene-encoded venom system as a primary chemical weapon for capturing prey [40,41]. Recent cryo-EM studies have resolved the atomic structures of several toxin-NaV channel complexes, showing that toxins engage with the channel mainly through protein-protein interactions [42–45]. However, we find that to tightly fix DIV-VSD in the resting configuration, scorpion α-toxin not only interact with this domain through a salt bridge and a cation-π interaction, but it also penetrates deeply into the lipid membrane and holds DIV-VSD against activation (Fig. 4D–E). These characteristics of α-toxins make the prey’s skeletal muscle sodium channels constitutively activated without any inactivation, resulting in muscle rigidity. We found the important role of DIV-VSD (Fig. 2E–J), especially the Asp/Lys-Tyr motif (Fig. 3B–D), which is embedded in the cell membrane at its resting state (Fig. 4A–E, Supplementary Fig. S4B, available as Supplementary Data at NSR online). Therefore, paralytic α-toxin obviously gained an upper hand in the predatory game between scorpions and their prey.

Scorpion stings containing these paralytic α-toxins also exhibit defensive roles against vertebrates, like rodents (Supplementary Movie S2, available as Supplementary Data at NSR online), by exploiting the same mechanisms. This poses, however, a different question of why rodents do not evolve tolerance. We first rule out the possibility that the rodents’ attack is swift enough to ingest scorpions directly and avoid scorpion stings (Supplementary Movie S2, available as Supplementary Data at NSR online), because scorpion sting events (in minisecond range) are too fast to dodge [46,47]. It is plausible that rodents have no need to exploit an otherwise new toxic food source and also do not get stung by scorpion frequently, given that they are not in the same food cycle. Supporting this argument, the projection behavior of frogs’ tongues may reduce the number of scorpion sting and envenomation capacity during the predation process. Moreover, at a molecular level, resistance to scorpion stings, especially in frogs, is more likely a receptor-benefit result...
of a long-term prey and predation game. In fNav1.4, the Asp/Lys-Tyr motif is mutated to the Asp/Glu-Tyr, one of attracting pair forces that mutually repel, which greatly reduces the affinity of the toxin for fNav1.4 (Fig. 4F). Except for scorpion-eating amphibians, such as the anuran species, we found that the Asp/Lys-Tyr motif is conserved in nonscorpion-eating vertebrates. Interestingly, the Asp/Lys-Tyr motif of nonscorpion-eating amphibians, such as salamanders, also is intact (Supplementary Fig. S4A, available as Supplementary Data at NSR online). Our results provide a clue that species-crossing interactions hide more delicate molecular mechanisms among ligands and receptors to support the interesting and intense coevolutionary game.

MATERIALS AND METHODS

Ethics statement

All of the animal experiments were performed in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of Kunming Institute of Zoology, Chinese Academy of Sciences. Experimental protocols using animals in this study were approved by the Institutional Animal Care and Use Committees at Kunming Institute of Zoology, Chinese Academy of Sciences (approval ID: SMKX-2018029).

Purification and protein sequencing of BmK-M9

A total of 1,000 (both sexes) adults Mesobuthus martensii were purchased from Shandong Province, China. As previously reported [48], crude venom was collected by stimulating the venom glands with a 3 V alternating current. BmK-M9 was purified from the crude venom by using a combination of a Sephadex G-50 gel filtration column and reverse-phase (RP)-HPLC. The purity and molecular weight of the toxin were analysed using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). The toxin with a purity of over 99.8% was collected and stored at −80°C. A Shimadzu protein sequencer (PPSQ-31A, Shimadzu, Japan) was used for the determination of the amino acid sequence of BmK-M9.

Preparation of recombinant toxin and fluorescent-labeled BmK-M9

Expression vector construction, protein expression and purification were performed as described previously [49,50] with fine tuning. In summary, the cDNA encoding BmK-M9 was synthesized with codons optimized for expression in Escherichia coli, and it was cloned into the modified expression vector pet32a (+) (Novagen). This vector (pet32a) encodes a His6 tag for affinity purification, a Trx-Tag for improving the solubility and activity of the expressed peptide, and a tobacco etch virus (TEV) protease recognition site for subsequent peptide cleavage release. The plasmid encoding BmK-M9 then was transformed into the E. coli strain BL21(DE3) for recombinant toxin production. Bacteria were grown in LB broth at 37°C with shaking at 180 rpm. BmK-M9 expression was induced with 500 μM IPTG at an OD600 of 0.8, and the cells were grown at 16°C with shaking at 100 rpm for a further 12 h before harvesting by centrifugation for 10 minutes at 10,000-fold gravitational acceleration. The fusion protein was extracted from the bacteria by cell ultrasonication and then captured by passing the extract (buffered in 20 mM Tris, 0.5 mM NaCl, pH = 8.0) over Ni-NTA resin (Qiagen 30230). Nonspecifically-bound proteins were removed by washing with 20 mM imidazole. The fusion protein was eluted with 500 mM imidazole. The eluted fusion protein was lyophilized and further purified by FPLC (Resource SGE 6 mL) to remove imidazole and obtain higher purity recombinant fusion protein. Added to 1 mg of the fusion protein was 10 U TEV protease, and then the cleavage reaction was allowed to proceed at 16°C for 12 h at a constant volume with TEV Protease buffer (50 mM NaH2PO4, 150 mM NaCl). The sample then was centrifuged at 12,000 rpm, and the supernatant was subjected to further purification using RP-HPLC (C8 XBridge OBD). Containing a nonnative N-terminal glycine residue, rBmK-M9 is one residue longer than native BmK-M9.

Given that rBmK-M9 with His-tag also works on sodium channels, we used a His-tag–specific dye, Invision (Invitrogen LC6030), to construct Fluorescent BmK-M9 (F-BmK-M9). Incubated with Invision for 24 h, rBmK-M9 was subjected to RP-HPLC purification. The single peak was collected and lyophilized for further imaging experiments.

F-BmK-M9 imaging

HEK293T cells transfected with sodium channels (rNav1.4 and fNav1.4) were incubated with F-BmK-M9 for 1 h in 2 mM Ca2+ Ringer’s solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 10 mM Glucose, 2 mM CaCl2, and 10 mM HEPES, pH = 7.4) before fluorescence imaging recordings. Fluorescence images of HEK293T cells incubated with F-BmK-M9 were acquired using an Olympus IX-71 microscope with a Hamamatsu R2 camera controlled by MetaMorph software. F-BmK-M9 was
excited by a LED light source (X-Cite 120LED, Lumenerg Dynamics) with a 560 nm excitation filter, while fluorescence emission was detected by a 590 nm emission filter.

**Insecticidal assays**

Dissolved in insect saline, 10 μM rBmK-M9 were injected into the abdomen region of adult American cockroaches (*Periplaneta americana*) and adult German cockroaches (*Blattella germanica*). Insect saline was used as control. Injections were made using a 1.0 mL syringe (B-D Ultra-Fine). A maximum volume of 1.5 mL was injected per *B. germanica* and 3.0 mL for *P. americana*. Thereafter, cockroaches were housed in closed 1 L conical flasks and provided with dry food and water. The lethal effects were then determined after a period of 24 h. For each acute toxicity assay, up to five doses of rBmK-M9 were injected (n = 5 insects per dose). The assay was repeated three times.

**Mutagenesis of toxin and sodium channels**

Chimeras rNaV1.4 and fNaV1.4 used in this study were generated by the overlapping extension method by using In-Fusion HD Cloning Kits and following the user manual (Clontech); all chimeras were verified by DNA sequencing [51]. Each sodium channel point mutation was constructed by using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent) and following the instruction manual; all point mutations were confirmed by DNA sequencing.

For each toxin mutant, site-directed mutagenesis was performed by PCR on expression plasmid first and then the corresponding peptide was expressed as described before. Final confirmation of toxin mutants was carried by CD spectra.

**Cut-open VCF and TEVC recordings**

Cut-open voltage-clamp fluorometry (VCF) was used to record ionic currents and fluorescence from oocytes [52,53]. *Xenopus laevis* oocyte preparation and cRNA injection was performed as described previously [54]. Briefly, the cRNA of BgNav1 was co-injected into oocytes with that of TipE at a 2:1 molar ratio (50 ng per cell total) for robust expression. Injected oocytes were incubated individually at 18°C for 5 d in ND-96 solution with 1% penicillin-streptomycin at a pH of 7.4. The temperature of three chambers was maintained at 19°C with a controller (HCC-100A; Dagan Corporation). The internal solution contained 113 mM NMG-Mes, 2 mM Na-Mes, 20 mM HEPES, and 2 mM EGTA, pH = 7.4. The external solution contained 95 mM NMG-Mes, 20 mM Na-Mes, 20 mM HEPES, and 2 mM Ca-Mes₂, pH = 7.4. The glass pipettes were filled with filtered 3 M KCl in 0.5% agarose with a resistance of 0.5 to 1.0 MΩ. For fluorescence measurement experiments, oocytes were labeled with 20 μM methanethiosulfonate-carboxytetramethylrhodamine in a depolarizing solution (110 mM KCl, 1.5 mM MgCl₂, 0.8 mM CaCl₂, and 10 mM HEPES, pH = 7.4) on ice for 40 min. Methanethiosulfonate-carboxytetramethylrhodamine was excited by a LED light source (Luminus, PT-121), while fluorescence emission was detected by a 40 × water-immersion objective with a numerical aperture of 0.8 (CFI Plan Fluor, Nikon). Gating currents were recorded with 1 μM TTX in the external solution.

**Cell culture, transient transfection, and electrophysiology**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C with 5% CO₂. Cells were plated on cover glasses before transfection. Transient transfection was conducted by using Lipofectamine 2000 (Invitrogen) and following the instruction manual.

Electrophysiological experiments were performed between 24–48 h after transfections as previously described [8]. The macroscopic currents were recorded by using a HEKA EPC10 amplifier with the PatchMaster software (HEKA). The borosilicate glass pipettes were pulled and fire-polished to a resistance of 3–4 MΩ. All recordings were performed at room temperature. To evoke sodium channel currents, a holding potential of −80 mV was used with a testing pulse to −10 mV. The association and dissociation traces were determined using a rapid solution changer (RSC-200, BioLogic) to deliver different concentrations of BmK-M9 and toxin mutants. The stable current amplitude before and after BmK-M9 application was recorded. For the sodium channels recording, the standard pipette solution contained 140 mM CsF, 1 mM EGTA, 10 mM NaCl, 3 mM KCl, and 10 mM MgCl₂, pH = 7.3. The standard bath solution was 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES, pH = 7.3.

**Lipid membrane interaction**

HEK293T cells were incubated with 10 μM rBmK-M9 for 24 h. The incubated cells were then
resuspended in 2 mL of PBS and then centrifuged at 18,000-fold gravitational acceleration for 1 h at 4°C. The supernatant was filtered and subjected to RP-HPLC detection.

Given that rBmK-M9 with His-tag also works on sodium channels, we used a His-tag specific antibody (CST) to prove the interaction between BmK-M9 and lipid membranes. Cells of HEK293T transfected with rNav1.4 and liposome were incubated with 10 μM rBmK-M9 for 24 h and then resuspended in 2 mL of PBS and centrifuged at 18,000-fold gravitational acceleration for 1 h at 4°C. The two kinds of cell pellets were washed three times with PBS to get rid of the residual free toxins, then lysed with RIPA and centrifuged at 18,000-fold gravitational acceleration for 1 h at 4°C. The two kinds of supernatants were collected and detected by western blot for the presence of the toxin.

Construction of BmK-M9 and rNav1.4 channel model

The structure of BmK-M9 was predicted by backrub protocol using the Rosetta molecular modeling suite version 2016.20. A partial model of rNav1.4 was constructed from L250 to E1600 by membrane-symmetry-loop modeling using the Rosetta molecular modeling suite version 2016.20. The cryo-EM structure of EeNav1.4 (5XSY) was used as the template, the S3–S4 linker and the S4–S5 linker were modeled de novo with the KIC loop modeling protocol. Each round generated 10000 models; among these models, the top 10 lowest-energy models were selected as the inputs for next round of loop modeling. After several rounds of KIC loop modeling, the top 10 models converged well. The lowest energy model was finally selected as the rNav1.4 model.

Docking of BmK-M9/rNav1.4 complexes

RosettaDock application from Rosetta program suite version 3.4 was used to dock BmK-M9 to rNav1.4 models. Model of the transmembrane domains of rNav1.4 were first relaxed in a membrane environment using the Rosetta-Membrane application. BmK-M9 was initially placed roughly in the center of the binding pocket defined by S3, S3–S4 linker and S4 segments. From the results of double mutation cycle experiments, the distances between D1428-K62 and K1432-Y42 were constrained to move within a 4 Å diameter sphere. After docking, the top 1,000 models with the lowest total energy score were first selected. They further were scored with the binding energy between the ligand and the channel. The top 10 models with the lowest binding energy were identified as the candidates. The model with the lowest binding energy among the largest cluster of the top 10 models was used as the representative model.

Data analysis

Offline data analysis was performed using IgorPro (WaveMetrics) as previously reported [55]. Voltage–activation relationships were obtained by measuring currents elicited by step depolarizations of 10 mV from a holding potential of −100 mV and calculating peak conductance (GNa) using the following equation: $G = I_{\text{Na}}/(V_m - E_{\text{rev}})$ where $G$ is peak conductance, $I_{\text{Na}}$ is peak inward sodium current, $V_m$ is the test potential and $E_{\text{rev}}$ is the reversal potential. The normalized conductance was fitted to a two-state Boltzmann function: $G/G_{\text{max}} = (1 + \exp(V-V_{1/2}/k))^{-1}$, where $V_m$ is the voltage potential of the pulse, $V_{1/2}$ is the voltage at half-maximal activation, and $k$ is the slope factor. The voltage dependence steady-state inactivation was determined using 200 ms inactivating prepulses from a holding potential of −120 to 40 mV in 10 mV increments and followed by test pulses to −10 mV for 50 ms. The peak current amplitude during each test pulse was normalized to the maximum current amplitude. The steady-state inactivation data were fitted using a Boltzmann equation: $I/I_{\text{max}} = [1 + \exp(V-V_{1/2}/k)]^{-1}$, where $V_{1/2}$, $V$ and $k$ represented the voltage at half-maximal activation, test potential and slope factor, respectively. Dose–response curves to determine LD50 values were fitted using the following form of the logistic equation: $y = 1/(1 + [x]/\text{Dose50})^{nH}$, where $x$ is the toxin dose and $nH$ is the Hill coefficient. Nonlinear curve-fitting of data were performed using IgorPro.

SUPPLEMENTARY DATA

Supplementary data are available at NSR online.

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