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A novel tarantula toxin stabilizes the deactivated voltage sensor of bacterial sodium channel

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ABSTRACT: Voltage-gated sodium channels (NaVs) are activated by transiting the voltage sensor from the deactivated to the activated state. The crystal structures of several bacterial NaVs have captured the voltage sensor module (VSM) in an activated state, but structure of the deactivated voltage sensor remains elusive. In this study, we sought to identify peptide toxins stabilizing the deactivated VSM of bacterial NaVs. We screened fractions from several venoms and characterized a cystine knot toxin called JZTx-27 from the venom of tarantula Chilobrachys jingzhao as a high-affinity antagonist of the prokaryotic NaVs nonselective voltage-gated, Bacillus alcalophilus (NsVBa) and bacterial sodium channel from Bacillus halodurans (NaChBac) (IC50 = 112 nM and 30 nM, respectively). JZTx-27 was more efficacious at weaker depolarizing voltages and significantly slowed the activation but accelerated the deactivation of NsVBa, whereas the local anesthetic drug lidocaine was shown to antagonize NsVBa without affecting channel gating. Mutation analysis confirmed that JZTx-27 bound to S3-4 linker of NsVBa, with F98 being the critical residue in determining toxin affinity. All electrophysiological data and in silico analysis suggested that JZTx-27 trapped VSM of NsVBa in one of the deactivated states. In mammalian NaVs, JZTx-27 preferably inhibited the inactivation of NaV1.5 by targeting the fourth transmembrane domain. To our knowledge, this is the first report of peptide antagonist for prokaryotic NaVs. More important, we proposed that JZTx-27 stabilized the NsVBa VSM in the deactivated state and may be used as a probe to determine the structure of the deactivated VSM of NaVs.—Tang, C., Zhou, X., Nguyen, P. T., Zhang, Y., Hu, Z., Zhang, C., Yarov-Yarovoy, V. DeCaen, P. G. Liang, S., Liu, Z. A novel tarantula toxin stabilizes the deactivated voltage sensor of bacterial sodium channel. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: peptide toxin · deactivated state · NsVBa

Mutations in NaVs cause a variety of diseases of the heart and central and peripheral nervous systems (e.g., long QT syndrome and epilepsy) (1, 2). Many of these mutations are found within the voltage-sensor modules (VSMs) of NaVs, which alter the voltage-dependent kinetics of the channel gating (3–5). Understanding structure and function of the voltage sensor of NaVs will provide insight into the molecular basis of electrical signaling in normal and diseased conditions.

Eukaryotic NaVs are large proteins with 24 transmembrane segments, making them challenging to study by using crystallographic techniques (6). Several laboratories have crystalized NaVs from bacteria, which are relatively small and homotetrameric (7–10). Four monomers of bacterial NaVs assemble to form a channel. Analogous to the 4 domains (I–IV) of eukaryotic NaVs, each bacterial channel monomer contains a voltage sensor and a pore domain. For prokaryotic NaVs, the first 4 transmembrane segments (S1–4) form a voltage sensor module (VSM) and the last 2 transmembrane segments (S5 and -6) comprise the pore-forming module. Both eukaryotic and prokaryotic NaVs are voltage sensitive due to an arrangement of several conserved arginine or lysine residues (called gating charges) located within the S4 segment. During voltage sensor
activation, the gating charges move toward the extracellular side in response to membrane depolarization (11).

Prokaryotic NaVs were thought to be Na\(^{+}\)-selective (12, 13). Recently, a nonselective member of this family was characterized as Bacillus alcalophilus (NsBa: nonselective voltage-gated B. alcalophilus) (14). This channel exhibits a unique selectivity filter (sequence: TLDWSGGG) which conducts K\(^{+}\) as well as Na\(^{+}\) ions, an adaptation that allows B. alcalophilus to grow in high-K\(^{+}\) conditions (15). Crystal structures from the full-length bacterial channels NsAb and NsRh exhibit voltage sensors in partially and fully activated states (7, 9). In conjunction with previous work, these structures have provided many mechanistic details of interactions within the voltage sensor that occur during the transition from the partially activated state to the fully activated state. However, the structure of the deactivated voltage sensor has not been solved and needs further investigation.

Several peptide toxins from venomous arachnids and insects stabilize deactivated or activated states of the VSM of ion channels (16–19). These toxins can be exploited by structural biologists to trap voltage sensors in specific states. Several peptide toxins acting on mammalian NaVs through different mechanisms have been characterized. The α and β scorpion toxins trap domain IV S4 and domain II S4 in the closed and activated conformation, respectively (20, 21). Crystal structures of the NaVMs (a prokaryotic NaV) pore with several brominated drugs depict a common binding site shared by eukaryotic NaVs (22), but whether a peptide neurotoxin binding site is shared by prokaryotic and eukaryotic NaVs is unknown. The present study describes the purification and characterization of a novel peptide toxin (JZTx-27) from the venom of the Chinese tarantula, Chilobrachys jingzhao. JZTx-27 binds to the extracellular S4-3 loop of the voltage sensor and prevents activation of NsBa by stabilizing the deactivated state. JZTx-27 also acts on the mammalian NaV1.1 superfamily with a variable potency. However, JZTx-27 appears to bind to mammalian NaVs and prevents the inactivation process. This novel tarantula toxin can be used as a molecular probe to further investigate the structural determinants of the deactivated state of sodium channel voltage sensors.

MATERIALS AND METHODS

Venom and toxin purification

The venom of Chilobrachys jingzhao was collected by electrical stimulation. The crude venom was lyophilized and preserved at −80°C before use. The crude venom was dissolved in double-distilled H\(_2\)O to a final concentration of 5 mg/mL and subjected to semipreparative reverse phase (RP)-HPLC purification (C18 column, 10 μM, 10 × 250 mm; Welch Materials, Inc., Shanghai, China). Venom components were eluted with a linear acetonitrile gradient (0–60% acetonitrile/0.1% TFA in 60 min) at a flow rate of 3.0 mL/min. The peak containing JZTx-27 was collected by lyophilization, and purified to homogeneity by analytical RP-HPLC (C18 column, 5 μM, 4.6 × 250 mm; Welch Materials, Inc.).

Constructs and transfection

cDNA of sodium channels from Paracoccus zaxanthinifaciencis (NaPZ) and Silicibacter pomeroyi (NaSP), NsBa and bacterial sodium channel from Bacillus halodurans (NaChBac) were cloned into a pTracer-CMV2 plasmid containing an enhanced green fluorescent protein that enables the confirmation of the transfected cells (12). Mutant and chimeric channels were generated with a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer’s instructions. NaV1.5-NaV1.8 chimeric channels were constructed by replacing human (h)NaV1.5 domains with those of rat (r)NaV1.8 by using a recombination strategy, as described in our previous study (23). Primers used for the linearization of hNaV1.5 channel were as reported (23), whereas rNaV1.8 transmembrane domains were amplified using the following primers. rNaV1.8 domain I: forward primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′, reverse primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′; rNaV1.8 domain II: forward primer 5′-AACGAGGGAGTGAAGGAGCTGTTGATTTATGGTTGA-3′, reverse primer 5′-AACGAGGGAGTGAAGGAGCTGTTGATTTATGGTTGA-3′; rNaV1.8, 1.8 DIII: forward primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′, reverse primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′; rNaV1.8, 1.8 IVA: forward primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′, reverse primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′; rNaV1.8, 1.8 IVA: forward primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′, reverse primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′; rNaV1.8, 1.8 IVA: forward primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′, reverse primer 5′-AAGACGGCTGTAGGTCTGCATCGTGA-3′. The joint sequences are underlined. Primers were designed with Primer Premier 5.0 software and were synthesized in Genscript (Genscript Corp., Nanjing, China). All constructs were sequenced to confirm that the appropriate mutations/chimeras had been made. Transfections of wild-type (WT), mutant, or chimeric channels into CHO-K1, ND7/23, and HEK293T cells were performed by using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s instructions. Six hours after transfection, the cells were seeded onto a glass coverslip (Thermo Fisher Scientific), and 24 h after seeding, they were ready for patch-clamp analysis.

Electrophysiological analysis

Cells transfected with WT/mutant/chimeric channels seeded in a glass coverslip were placed in a perfusion chamber, in most cases, rapid exchange of the bath solutions around the cells was performed (24). For determining the binding kinetic of toxin with channel, toxin was applied by a pipet located close to the recording cell to achieve fast solution exchange. For electrophysiological recording, the pipet solution contained 27 mM CsCl, 120 mM methane sulfonate, 8 mM NaCl, 10 mM EGTA, 2 mM Mg-ATP, and 20 mM HEPES (pH = 7.4). The bath solution contained 140 mM NaCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM KCl, 20 mM HEPES (pH = 7.4), and 10 mM glucose. All experiments were conducted at room temperature (20–25°C). All chemicals were products of Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile double-distilled H\(_2\)O. Data were collected by the PatchMaster software in an HEKA EPC-10 USB patch-clamp system (HEKA Elektronik, Ludwigshafen, Germany) and analyzed by Igor Pro 6.00, Prism (GraphPad Software, La Jolla, CA, USA), Sigmastat 10.0 (Systat Software, Inc., San Jose, CA, USA), and OriginPro.
8 (Northampton, MA, USA); voltage errors were minimized by using 80% series resistance compensation; and the capacitance artifact was canceled by using the computer-controlled circuitry of the patch-clamp amplifier. The dose-response curves of the toxin on WT, chimeric, and mutant channels were fitted to a Hill equation to estimate the potency of the toxin (IC_{50}). The G–V and SSI curves were fitted by using a Boltzmann equation (Eq. 1):

\[ y = y_{\text{steady}} + \left( y(0) - y_{\text{steady}} \right)/(1 + \exp((V - V_{1/2})/K)) \]  

(1)

where \( V_{1/2}, V_c \) and \( K \) represents midpoint voltage of activation or inactivation, test potential, and slope factor, respectively. The current recovery of Ns\textsubscript{v}Ba and Na\textsubscript{ChBac} upon washing off the toxin were fitted by Eq. 2:

\[ y = y(0) + a(1 - e^{-t/\tau}) \]  

(2)

the decreases in Ns\textsubscript{v}Ba and Na\textsubscript{ChBac} currents after toxin application were fitted by Eq. 3:

\[ y = y_{\text{steady}} + ae^{-t/\tau} \]  

(3)

where \( \tau \) represents the time constant. Free energy of toxin-channel association was derived from Eq. 4:

\[ \Delta G = -RT \ln (IC_{50}) \]  

(4)

where (IC_{50}), \( R \) and \( T \) are half maximal inhibitory concentration, the universal gas constant and absolute temperature, respectively.

**Statistics**

Data are presented as means \pm SD; \( n \) = separate experimental cells. Statistical significance was assessed with GraphPad Prism using the paired Student’s t test or 1-way ANOVA. Statistical significance was accepted at \( P < 0.05 \).

**Structural modeling of Ns\textsubscript{v}Ba–JZTx-27 complex**

Homology modeling of Ns\textsubscript{v}Ba voltage-sensing module (VSM) was performed with Rosetta cyclic coordinate descent (25) and kinematic (26) loop modeling applications with membrane-environment–specific energy function (27–29) and a bacterial Na\textsubscript{v}Ab channel structure (Protein Database ID: 3RYY) (7) as a template. The activated-state Ns\textsubscript{v}Ba VSM model was generated by sequence alignment with the Na\textsubscript{v}Ab VSM. The resting-state models of Ns\textsubscript{v}Ba were generated by shifting the Ns\textsubscript{v}Ba S4 sequence down by 3 (resting state 2) or 6 (resting state 1) residue positions, with respect to the Na\textsubscript{v}Ab S4 sequence, to simulate a 1- or 2-helix-turn sliding transition of the S4 gating-charge–carrying arginines from the activated to the deactivated state (27, 30). The S1-2 and S3-4 loops were rebuilt de novo. For each VSM state, 20,000 models were generated, and the 1000 best scoring models were clustered to identify the most frequently sampled conformations. Models representing the top 20 clusters were visually evaluated based on experimental data to select the best models for toxin docking. Homology models of JZTx-27 were generated with the Rosetta Relax application (31), and the Hwuentoxin-I NMR structure (ID: 1QK6) (32) served as a template. Docking of JZTx-27 to the Ns\textsubscript{v}Ba VSM models was performed with the Rosetta Dock application (33) with membrane-environment–specific energy function (27–29). For each toxin–channel pair, 10,000 other models were generated. The 1000 best scoring models were evaluated based on the difference in free energy between the unbound and bound states (\( \Delta G \)). The best 1,000 models by \( \Delta G \) score were clustered, and the top 20 clusters were evaluated based on agreement with experimental data to select the best models. Structural model of Ns\textsubscript{v}Ba–JZTx-27 complex shown in Fig. 7 represents 1 of the top 20 clusters of Ns\textsubscript{v}Ba in resting state 1. This model had the best agreement with the data, based on mapping of F98 and H102 at the channel–toxin interface.

**RESULTS**

**Isolation and characterization of JZTx-27 as an antagonist of Ns\textsubscript{v}Ba and Na\textsubscript{ChBac}**

To identify peptide modifiers of bacterial Na\textsubscript{v}s, we collected RP-HPLC fractions of venoms of several species of spider and tested their effect on Na\textsuperscript{+} currents conducted by 4 bacterial Na\textsubscript{v} channels: Na\textsubscript{v}PZ, Na\textsubscript{v}SP, Ns\textsubscript{v}Ba, and Na\textsubscript{ChBac}. We identified and purified a peptide (JZTx-27 or U24-theraphotoxin-Cg1a) from the venom of Chilobrachys jingzhuao as an antagonist of Ns\textsubscript{v}Ba and Na\textsubscript{ChBac} (Supplemental Fig. S1A, B). The molecular mass of JZTx-27 is 4086.81 Da (M+H\(^{+}\)), as determined by matrix-assisted laser desorption/ionization mass spectrometry (Supplemental Fig. S1D). The full amino acid sequence was determined by Edman degradation, which was also confirmed by its cDNA sequence (Supplemental Fig. S1C). The observed molecular mass of JZTx-27 (4085.81 Da) was 1 Da less than the theoretical one (4086.78 Da) based on the mature peptide sequence, indicating C-terminal amidation in the toxin to delete the C-terminal glycine residue in the propeptide (Supplemental Fig. S1C, D). This 34-residue peptide contains 6 cysteines whose localization was conserved among venom peptides containing an inhibitor cystine knot (ICK) motif (Fig. 1A). It was assumed that JZTx-27 would adopt the ICK scaffold and share the same disulfide linkage pattern (1–4, 2–5, and 3–6; the numbers indicate the relative positions of cysteines in the sequence). JZTx-27 inhibited Na\textsuperscript{+} currents from the bacterial Na\textsubscript{v}s Ns\textsubscript{v}Ba and Na\textsubscript{ChBac} (Fig. 1B, C), whereas little or no inhibition was observed for the Na\textsubscript{v}PZ and Na\textsubscript{v}SP channels (Fig. 1D, E). The inhibition of Ns\textsubscript{v}Ba and Na\textsubscript{ChBac} currents by JZTx-27 was dose dependent. The IC\(_{50}\) was 112 nM for Ns\textsubscript{v}Ba at the depolarizing voltage of 0 mV, and it was 30 nM for Na\textsubscript{ChBac} at the depolarizing voltage of −20 mV (Fig. 1F). These potencies translate to a very high energy of interaction between the sodium channels and the toxin (\( \Delta G = −9.9 \text{ kcal/mol and } −10.7 \text{ kcal/mol for Ns}^{\text{v}} \text{Ba and Na}^{\text{ChBac}}, \) respectively). The binding of JZTx-27 to the channel was very rapid (\( t_{\text{on}} = 3.1 \pm 1.6 \text{ s for Ns}^{\text{v}} \text{Ba} \) and \( t_{\text{on}} = 5.9 \pm 1.5 \text{ s for Na}^{\text{ChBac}}, \) whereas dissociation was relatively slow after toxin removal (\( t_{\text{off}} = 41.8 \pm 5.4 \text{ s for Ns}^{\text{v}} \text{Ba} \) and \( t_{\text{off}} = 105.8 \pm 2.4 \text{ s for Na}^{\text{ChBac}}, \) Fig. 1G).

**Voltage-dependent inhibition of Ns\textsubscript{v}Ba by JZTx-27**

A common feature of Na\textsubscript{v} gating modifier toxins is their ability to alter channel gating by trapping voltage sensors in a certain state, which affects voltage-dependent conformation transitions in response to membrane depolarizations. In contrast, pore blockers function by interacting with the outer or inner pore of Na\textsubscript{v}s. Although some pore
and NaChBac (IC50 = 30 nM) by JZTx-27 (5.4 s for NsVBa; NaChBac (0.2 M) inhibited NsVBa currents, whereas the current (disul
inhibition of the NsVBa currents by 150 or 750 nM JZTx-27
was most pronounced at a depolarizing voltage of 40 mV, which suggests that the action of toxin on NsVBa was positively shifted by JZTx-27 but not by lidocaine (Fig. 2A). In addition, JZTx-27 did not change the steady-state inactivation of NsVBa markedly (V1/2 = −52.7 ± 5.9 mV for control and V1/2 = −52.1 ± 5.7 mV for 150 nM JZTx-27 treatment). These data suggested that the inhibition of NsVBa currents by JZTx-27 was voltage dependent, and the mechanism seemed to be the reopening of toxin-occupied channels, as judged from the distinct steady-state activation kinetics of NsVBa after JZTx-27 treatment.

**JZTx-27 stabilizes NsVBa in the resting state**

The interaction of peptide toxins with ion channels may be state dependent. To determine whether JZTx-27 prefers the NsVBa channel in one state in its gating pathway, the effects of JZTx-27 on the activation and deactivation of NsVBa channels were explored. The current of NsVBa was elicited by a 20 ms depolarization to 60 mV, followed by a −50 mV holding for 500 ms (tail voltage). This tail voltage was too weak to activate the closed NsVBa channels, and those already opened in the depolarization step were allowed to endure the deactivation process. Representative current traces in response to this voltage protocol are shown in Fig. 3A (inset). The activation phases of the outward currents before and after JZTx-27 or lidocaine blockers were shown to affect the voltage-dependent activation or inactivation of NsVs, the underlying mechanism may be an allosteric effect, not direct hindering or facilitating the voltage sensor movement (34). The NsVs blocker lidocaine antagonizes NaChBac in a concentration range similar to resting blockade on eukaryotic NaVs (35, 36). In the present study, the kinetics of NsVBa channel after treatment with lidocaine and JZTx-27 were analyzed and compared. We tested the activity of lidocaine on NsVBa and found that it dose dependently antagonized NsVBa with an affinity similar to NaChBac (IC50 = 100 μM; Supplemental Fig. S2A and Fig. 2E). As shown in Fig. 2A, B, 150 nM JZTx-27 and 100 μM lidocaine effectively inhibited NsVBa currents, whereas the current–voltage (I–V) relationship was the same as the control after lidocaine treatment, but it was positively shifted by JZTx-27 (Fig. 2A, B). In addition, the conductance–voltage (G–V) curves showed that the voltage-dependent activation of NsVBa was positively shifted by JZTx-27 but not by lidocaine (Fig. 2C and Supplemental Fig. S2B; V1/2 = −20.6 ± 9.8 mV for control and V1/2 = 8.4 ± 7.0 mV for 150 nM JZTx-27 treatment; V1/2 = −20.6 ± 8.3 mV for control and V1/2 = −20.5 ± 9.2 mV for 100 μM lidocaine treatment), which supports that JZTx-27 may act on NsVBa by modifying channel gating rather than physically blocking the pore.

The I–V curves in Fig. 2A clearly show that the JZTx-27 did not efficiently block NsVBa current at a depolarizing voltage of 40 mV, which suggests that the action of toxin on NsVBa channel is voltage dependent. At voltages evoking large inward NsVBa currents (from −20 to 30 mV), the inhibition of the NsVBa currents by 150 or 750 nM JZTx-27 was most pronounced at −20 mV, and the inhibition ratio decreased at more depolarized voltages (Fig. 2D). A higher dose of JZTx-27 (750 nM) resulted in much more inhibition of NsVBa currents, but the slope of the curve was significantly smaller than that of 150 nM JZTx-27 (K = 0.003 ± 0.001 and K = 0.010 ± 0.002 for 750 nM and 150 nM JZTx-27, respectively; P < 0.001, 1-way ANOVA, n = 5–9; Fig. 2D), showing less dependence on voltage when saturating channels with toxins. The apparent affinity (IC50) of JZTx-27 with NsVBa was 103 and 220 nM at 0 and 30 mV, respectively (Fig. 2E), whereas lidocaine exhibited nearly the same apparent affinity with NsVBa at 2 voltages (IC50 ≈ 100 μM) (Fig. 2E). In addition, JZTx-27 did not change the steady-state inactivation of NsVBa markedly (V1/2 = −52.7 ± 5.9 mV for control and V1/2 = −52.1 ± 5.7 mV for 150 nM JZTx-27 treatment). These data suggested that the inhibition of NsVBa currents by JZTx-27 was voltage dependent, and the mechanism seemed to be the reopening of toxin-occupied channels, as judged from the distinct steady-state activation kinetics of NsVBa after JZTx-27 treatment.
PEPTIDE TOXIN ANTAGONIZES PROKARYOTIC SODIUM CHANNEL

Figure 2. JZX27 antagonizes NsVBa voltage dependently. A, B) I-V relationships of NsVBa before and after treatment with JZX27 or lidocaine. Cells were held at −100 mV, and a cluster of depolarizing pulses (from −100 to 70 mV, in 10 mV increments) were applied. Currents evoked by different depolarizing voltages were normalized to the maximum peak current before drug treatment (solid lines). If currents from a drug-treated cell were normalized to its own maximum peak current (normalization to drug-treated channels (solid lines). The I-V curve is shown as the red dashed lines, which indicate the shape comparison of I-V curves from the control and the drug-treated channels (n = 8–12). C) The steady-state activation curve of NsVBa was positively shifted by JZX27 (V1/2 = −20.6 ± 9.8 mV for control and V1/2 = 8.4 ± 7.0 mV for JZX27-treated NsVBa channels; n = 8–12). D) In every depolarizing voltage (ranging from −20 to 30 mV), the NsVBa current after 150 or 750 nM JZX27 treatment was normalized to that before drug application and plotted, showing voltage-dependent inhibition of NsVBa by JZX27 (n = 5–9). E) Dose–response curves for JZX27 or lidocaine blocking NsVBa currents, IC50 were determined as 103 nM and 220 nM for JZX27 and 100 μM for lidocaine, at 0 and 30 mV, respectively (n = 7–9).

treatment were fitted as shown in Fig. 3A, B, and the activation time constant (τactivation) was calculated. The τactivation of NsVBa channel before and after 150 nM JZX27 treatment was determined to be 2.9 ± 0.7 and 5.5 ± 1.6 ms, respectively, showing a significant slowing of channel activation by toxin (representative traces in Fig. 3A and statistics in Fig. 3C). We also tested a higher dose of toxin, 750 nM JZX27, which increased the τactivation significantly as well (Fig. 3C). τactivation = 3.4 ± 0.5 ms for control and 6.2 ± 1.7 ms for toxin treatment). In contrast, as shown in Fig. 3B, traces before and after the 50 μM lidocaine treatment were superimposed, suggesting no alteration of the NsVBa activation by lidocaine. Data in Fig. 3C also show that the τactivation of NsVBa channels after varied doses of lidocaine treatment (20 μM, 50 μM, and 1 mM) were not greatly changed when compared with that of the control. This evidence, as well as the unaltered I-V relationship of NsVBa by lidocaine treatment, strongly suggests that the residual currents after lidocaine treatment are contributed by the population of lidocaine-free channels.

Another characteristic of NsVBa after JZX27 treatment is that channels deactivated much faster than that of the control, as shown by normalizing the tail current magnitudes in Fig. 3D. Fitting the falling phase of the tail currents showed the τdeactivation to be 57.3 ± 14.8 ms for control and 30.2 ± 15.2 ms for 150 nM toxin-treated channels (Fig. 3D). 0.750 nM JZX27 also reduced the deactivation time constant from 57.7 ± 15.2 to 15.2 ± 5.0 ms (Fig. 3D). These data suggest that toxin remains bound to NsVBa channel during the activation and the deactivation process, altering the kinetics of channel state transition.

We further compared the effects of JZX27 and lidocaine on NsVBa channel activation at much stronger depolarizations. Cells were held at −100 mV and NsVBa channels were activated by applying a series of 20 ms depolarizations ranging from 60 to 100 mV (10 mV/step), followed by a −50 mV holding of tail voltage for 500 ms. The change of the tail current amplitudes in response to the depolarizations (60–100 mV) reflects the change of the number of activated channels. As shown in Fig. 3E, at the voltages tested, the activation of NsVBa was significantly slowed by 750 nM JZX27: τactivation = 2.6 ± 0.3 ms for control and 5.6 ± 1.8 ms for toxin treatment at 70 mV; 2.1 ± 0.3 ms for control and 4.9 ± 1.7 ms for toxin treatment at 80 mV; 1.7 ± 0.4 ms for control and 4.4 ± 1.7 ms for toxin treatment at 90 mV; and 1.4 ± 0.3 ms for control and 3.9 ± 1.7 ms for toxin treatment at 100 mV. However, 1 mM lidocaine did not markedly alter the activation of NsVBa at every depolarizing voltage (Fig. 3F). The traces in Fig. 3G showed the tail currents from 2 representative cells in
response to voltage protocol described. In the control and the lidocaine groups (Fig. 3G), the tail current amplitudes of NsVBa at 60 and 100 mV depolarizations were almost the same, indicating that all channels available in the membrane were activated at 60 mV. However, the amplitudes of tail currents increased with the increment of depolarizing voltages in cells treated with JZTx-27 (Fig. 3G), indicating more toxin-occupied channels were activated by strengthening depolarization (see also statistics in Fig. 3H).

These data show that the activation of NsVBa was significantly slowed by JZTx-27 and that the toxin-occupied channels required much stronger depolarization to be activated and display accelerated deactivation, which suggests that JZTx-27 traps NsVBa in the resting state. We proposed that the toxin delays the closed-to-open state transition, possibly by trapping the deactivated state of the voltage sensor.

**JZTx-27 interacts with the S3-4 extracellular loop of NsVBa channel**

Our data suggest that JZTx-27 acts as a gating modifier, sharing a similar manner of action with some mammalian...
Na\textsubscript{Va}s site 4 toxins which cause a positive shift of the steady-state activation. These toxins are proposed to bind to the voltage sensor of Na\textsubscript{Va}s by interacting with the S1-2 and S3-4 extracellular loops, where voltage-dependent movement of the S4 voltage sensor is impaired. To test this hypothesis, we constructed NsVBa chimeric channels by replacing its S1-2 (chimera 1) and S3-4 (chimera 2) extracellular loops with those of NaVPZ (Figs. 4A and 5A). Chimera 2 but not chimera 1 was functionally expressed in CHO-K1 cells (Fig. 4B). The affinity of JZTx-27 with chimera 2 was dramatically reduced when compared to WT channel as 3 \mu M toxin only inhibited its peak current by 19.4 \pm 3.6\% (Fig. 4B, C). The apparent IC\textsubscript{50} of JZTx-27 on chimera 2 was assessed at the voltage evoking its maximum peak current (Fig. 4C). These data highlight the crucial role of S3-4 loop of NsVBa in determining the JZTx-27-NsVBa interaction.

**F98 in NsVBa is critical for JZTx-27-channel interaction**

To determine the key amino acids responsible for JZTx-27-NsVBa interaction, we used scanning mutagenesis of the S1-2 and S3-4 loops. Because some mutations altered channel activation kinetics, the apparent IC\textsubscript{50} of JZTx-27 against each mutant was measured at the voltage for their maximum peak current (Supplemental Table S1). Each amino acid positioned in the S1-2 loop, as well as the S3b paddle motif. All of these mutations reduced the affinity of JZTx-27 with NsVBa by less than 3-fold when compared to WT channel. These data suggest that the S3-4 loop of NsVBa forms a key receptor site for JZTx-27. Taking into account the disruptive effect of F98P mutation on the spatial organization of the S3-4 loop, we further tested the effect of mutating a conserved F103 (analogous to F98 in NsVBa) in NaChBac on the affinity of toxin with channel. Consistent with the NsVBa F98P mutant, the NaChBac F103P mutant was resistant to JZTx-27 treatment with 1.5 \mu M toxin inhibiting little of its peak current (Fig. 5E). However, in contrast to NsVBa F98A and NsVBa F98L mutants, the affinity of JZTx-27 to NaChBac F103A mutant was reduced by \sim 10-fold when compared with that of the WT channel (Fig. 5E, F; IC\textsubscript{50} = 30 nM and 308 nM for WTNaChBac and NaChBac F103A mutant, respectively). These data validate the critical role of F98 in NsVBa in the interaction of toxin with channel. The observation that mutating F98 to alanine (F98A) or leucine (F98L) in NsVBa barely affected the affinity of toxin with channels may be explained by a compensatory binding site for JZTx-27 in the channel sequence.

**The effect of JZTx-27 on mammalian Na\textsubscript{Va}s**

The activity of JZTx-27 on endogenous sodium currents from rat dorsal root ganglion (DRG TTX-R INa) and on 6 heterologously expressed mammalian Na\textsubscript{V} subtypes (hNaV1.1, hNaV1.3, rNaV1.4, hNaV1.5, hNaV1.7, and rNaV1.8) were assessed. The endogenous DRG TTX-R Na\textsuperscript{+} channels, hNaV1.1 and rNaV1.8, were resistant to JZTx-27 (Fig. 6A, C). However, JZTx-27 antagonized the peak currents as well as the inactivation of hNaV1.3, rNaV1.4, hNaV1.5, and hNaV1.7 (Fig. 6A). Of the Na\textsubscript{V} subtypes tested, hNaV1.5 exhibited the greatest sensitivity to JZTx-27, with an EC\textsubscript{50} of 700 nM (measured by the I_{3ms}/I_{peak} ratio; Fig. 6B). Na\textsubscript{V} chimeras were constructed to explore the primary receptor site in hNaV1.5 for JZTx-27. Functional channels were constructed by replacing DII, DIII, and DIV, but not DI, of hNaV1.5 with those of rNaV1.8 [Fig. 6D–F, referred to as DII (NaV1.8), DIII (NaV1.8), and DIV (NaV1.8)].

**Figure 4.** Determination of the key region in NsVBa for binding JZTx-27. A) The strategy for NsVBa/NavPZ chimera construction. B) Representative traces showed that chimera 2 was resistant to JZTx-27 compared with the WT NsVBa channel (n = 4–6). C) Dose–response curves show that replacing NsVBa S3-4 extracellular loop with that of NavPZ (chimera 2) attenuated toxin affinity, with 3 \mu M JZTx-27 inhibiting its peak current by only 19.4 \pm 3.6\% (n = 5–8).
Currents of each chimera were elicited by depolarization to 10 mV from a holding potential of −80 mV. DII (NaV1.8) chimera responded robustly to JZTx-27, with 1 μM toxin inhibiting its inactivation and activating its peak current (Fig. 6D); DIII (NaV1.8) chimera was sensitive; but DIV (NaV1.8) chimera was resistant to JZTx-27 (Fig. 6E, F), which suggests that the primary toxin binding site for JZTx-27 is located in DIV of NaV1.5. In contrast to NaV1.5, JZTx-27 activated the peak current of DII (NaV1.8) and did not affect that of DIII (NaV1.8), suggesting multiple binding sites in hNaV1.5 for JZTx-27. In addition, the activation of the peak current of DII (NaV1.8) by JZTx-27 may be partially contributed by toxin rendering DII (NaV1.8) refractive to inactivation by positively shifting its steady-state inactivation (Supplemental Fig. S3). JZTx-27 could partially resemble α-scorpion toxins in acting on mammalian NaV1s, in stabilizing the deactivated voltage sensor of DIV to reduce the rate of channels’ inactivation.

**Structural model of JZTx-27 interaction with NsV1Ba voltage sensor**

We used Rosetta structural modeling software to dock JZTx-27 to multiple states of the NsV1Ba VSM. Based on experimental data presented in this study (Fig. 5D),
mutations of the S3-4 loop residues F98 and H102 had the biggest effect on JZTx-27 binding. Figure 7 shows one of the most energetically favorable models of JZTx-27 in complex with NsVBa VSM in deactivated state 1, which has F98 and H102 at the toxin–channel interface. F98 on the channel is in proximity to F6 and W28 on the toxin, and H102 on the channel is in proximity to K30, D32, and I33 on the toxin. Experimental data also show a 2–3-fold change in IC_{50} for Y40A/P41A and R44A/H45A NsVBa double-site mutants (Fig. 5D). Y40 and H45 sidechains are pointing away from the toxin in our model (not shown). P41 is in proximity to W34 on the toxin in our model (Fig. 7), but the P41A mutant may also affect the secondary structure of the S1-2 loop and therefore reduce toxin binding. R44 is in proximity to D32 on the toxin in our model. Our model also suggests that G100 and G101 within the S3-4 loop of NsVBa allows for shape complementarity of C-terminal region of the toxin. None of the top 20 clusters of the NsVBa–JZTx-27 complex in activated or deactivated state 2 (Supplemental Fig. S4) captured F98 and H102 at the channel–toxin interface.

DISCUSSION

**JZTx-27 stabilizes the resting conformation of NsVBa**

The voltage sensor–trapping model is a common mechanism underlying gating-modifier peptide toxins acting on voltage-gated ion channels (16). These toxins bind to the extracellular loops and trap the voltage sensors in a certain conformation, affecting the channels’ gating kinetics. In the present study, JZTx-27 trapped NsVBa VSM in the deactivated state and consequently rendered the channel...
refractory to be activated by depolarization. Our conclusion is based on the following observations: 1) JZTx-27 blocks NsVBa voltage dependently, with less inhibition at more depolarized voltages, and positively shifts the steady-state activation (Fig. 2); 2) the toxin-occupied channels can be reopened with decelerated activation but accelerates deactivation kinetics when compared with toxin-unoccupied channels (Fig. 3); and 3) the activation of toxin-occupied channels requires much stronger depolarization (Fig. 3). In contrast, lidocaine-treated NsVBa channels did not have these gating properties. This difference may originate from the different molecular mechanisms by which they block NsVBa. JZTx-27 directly interacted with the S3-4 extracellular loop of NsVBa to impede VSM activation, and enhanced depolarization could have triggered the outward movement of the toxin-VSM complex to reopen the channel, whereas lidocaine antagonized NaChBac by stabilizing channels in the inactivated state (35). Furthermore, the binding site of lidocaine in bacterial NsVBa may partly overlap with PII, whose binding area in NaVbMs was determined to be the pore cavity region (37). One possibility is that lidocaine interacts with the pore of NsVBa to stabilize the inactivated state, hence the lidocaine-bound NsVBa channels could not be activated by simply driving outward movement of the voltage sensors by strengthening depolarizations. The observation that JZTx-27 significantly accelerates the deactivation of NsVBa suggests that toxin-occupied channels also contribute to the tail current elicited by tail-voltage holding after strong depolarization (Fig. 3D), which indicates that the toxin forms a stable complex with the deactivated NsVBa voltage sensor. In other words, NsVBa voltage sensor activation is impaired, but its deactivation is facilitated by the cargo toxin, which is reminiscent of hanatoxin’s effect on the Kv2.1 channel (38–40).

Our structural model of JZTx-27–NsVBa voltage sensor interaction suggests that the hydrophobic surface of the toxin interacts with the membrane and S3-4 loop of the channel. The Rosetta model predicts that NsVBa residues F98 and H102 interact with several residues on the toxin and that the S3-4 loop has significant shape complementarity with the C-terminal region of the toxin. The activated and deactivated state 2 of the NsVBa VSM showed significantly different conformation of the S3-4 loop region (Supplemental Fig. S4). We suggest that in addition to interactions between JZTx-27 and F98 and H102 on the NsVBa VSM, shape complementarity between the JZTx-27 and NsVBa VSM S3-4 loop region plays an important role in stabilization of the specific NsVBa VSM deactivated state upon JZTx-27 binding.

A homologous neurotoxin binding site in mammalian and bacterial NaVs

Eight neurotoxin binding sites have been characterized in mammalian NaVs (41). However, little is known about whether peptide toxin binding sites are conserved in prokaryotic NaVs. Previous studies showed that local anesthetics inhibit bacterial NaVs as well as their mammalian counterparts (35); fenestration between adjacent subunits of bacterial NaVs provides an entry route for small neutral or hydrophobic drugs (22, 42, 43). The present study proved that the S3-4 extracellular loop of NsVBa channel is the primary receptor site for JZTx-27, which is the first report that there is a mammalian NaVs homologous peptide toxin binding site in bacterial NaVs. Of the 8 neurotoxin-binding sites in mammalian NaVs, site 3 and 4 are composed of extracellular loops of domains IV and II, respectively. Peptide toxins targeting sites 3 and 4 affect channel activation and inactivation, respectively (41). The effect of JZTx-27 on mammalian NaVs was similar to α-scorpion toxins. Using NaV chimeras, we have clearly demonstrated that JZTx-27 targets DIV of NaV1.5 (Fig. 6D–F). The different responses of mammalian NaVs and bacterial NaVs to JZTx-27 resemble that of NaV1.5 and Kv2.1 to JZTx-I (44, 45) and can be interpreted as follows: JZTx-27 targets the DIV VSM of mammalian NaVs that is responsible for inactivation (46–49). However, bacterial NaVs are homotetramers that lack a fast inactivation gate structure and a fast inactivation process. JZTx-27 traps the bacterial NaVBa VSM in the deactivated state leading to inhibition of peak current.

JZTx-27 as a probe for the determination of NsVBa structure in the resting state

Many neurotoxins bind to ion channels and trap them in specific states. This feature is useful for ion channel structure determination, as channel-toxin complexes often immobilize/stabilize labile channel motifs that enhances their structural refinement. In some cases, channel-toxin complex structure determination can reveal the channel structure in states that could not be captured from unbound channels, such as those channel states found in the cocystal structure of acid-sensing ion channel
The simpler bacterial NaVs are used by biologists to understand the biophysical determinant of mammalian NaV conformational changes and function (53). Comparing the NaVMs open pore with that of NaVAb closed-pore structures has defined the mechanism of pore–module opening and closing (8). Most recently, 4 Na⁺ occupancy sites (S₁–S₄) within the selectivity filter of NaVMs has been resolved in a crystal structure (54), challenging the 3-site model (siteOUT-siteCEN, and siteEN) proposed from the unoccupied filter of the NaVAb structure (7). Thus, it would be interesting to see whether or not toxin binding to the voltage sensor domain could alter Na⁺ occupancy within the selectivity filter. Such observations would undoubtedly enhance our understanding of mechanical communication between the voltage sensor and the ion conducting site found in the pore modules. Compared with potassium selectivity in K⁺-channels (55, 56), the mechanism of Na⁺-selectivity in Na⁺-channels remains poorly understood. NaV-toxin complexes may provide a unique tool to determine which Na⁺ selectivity filter site are most affected by toxin binding and thus which sites are essential to sodium conductance. The X-ray crystal structures of the NaVAb and NaVRh channels have captured the VSM in 2 different activated states (7, 9). However, no structure with the voltage sensor in the deactivated state has been reported so far. Although interactions which stabilize the fourth transmembrane segment (S4) in the deactivated and activated states have been described by modeling and functional analysis (27, 57–59), the structure of a sodium channel with a deactivated voltage sensor would certainly provide additional insight. For example, a deactivated voltage sensor structure would set the lower limit of S4 movement during the activation process and define the level of hydration within the gating pore. Because the resting membrane potential is absent in crystallographic conditions (60), the resting state (which requires ~100 mV of membrane potential) will continue to elude structural biologists unless an agent (such as a toxin or channel modifier) can be used to provide molecular constraints to capture this conformation. The tarantula toxin JZTx-27 is able to stabilize NsVBa channel in the resting conformation (7, 9). Thus, it would be interesting to see whether or not toxin binding to the voltage sensor domain could alter Na⁺ occupancy within the selectivity filter. Such observations would undoubtedly enhance our understanding of mechanical communication between the voltage sensor and the ion conducting site found in the pore modules.

REFERENCES


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AUTHOR CONTRIBUTIONS

C. Tang and Z. Liu developed the concept and designed the study; C. Tang, X. Zhou, Y. Zhang, Z. Hu and C. Zhang performed the experimental studies and data acquisition; P. T. Nguyen and V. Yarov-Yarovoy were responsible for the structure modeling; and C. Tang, Z. Liu, P. G. DeCaen, V. Yarov-Yarovoy, and S. Liang prepared the manuscript.
A novel tarantula toxin stabilizes the deactivated voltage sensor of bacterial sodium channel

Cheng Tang, Xi Zhou, Phuong Tran Nguyen, et al.

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SUPPLEMENTAL MATERIAL

A novel tarantula toxin stabilizes the deactivated voltage sensor of bacterial sodium channel
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Supplemental Fig.S1. (A) RP-HPLC profile of the venom from *Chilobrachys jingzhao*. Red arrow indicates the fraction containing JZTx-27. (B) The peak containing JZTx-27 in Supplemental Fig.S1A was further purified by analytical C18 RP-HPLC. Red arrow indicates the purified JZTx-27. (C) The molecular weight of JZTx-27 was determined as 4086.81Da (M+H+) by MALDI-TOF MS. (D) cDNA and amino acid sequence of JZTx-27. The mature sequence (shaded in black) was also determined by Edman degradation; the signal peptide is shown in gray, and the propeptide is underlined.
Supplemental Fig. S2. (A), Representative traces showing lidocaine blocks NsV_Ba current dose-dependently. (B), Lidocaine treatment did not alter the steady-state activation of NsV_Ba.

Supplemental Fig. S3. Steady-state inactivation curves of NaV_1.5, DII(NaV_1.8), DIII(NaV_1.8) and DIV(NaV_1.8) chimeric channels before and after application of 1 µM JZTX-27. Toxin dramatically modified the voltage dependence of inactivation of DII(NaV_1.8) but not others by shifting the V_h from $-72.1 \pm 3.0$ mV to $-53.1 \pm 11.4$ mV ($p = 0.00685$) and increasing the K_h from $-7.4 \pm 2.5$ mV to $-14.9 \pm 1.6$ mV ($p = 0.00044$).

Supplemental Fig. S4. Transmembrane view of structural models of NsV_Ba in resting and activated states. Transmembrane segments S1 through S4 are colored by rainbow.
Supplemental table S1. The voltage evoking the maximum peak current of each mutant/chimeric channel. Data was presented as MEAN ± SD (n = 4 - 12).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$V_{\text{peak}}$</th>
<th>Mutant</th>
<th>$V_{\text{peak}}$</th>
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<tbody>
<tr>
<td>NsV Ba Y40A/P41A</td>
<td>-32.5 ± 22.2</td>
<td>NsV Ba F98A</td>
<td>-44 ± 8.9</td>
</tr>
<tr>
<td>NsV Ba L43A</td>
<td>-32.5 ± 5.0</td>
<td>NsV Ba F98P</td>
<td>8.3 ± 4.1</td>
</tr>
<tr>
<td>NsV Ba R44A/H45A</td>
<td>-46.0 ± 11.4</td>
<td>NsV Ba V99A</td>
<td>2.9 ± 7.6</td>
</tr>
<tr>
<td>NsV Ba E46A/Y47A</td>
<td>-34.0 ± 5.5</td>
<td>NsV Ba H102Q</td>
<td>-58.0 ± 8.4</td>
</tr>
<tr>
<td>NsV Ba S94A</td>
<td>-30.0 ± 10.0</td>
<td>NsV Ba F103G</td>
<td>-48.3 ± 11.7</td>
</tr>
<tr>
<td>NsV Ba S95A</td>
<td>5.0 ± 10.5</td>
<td>NsV Ba I104L</td>
<td>-17.5 ± 5.0</td>
</tr>
<tr>
<td>NsV Ba H96A</td>
<td>4.0 ± 5.5</td>
<td>wt NsV Ba</td>
<td>-3.4 ± 6.2</td>
</tr>
<tr>
<td>NsV Ba I97A</td>
<td>-35.0 ± 13.8</td>
<td>Chimera 2</td>
<td>-20.0 ± 11.0</td>
</tr>
</tbody>
</table>

$V_{\text{peak}}$: the voltage evoking the maximum peak current.