Voltage-gated sodium channels (VGSCs) are trans-membrane proteins distributed widely in the most excitable tissue. Similar to the shaker potassium channel (1), the three-dimen-sional structure of sodium channel is a bell-shaped molecule determined by helium-cooled cryo-electron microscopy and single particle image analysis (2). In terms of tetrodotoxin (TTX), they can be classified into TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) types. More recently, from mammals includ-ing human, more than 10 mammalian (Nav1.1–Nav1.9 and Na\(_{x}\)) subtypes have been identified, cloned, functionally expressed, and characterized (3). Most of them can express in dorsal root ganglion (DRG) neurons, except for Nav1.4 in skeletal muscles and Nav1.5 in cardiac myocytes (4). These subtypes have been highly conserved during evolution (5, 6). With more than 75% sequence identity among one another, they exhibit relatively similar pharmacological properties in different expression systems. However, it is the divergent residues among the sequences of these VGSC isoforms that determine their response to distinct ligands. For instance, after tyrosine 371 is substituted by serine in rNav1.6 and rNav1.3 (wild types), which are TTX-S phenotypes, the mutants become re-sistant to TTX (7, 8).

As the major contributors to the initiation and propagation of action potentials, VGSCs become the main targets attacked by many spider toxins. With specific pharmacological properties and higher affinity with VGSCs, spider peptides have attracted the interests of many scientists. Until now, more than 30 sodium channel toxins have been purified and well characterized from venom of various species. NMR and homology modeling techniques indicate that, irrespective of the different composition of amino acids, most of them adopt a typical inhibitor cystine knot (ICK) fold distinct from the \(\alpha/\beta\) scaffold emerging in scorpion toxins (9, 10). Most residues in their primary structure are believed to support the peptide framework, whereas only a few charged residues situated at the loop domains of ICK motifs are critical to interact with sodium channels. Recently, scorpion toxin determinants demonstrate that some conserved aromatic residues (Phe, Tyr, and Trp) also play an important role in modifying the sodium channel activities (11). Spider toxins exhibit limited sequence identity in the sodium channel toxins from other origins, such as marine animals, scorpions, and snakes, revealing that there is a perspective for searching for new valuable ligands to dissect variant VGSCs. Furthermore, spider toxins have been shown to lead to new insecticides and pharmaceuticals. On the functional \(\alpha\) subunit of VGSCs, more than six sites (sites 1–6) have been disclosed to bind certain ligands (12). Spider toxins mainly interact with three of them, corresponding to blocking channel pore (site 1, hainantoxin-IV (HNTX-IV) and Huwentoxin-IV (HWTX-IV)), slowing channel inactivation (site 3, \(\mu\)-agatoxins and \(\delta\)-urotoxins), and inhibiting channel activation (site 4, Magi 5 and ProTx I–II), respectively (13–18). Most of them are found to have high affinity with the subtypes of VGSCs localizing on sensory neurons, but a few affect the cardiac isoform.

In this study, we report the isolation, cDNA sequence clone, and functional characterization of a novel cardiotoxin from the spider *Chilobrachys mingzhao*, which was identified as a new species recently (19). The crude venom is lethal to mice with an intraperitoneal LD\(_{50}\) of 4.4 mg/kg. The spider toxin, denoted
jingzhaotoxin-III (JZTX-III), is composed of 36 amino acid residues including 6 cysteine cross-linked in a pattern of I-IV, II-V, and III-VI. The toxin shows no effect on voltage-gated potassium channels (K, 1.1–1.3) expressed in Xenopus laevis oocytes or VGSCs and voltage-gated calcium channels (VGCCs) distributed in DRG neurons. However, it can selectively inhibit activation of TTX-R VGSCs in cardiac myocytes followed by slow afterpotential voltage in a depolarization direction. We further assume that JZTX-III binds to site 4 on sodium channel proteins, which is formed by amino acid residues in the extracellular linker between domain II-S3 and domain II-S4.

**MATERIALS AND METHODS**

**Toxin Purification and Sequencing**—The venom from female C. jingzhao spiders was collected as described earlier (16). Lyophilized venom (1 mg in 0.2 ml distilled water) was applied to a Vydac C18 analytical reverse-phase (RP)-HPLC column (218TP54, 4.6 × 250 mm) and eluted at a flow rate of 1 ml/min by a linear gradient of 0–40% of buffer B (acetonitrile containing 0.1% v/v trifluoroacetic acid) over 50 min after an equilibrium period of 3 min with buffer A (distilled water containing 0.1% v/v trifluoroacetic acid). The fraction containing JZTX-III was purified further on the same RP-HPLC column by a slower linear gradient of 30–55% buffer B over 20 min. Once purified to >99% homogeneity assessed by RP-HPLC and mass spectrometry, the peptide sample was lyophilized and stored at −20 °C until use. The molecular mass was determined by MALDI-TOF mass spectrometry on a Voyager-DE™ STR Biospectrometry™ work station. The entire amino acid sequence was obtained from a single sequencing run on an Applied Biosystems/PerkinElmer Life Sciences Procise 491-A protein sequencer.

**Identification of JZTX-III cDNA**—The characterization of JZTX-III cDNA was performed using 3′- and 5′-RACE methods described previously (20). First, according to the manufacturer’s instructions, the total RNA was extracted from 0.1 g of fresh venom glands of female spiders using the TRIzol reagent kit. 5 RNA was extracted from 0.1 g of fresh venom glands of female spiders lyophilized and stored at −20 °C. The RNA samples were precipitated and cloned into the pGEM-T easy vector for RACE as follows: the gene-specific primer 2 (5′-GGCA GCCCGTCCGCTGACTAC-3′) was used with the mode isoform of the Boltzmann equation: Inhibition% = 100(1 + eIC50/km), in which IC50 is the concentration of toxin at half-maximal inhibition, k is the slope factor, and C is the toxin concentration.

**RESULTS**

**Purification and Sequence Analysis of JZTX-III**—A typical RP-HPLC chromatogram of the female spider venom was shown in Fig. 1A, in which more than 20 fractions eluted were monitored at 280 nm. The fraction with the retention time of 39 min, containing JZTX-III, was further purified by a repeated RP-HPLC (Fig. 1B). Two purifications yielded about 0.05 mg of JZTX-III/mg of crude venom with a purity over 99%. Its molecular mass was determined to be 3919.4 Da by MALDI-TOF mass spectrometry. The complete amino acid sequence of the toxin was obtained by Edman degradation and found to contain 36 residues including 6 cysteines (see Fig. 4A). After being reduced by dithiothreitol and then alkylated with iodoacetamide, the molecular mass of JZTX-III increased 348 Da (58 Da × 6), implying that all 6 cysteines were involved in forming three disulfide bridges. Since the primary structure had mass of 3919.52 Da, consistent with the measured mass, the C-terminal residue could not be amidated. JZTX-III is a basic peptide sharing less than 50% sequence identity with any known peptides, although its 6 cysteines were highly conserved at corresponding positions in many toxins, such as HWTX-IV, ProTx-I, and ProTx-II (16, 17).

**Determination of the Disulfide Bridges in JZTX-III**—Because vicinal cysteines (18CC19) emerge in the primary structure of JZTX-III, a chemical strategy composed of partial reduction and sequence analysis was introduced instead of a traditional enzymatic method. As shown in Fig. 2, four main peaks were obtained from the RP-HPLC separation of the partial reduced mixture of JZTX-III by TCEP. MALDI-TOF mass spectrometry analysis points out that the two intermediates yielded were resolved to contain one or two disulfide bridges in peak II and I, respectively, whereas peaks R and N represent completely reduced peptide and intact peptide, respectively. Peaks I–II were collected and alkylated rapidly with iodoacetamide followed by further purification using analytical RP-HPLC. Molecular mass determination and sequencing indicated that the free thiols of these two peptides had not been alkylated.

In Fig. 3A, Pht-CM-Cys signals were observed at the 4th and 19th cycles in the sequencing chromatograms of alkylated peak
I, whereas no signals emerged at other cysteine cycles. The result indicates that the only reduced disulfide bond is Cys 4 – Cys19. When sequencing alkylated peak II, Pth-CM-Cys signals were observed at the 4th, 11th, 19th, and 24th cycles in the profiles of cysteine cycles (Fig. 3B), indicating that Cys 18 was still linked to Cys 31 by a disulfide bond. The above results indicate that two of three disulfide bridges in JZTX-III were determined to be Cys 4 – Cys19 and Cys 18 – Cys31. Accordingly, the third one is cross-linked between Cys 11 and Cys24. Thus, JZTX-III has a conserved disulfide connectivity emerging among ICK motifs where 6 cysteines were linked in a pattern of I-IV, II-V, and III-VI (9).

Cloning and Sequencing of JZTX-III cDNA—The full-length cDNA sequence of JZTX-III was completed by overlapping two fragments resulting from 3’- and 5’-RACE. As shown in Fig. 4B, the oligonucleotide sequence of the cDNA was a 373-bp bond in which the first ATG was assumed to serve as the translation start codon. The open reading frame, ending before the first stop codon TGA at 3’-terminal position, encoded 63 residues corresponding to the JZTX-III precursor. It comprised a signal peptide of 21 residues, a pro-peptide of 5 residues, and a mature peptide of 36 residues. The deduced mature peptide sequence was consistent with that of native JZTX-III determined by Edman degradation. Unlike huwentoxin-IV, JZTX-III had no extra Gly or Gly + Arg/Lys residues at the C terminus, which are known to allow “post-modification” α-amination at the C-terminal residue (20). The prepro-regions common to all spider toxins are a hydrophobic peptide and can be processed at a common signal site -X-Arg- before mature peptide sequences, which is recognized by special endoprotelytic enzymes. In general, this region is composed of over 40 residues. Interestingly, further analysis indicated that JZTX-III had a very small pre-pro-region that exhibits no similarity to those of other spider toxins from diverse species including the Chinese bird spider Selenocosmia huwena Wang (also known as Ornithoctonas huwena Wang) (20, 21). Furthermore, it is worth noting that the signal site anterior to mature JZTX-III was an uncommon one (-X-Ser-) (20, 23–25). A polyadenylation signal, AATAAA, was found in the 3’-untranslated region at position 16 upstream of the poly(A).

Effects of JZTX-III on VGSCs—Using whole cell patch clamp technique, the actions of JZTX-III were characterized on VGSCs in rat DRG neurons and ventricular myocytes, in which both TTX-S and TTX-R types are co-expressed. TTX (200 nM) was added to the external bath solution to separate TTX-R type...
from mixture currents. Although Maier et al. (26) suggested that some brain TTX-S subtypes were situated in transverse tubules of ventricular myocytes, in our experiments, the induced sodium currents were not changed in the absence or presence of TTX at 0.2 μM (data not shown, n = 4). Therefore, the effects of JZTX-III on cardiac myocytes were assayed in bath solution without TTX.

After establishing whole cell configuration, the experimental cells were held at −80 mV for over 4 min to allow adequate equilibration between the micropipette solution and the cell interior, and then the current traces were evoked using a 50-ms step depolarization to −10 mV every second. As shown in Fig. 5, A and B, 1 μM JZTX-III showed no evident effects on the normal activities of both TTX-S and TTX-R VGSCs in rat cardiac myocytes. However, cardiac TTX-R currents were sensitive to the novel toxin. 1 μM JZTX-III reduced the control peak amplitude to a maximum effect by 64.7 ± 4.7% (Fig. 5C, n = 8). JZTX-III up to 10 μM completely eliminated the remaining currents within less than 1 min (Fig. 5D and E, n = 4). The rapid inhibition was dose-dependent with an IC50 value of 0.38 ± 0.04 μM (Fig. 5F). It was observed that similar to ProTx I–II (15), JZTX-III failed to alternate channel inactivation, although most spider toxins (e.g. δ- and μ-toxins) identified to date share a common mode of slowing channel inactivation similar to scorpion α-toxins (7). ProTx I–II have been suggested to bind to VGSC site 3 or site 4. To further determine the detailed site for the toxin of interest, a simple competitive assay was introduced between JZTX-III and site 3 toxins. *Buthus martensi* Karsch I (BMK-I), acting on site 3, is a typical α-like scorpion toxin isolated from the Asian scorpion, *B. martensi* Karsch. It can slow the inactivation of VGSCs expressing in both mammalian sensory neurons and ventricular myocytes without significantly affecting the peak amplitudes (10, 27). Exposed to 10 μM JZTX-III, the slowing currents induced by 10 μM BMK-I were eliminated completely, suggesting that the spider toxin modulated cardiac VGSCs through a mechanism distinct from site 3 toxins.

Fig. 6 shows the current-voltage (I-V) curve of cardiac TTX-R VGSCs, yielding that initial activated voltage and reversal potential are −50 mV and +25 mV, respectively (n = 4). After 1 μM JZTX-III treatment for 1 min, the inhibition of currents could be observed at tested potential from −40 mV to +20 mV. JZTX-III shifted the threshold of initial activation more than +10 mV in a depolarizing direction, but no change was observed significantly in the membrane reversal potential, implying that it did not change the ion selectivity of channels.

Effects of JZTX-III on VGCCs and Voltage-gated Potassium Channels (VGPCs)—There are two main categories of VGCCs distributed in rat DRG neurons: high voltage-activated channels and low voltage-activated channels, which can be discriminated by their voltage dependence and kinetics. JZTX-III (1 μM) was not found to affect VGCCs (Fig. 7, n = 3). Three different VGPC isoforms (K1.1, K1.2, and K1.3) were expressed in *Xenopus laevis* oocytes and checked for toxins using the two-electrode voltage clamp technique as described previously (28). No effects were detected with JZTX-III at 1 μM (Fig. 8, n = 4).
In this work, we have isolated and characterized a 3.9-kDa toxin named JZTX-III from the Chinese spider *C. jingzhao* (19). The full sequence of the toxin was performed by Edman degradation and found to contain 36 residues including 6 cysteines. No amidation at its C-terminal residue is detected by MALDI-TOF mass spectrometry and its cDNA sequence analysis. Although it exhibits less than 50% sequence identity to any known peptides, it contains a conserved disulfide connectivity frequently emerging in ICK peptide toxins from diverse species, such as spiders and marine snails, cross-linked in a pattern of I–IV, II–V, and III–VI. Based on the analysis of precursor organization and gene structure combined with a three-dimensional fold, Zhu et al. (29) suggested that these ICK peptides from animals shared a common evolutionary origin. The molecular scaffold is highly stabilized by the three disulfide bridges, especially the third (III–VI) (9). Huwentoxin-II, from the Chinese bird spider *S. huwena*, adopts a scaffold distinct from ICK motif for having a unique disulfide connectivity of I–V, II–III, and IV–VI (30). The residue numbers between 2 cysteines in JZTX-III also conform exactly to the ICK definition described as a consensus sequence CIX3–7CIIX4–6CIIIX1–4CVI (where X is any residue, with the number indicated by the range) (9).

The amino acid sequence of JZTX-III is verified further by its cDNA, which produces a precursor comprising a signal peptide, an intervening pro-peptide, and a mature peptide. Concerning the structural organization, JZTX-III should be matured through a post-translational cleavage during the course of se-

Fig. 5. Effects of JZTX-III on VGSCs. All current traces were evoked by a 50-ms step depolarization to −10 mV from a holding potential of −80 mV at every 2 s. Both TTX-S (A) and TTX-R (B) VGSCs were significantly unaffected by 1 μM HuNTX-I on DRG neurons, isolated from adult rat by the method described in Ref. 18. C, effects of TTX-R sodium currents in cardiac myocytes. 1 μM JZTX-III evidently reduced the control current amplitude by 64.7 ± 4.7% (n = 8), whereas at 10 μM, the toxin eliminated the slowing inward current induced by 1 μM EMK-I (D, n = 4) in a time-dependent manner (E, n = 4). F, the concentration-dependent inhibition of TTX-R sodium currents in cardiac myocytes. Every data point (mean ± S.E.) coming from 3–8 cells shows current relative to control. These data points were fitted according to Boltzmann equation (see “Materials and Methods”).

Fig. 6. Effects of JZTX-III on the current-voltage (I-V) relationship of TTX-R VGSCs in cardiomyocytes. A family of currents was elicited by 50-ms depolarizing steps to various potentials from a holding potential of −80 mV. Test potentials ranged from −80 mV to +50 mV at increments of +10 mV. The I-V curve (B) of sodium currents showed the relationship between current traces before (above) and after (below) adding 1 μM JZTX-III in A. In B, the data points obtained from four separated experimental cells are shown as mean ± S.E.

Fig. 7. Effects of JZTX-III on VGCCs on rat DRG neurons. A, high voltage-activated currents were elicited by a 150-ms depolarizing voltage of 0 mV from a holding potential of −40 mV, and current traces were not changed before and after the application of 1 μM JZTX-III. B, low voltage-activated currents were induced by a 150-ms depolarizing potential of −30 mV from a holding potential −90 mV, and current traces were not changed before and after the application of 1 μM JZTX-III.

Fig. 8. Effects of JZTX-III on VGPCs expressed in *X. laevis* oocytes. Kv1.1 (A), Kv1.2 (B), and Kv1.3 (C) current traces were evoked by depolarizations to +10 mV from a holding potential of −90 mV. After exposure to 1 μM JZTX-III, no changes of the currents were detected.
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cretion. Many works have demonstrated that there is a common endoproteolytic site \((-\text{X-Arg})\) between the sequences of prepro and mature peptide (20, 23–25). Different from known spider toxins, JZTX-III precursor contains an uncommon site \((-\text{X-Ser})\), suggesting that the processing to endoproteolysis prepro-peptide should be accordingly different from that of them. Another intriguing finding in this study is that the intervening pre-peptide region of JZTX-III is the smallest one identified to date in the field of spider toxins. The region, generally rich in glutamate residues, emerges in cDNA sequences of most animal toxins from diverse sources, but it is missing in some scorpion toxins. Until now, its action in forming toxins is not yet well defined, although Diao et al. (20) inferred that it might contribute to stabilizing the toxin pre-cursor and prevent the mature toxins from interacting with other molecules in the cytoplasm. Furthermore, the analysis of prepro-regions can provide new proof for interpreting the evolutionary relationship in animal toxins. Around 50,000 conotoxins, although targeting different receptors, can be grouped into seven superfamilies (24). However, no similar description about spider toxins demonstrated that their prepro-peptides share higher sequence identity with one another, until in our recent work, seven distinct cDNAs from the gland of \(S.\ huvena\) were classified into two superfamilies (20). Having two distinct characterizations, an uncommon endoproteolytic site \((-\text{X-Arg})\) and a very small pre-region exhibiting limited sequence identity to others, JZTX-III defines a novel superfamily distinct from the previously reported two superfamilies.

To date, more than 30 spider toxins from BLAST databases are found to target neuronal VGSCs, but few are found to target the cardiac subtype. According to their distinct pharmacological characterization, these toxins can be classified into two groups: excitatory toxins and depressant toxins (21). ProTx-I and ProTx-II are the only agents reported in the both groups to inhibit \(\text{Na}_{1.5}\), a TTX-R subtype expressing especially in cardiac myocytes (17). A similar inhibition of channel activation is observed after the application of JZTX-III, and it belongs to the depressant toxins. It seems that its selectivity for sodium channel isoforms is even higher than that of ProTxOs, which inhibit some neuronal VGSC subtypes (\(\text{Na}_{1.2, 1.8–1.9}\)) with \(\text{IC}_{50}\) values of less than 0.1 \(\mu\)M. Moreover, ProTxOs target outward delayed-rectifier VGPCs and T-type VGCCs (17). We also checked the effects of JZTX-III on neuronal VGSCs isoforms and VGCCs as well as VGPCs (K,1.1–1.3) expressed in \(Xenopus laevis\) oocytes, but no evident effects were observed. It is very likely that \(\text{Na}_{1.4}\) is not the target for JZTX-III because the peptide did not affect the normal contractions of mouse diaphragm induced by direct electrical stimulus. The properties of JZTX-III in \(\text{Na}_{1.5}\) are similar to those of scorpion \(\beta\)-toxins. They inhibit channel activation without affecting the inactivation kinetics or the ion selectivity of \(\text{Na}^+\) (10). This mechanism is different from that of excitatory spider toxins, such as \(\delta\)-a-tracotoxin-Ar1, in which, binding to the extracellular S3-S4 loop of domain IV, modify the conformation of channel peptides and cause an uncoupling of channel activation and inactivation in a similar manner to scorpion \(\alpha\)-toxins or sea anemone toxins (14). In our experiments, JZTX-III inhibited the slowing currents induced by site 3 toxin (BMK-I, a scorpion \(\alpha\)-like toxin) completely, suggesting that the binding site for the spider toxin is not site 3. The mechanism of JZTX-III is also different from that of other depressant toxins, such as HNTX-IV. They block neuronal TTX-S VGSCs with no shift in the I-V curve and are assumed to be site 1-like toxins (16). According to the distinct effects on the VGSCs when toxins selectively bind to six sites of the channels (12), JZTX-III can be reasonably inferred to interact with site 4 located at the extracellular S3-S4 loop of domain II of the channel molecules. Furthermore, it is worth noting that although both \(\beta\)-scorpion toxin and JZTX-III inhibit channel activation, they cause a shift of the voltage dependence in different directions, implying that these toxins do not overlap the same active residues at site 4 of the VGSC protein. Thus, JZTX-III hopefully represents a useful probe for discriminating rat cardiac TTX-R VGSC isoform, although it has a lower affinity (\(\text{IC}_{50} < 0.4 \mu\)M).

Naturally occurring toxin determinants are helpful for insight into the underlying mechanism of peptides responding to distinct receptors. NMR structures of hainantoxin-I (HNTX-I) and ProTxOs reveal that a hydrophobic patch formed by Phe, Tyr, Trp, and Val acts as an ion channel binding site anchor and charged residues can be responsible for their pharmacological specificity (17, 21). Sequence alignment in Fig. 4A indicates that JZTX-III shows limited sequence identities with other sodium channel toxins (e.g., HNTX-I and ProTxOs). However, interestingly, several hydrophobic residues (Phe\(^7\), Tyr\(^22\), Trp\(^30\), and Val\(^33\)) in JZTX-III are strictly conserved at the corresponding positions in other sodium channel toxins. HNTX-IV is a potent blocker of neuronal TTX-S VGSC in DRG neurons with an \(\text{IC}_{50}\) value of 44.6 nm (18). Substitutions of Lys\(^{27}\) or Arg\(^{29}\) with Ala reduce HNTX-IV sensitivity of TTX-S VGSC in DRG neurons by over 10-fold.\(^2\) The 2 positive residues are also conserved in HWTX-IV, ProTxOs, and HNTX-I, which are proved to inhibit \(\text{Na}_{1.2}\), whereas they are missing in JZTX-III. It is likely that the 2 residues may be responsible for binding \(\text{Na}_{1.2}\) but not \(\text{Na}_{1.5}\). JZTX-III has 8 charged residues, and most of them, except for Asp\(^7\) and Arg\(^{13}\), can be found at corresponding positions in neurotoxins. From the listed sequences, it is still difficult to infer the crucial residues responsible for Nav1.5, but we can assume that Asp\(^7\) and Arg\(^{13}\) may result in the subtle difference in pharmacological characterization between JZTX-III and other toxins.

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REFERENCES
