Huwentoxin-X (HWTX-X) is a novel peptide toxin, purified from the venom of the spider Ornithoctonus huwena. It comprises 28 amino acid residues including six cysteine residues as disulfide bridges linked in the pattern of I–IV, II–V, and III–VI. Its cDNA, determined by rapid amplification of 3’ and 5’ cDNA ends, encodes a 65-residue prepropeptide. HWTX-X shares low sequence homology with ω-conotoxins GVIA and MVIIA, two well known blockers of N-type Ca\(^{2+}\) channels. Nevertheless, whole cell studies indicate that it can block N-type Ca\(^{2+}\) channels in rat dorsal root ganglion cells (IC\(_{50}\) 40 nM) and the blockage by HWTX-X is completely reversible. The rank order of specificity for N-type Ca\(^{2+}\) channels is GVIA ≈ HWTX-X > MVIIA. In contrast to GVIA and MVIIA, HWTX-X had no detectable effect on the twitch response of rat vas deferens to low frequency electrical stimulation, indicating that HWTX-X has different selectivity for isoforms of N-type Ca\(^{2+}\) channels, compared with GVIA or MVIIA. A comparison of the structures of HWTX-X and GVIA reveals that they not only adopt a common structural motif (inhibitor cystine knot), but also have a similar functional motif, a binding surface formed by the critical residue Tyr, and several basic residues. However, the dissimilarities of their binding surfaces provide some insights into their different selectivities for isoforms of N-type Ca\(^{2+}\) channels.

Ca\(^{2+}\) entry into cells through voltage-gated Ca\(^{2+}\) channels mediates many physiological processes including neurotransmitter release, neurosecretion, neuronal excitation, survival of neurons, and regulation of gene expression. Currently, five main types of Ca\(^{2+}\) channels (T, L, N, P/Q, and R) in vertebrate cells have been defined by their physiological and pharmacological properties (1–3). Among the multiple types of Ca\(^{2+}\) channels, N-type Ca\(^{2+}\) channels are sensitive to ω-conotoxin GVIA that is derived from the sea snail Conus geographus, and are concentrated at presynaptic nerve terminals, regulating the influx of Ca\(^{2+}\) necessary for neurotransmitter release in both the central and peripheral nervous systems (4–6). Several different isoforms of N-type Ca\(^{2+}\) channels have now been cloned and functionally characterized (7, 8). Pharmacological and gene knock-out studies implicate N-type Ca\(^{2+}\) channels as key mediators of nociceptive signaling in dorsal root ganglion (DRG) cells, and therefore as potential targets for the development of analgesic drugs (9). Indeed, ziconotide, a synthetic analog of ω-conotoxin MVIIA that is also a specific N-type Ca\(^{2+}\) channel blocker isolated from the venom of the marine snail Conus magus, has received approval for severe chronic pain resistant to other procedures (10, 11). Another more selective blocker of N-type Ca\(^{2+}\) channels, ω-conotoxin CVID isolated from C. magus, is currently in Phase II clinical trials in Australia. It is hoped that this peptide may overcome some of the side effects associated with MVIIA use (12–15).

The most specific blockers of N-type Ca\(^{2+}\) channels known to date are isolated from the venoms of cone snails. Only a few peptide toxins from other animals have been reported to be able to act specifically on this type of channels, such as Ptu1, isolated from the venom of assassin bugs Peirates turpis (16), and HWTX-I, isolated from the venom of spider Ornithoctonus huwena (17). It is well known that spiders are among the oldest animals on the earth. There are 38,000 described spider species, with at least a similar number uncharacterized. A very conservative estimate of 20 pharmacologically distinct peptides per species leads to an estimated total of ~1.5 million spider venom peptides, which is much larger than the ~50,000 peptides estimated to be present in venoms of cone snails (18). Thus it is reasonable to believe that the spider venoms might contain a number of peptide probes that are more specific for isoforms of N-type Ca\(^{2+}\) channels. These peptides might aid to discriminate among their isoforms and would become ideal drug candidates for treatment of N-type Ca\(^{2+}\) channel-related disorders.

The Chinese bird spider, O. huwena, is found mainly in the hilly areas of Yunnan and Guangxi provinces in the south of China (19). More than 10 peptide toxins have been isolated from this spider venom, including the N-type Ca\(^{2+}\) channel inhibitor (HWTX-I) (17), insecticidal neurotoxins (HWTX-II, HWTX-VII, and HWTX-VIII) (20, 21), tetrodotoxin-sensitive Na\(^{+}\) channel blocker (HWTX-IV) (22), the smallest lectin-like peptide (SHL-I) (23), and others. In the present study, we report the isolation and characterization of huwentoxin-X (HWTX-X) from the venom of O. huwena, a novel specific blocker of N-type Ca\(^{2+}\) channels in rat DRG cells. cDNA sequencing by rapid amplification of the 3’ and 5’ cDNA ends (RACE) method indicates HWTX-X is initially expressed as a prepropeptide, an expression pattern similar to other spider peptide toxins and conotoxins. The determination of the solution structure of HWTX-X by two-dimensional \(^1\)H NMR with distance geometry and simulated annealing reveals that HWTX-X adopts the same inhibitor cystine knot motif seen in ω-conotoxins (e.g. GVIA or MVIIA).

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The atomic coordinates and structure factors (code 1Y29) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2. The abbreviations used are: DRG, dorsal root ganglion; HWTX-X, huwentoxin-X; RACE, rapid amplification of cDNA ends; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; HVA, high voltage-activated; HOBt/TBTU/NMM, hydroxybenzotriazole monohydrate/2-(1H-benzotriazol-1-yl)-1,2,3-tetramethyl-uronium tetrafluoroborate/N-methylmorpholine.
**EXPERIMENTAL PROCEDURES**

**Materials and Animals**—Kunming albino mice and Sprague-Dawley rats were purchased from the Xiayga School of Medicine, Central South University. Cockroaches were from our laboratory stock colonies. All sequencing reagents were purchased from Applied Biosystems (Foster City, CA). The 3′ and 5′ RACE kits and TRIZol reagent were purchased from Invitrogen. Restriction enzymes, Taq DNA polymerase, and pGEMT Easy Vector system were from Promega. All synthesis reagents were purchased from Chemassist Corp. Trifluoroacetic acid and α-cyano-4-hydroxy cinamic acid were from Sigma. All other reagents are analytical grade.

**Toxin Purification**—The venom was obtained by electrical stimulation of female spiders, and the freeze-dried crude venom was stored at −20° C prior to analysis. Lyophilized venom, dissolved in double-distilled water, was applied onto a reverse-phase high performance liquid chromatography (RP-HPLC) Vydac C18 column (300 A, 4.6 × 250 mm) using a Waters Alliance system. Venom components were eluted using a linear acetonitrile gradient (0–60% acetonitrile, 0.1% trifluoroacetic acid in 60 min) at a flow rate of 1.0 ml/min. Elution of peptides was monitored at 215 nm.

**Mass Spectrometry**—The molecular masses of peptides were determined using MALDI-TOF MS (Applied Biosystems, Voyager-DE STR Biospectrometry work station). Ionization was achieved by irradiation with a nitrogen laser (337 nm), with a 20-kV acceleration voltage. α-Cyano-4-hydroxycinnamic acid was used as matrix. Prior to each analysis in the reflection mode, the masses were calibrated internally using HWTX-I (MH+, 3751.45 Da).

**Amino Acid Sequencing by Automated Edman Degradation**—The native or carboxymethylated peptide was submitted to automatic N-terminal sequencing on an Applied Biosystems model 491 gas-phase sequencer. Edman degradation was performed with a normal automatic cycle program.

**HWTX-X cDNA Isolation and Characterization**—The full-length HWTX-X cDNA was obtained using the RACE method as described previously (24). Briefly, 5 μg of mRNA, extracted from the venomous glands of the spider O. huwena, was taken to convert mRNA into cDNA by using the 3′ RACE kit supplied with Superscript II reverse transcriptase and a universal adapter primer (5′-GGCCACCGC- TCCAGTATGC(dT)3′). The cDNA was then used as a template for PCR amplification using the abridged universal adapter primer (5′- CGAAAGCTTGGCCACCGGTCGACTAGTAC-3′) and the gene specific primer (5′-GG(A/G/C/T)AA(A/G)CC(A/G/C/T)TG(C/T)TA(C/T)GG(A/G/C/T)-3′) designed corresponding to the N-terminal residues (Gly6-Lys-Pro-Cys-Tyr-Gly11) of HWTX-X. Based on the partial cDNA sequence of HWTX-X determined by 3′ RACE, an antisense primer was designed and synthesized for 5′ RACE. With the strategy described by the RACE kit supplier, the 5′-end cDNA of HWTX-X was cloned by using the gene-specific primer (5′-GTGAACACACTCCGGCTCGAGAC-3′) and nested primer, respectively. The amplified products were then precipitated and cloned into the pGEM-T EASY vector for sequencing.

**Peptide Synthesis, Folding, and Purification**—HWTX-X was synthesized using an Fmoc (N-(9-fluorenylethoxycarbonyl) tert-buty l strategy and HOBt/TBTU/NMM coupling method on an automatic peptide synthesizer (PerSeptive Biosystems) (25). The crude linear peptide was diluted to a final concentration of 30 μM by 0.1 M Tris-HCl solution (pH 8.0) containing 5 mM reduced glutathione and 0.5 mM oxidized glutathione. The solution was stirred slowly at room temperature for 24 h and the folding reaction was monitored by RP-HPLC and MALDI-TOF MS. The oxidized product was purified by semiprepara-

tive RP-HPLC using a 40-min linear acetonitrile gradient (10–30% acetonitrile, 0.1% trifluoroacetic acid) on a column (C18, 1.0 × 25 cm) at 3 ml/min flow rate. The purity of the synthetic HWTX-X was confirmed by analytical RP-HPLC and MALDI-TOF MS.

**Biological Assays**—The toxicity of HWTX-X was quantitatively assayed by intraperitoneal injection into 18–20-g mice of both sexes and intra-abdominal injection into adult male cockroaches (Periplaneta americana) with body weights of 0.3–0.5 g using 20-μl solutions (in 0.9% (w/v) normal saline). Vas deferens were performed according to the method of Liang (26). Briefly, adult male Sprague-Dawley rats were killed by CO2 anesthesia followed by decapitation. Vas deferentia were mounted in 5-ml organ baths, with the top of each tissue attached to an isometric force transducer and the bottom attached to a movable support and straddled with platinum stimulating electrodes. The vasa were immersed in Krebs solution and stretched by a passive force of about 10 millinewtons. After an equilibration period of 30 min with frequent changes of medium, the vasa were stimulated with single electrical field pulses (30 V, 0.1 ms duration) every 20 s. The resulting twitch responses mediated by sympathetic nerves were recorded on a chart recorder (RM6240B).

**Electrophysiological Assays**—Acutely dissociated DRG were prepared from 4-week-old Sprague-Dawley rats and maintained in short-term primary culture using the method described by Hu et al. (27). The dissociated cells were suspended in essential Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cells were plated on a poly-L-lysine-coated dish (35 × 10 mm) and incubated at 37 °C in an atmosphere of 5% CO2. Cells in culture for 3–24 h were used in the patch experiments. Experiments were conducted at room temperature (20–25 °C).

**Cell current recordings** were made with the whole cell patch clamp technique using an EPC-9 patch clamp amplifier (HEKA Electrond, Lambrecht, German). Voltage steps and data acquisition were controlled using a PC computer with software Pulsefit+ Pulse 8.0 (HEKA Electronics, Lambrecht, Germany). The patch pipettes with DC resistances of 2–3 MΩ were fabricated from borosilicate glass tubing (VWR micropipettes, 100 μl, VWR Company) using a two-stage vertical micropipette puller (PC-10, Narishige, Japan) and fire-polished by a heater (Narishige, Japan). Data were sampled at 10 KHz and filtered at 3 KHz. Under the voltage clamp 70–80% series resistance compensation was applied.

Ca2+ channel currents (I_{Ca}, -) were measured using Ba2+ as a charge carrier. For these experiments, external solutions contained (in mM): 160 triethanolamine-CI, 10 HEPES, 2 BaCl2, 10 glucose, and 200 nM tetrodotoxin, adjusted to pH 7.4 with triethanolamine-OH. The internal solution contained (in mM): 120 CsCl, 5 Mg-ATP, 0.4 Na2-GTP, 10 EGTA, 20 HEPES-CsOH (pH 7.2) (13–15). I_{Ca} was evoked at −50 or 0 mV from a holding potential of −90 or −40 mV. HWTX-X, ω-conotoxin GVIA, MVIIA, nifedipine, and NiCl2 were applied to the extracellular environment by low pressure ejection from a blunt pipette positioned about 50–100 μm away from the cell being recorded. Data were given as mean ± S.E. and statistical significance (p < 0.05) was determined using a paired or independent Student’s t test as appropriate.

**NMR Spectroscopy of HWTX-X**—The NMR sample was prepared by dissolving HWTX-X in 450 μl of 20 mM deuterium sodium acetate buffer (H2O/D2O, 9.1, v/v) containing 0.002% NaN3 and 0.01 mM EDTA with a final concentration of 6 mM HWTX-X and a pH of 4.0. Sodium 3-(trimethylsilyl) propionate-2,3,3-d4 was added to a final concentration of 200 μM as an internal chemical shift reference. For experiments in D2O, the sample used in H2O experiments was lyophilized and then.
Purification Characterization and Synthesis of HWTX-X—Crude venom from the spider O. huwena was fractionated by RP-HPLC on a VydyC C18 column (Fig. 1A). More than 20 peaks were observed in the chromatogram. The sharp peak labeled HWTX-X, eluted at 13 min at a point in the gradient of about 18% acetonitrile, 0.1% trifluoroacetic acid, containing 0.5% acetonitrile, 0.1% trifluoroacetic acid and 0.2% triethylamine, was a major product as revealed by RP-HPLC and MALDI-TOF spectrometry (Fig. 1, inset). The purified product was homogeneous on the analysis by reverse-phase HPLC, and its mass (2931.45 Da) was in good accordance with the theoretical mass for the oxidized analog. Furthermore, the synthetic HWTX-X was able to block N-type Ca\textsuperscript{2+} channels in DRG cells similarly to the native peptide as determined by the patch clamp technique. Because the synthetic HWTX-X seemed to be structurally and functionally identical to the native toxin, it might be used for the further studies.

The cDNA Sequence of HWTX-X—The full-length cDNA sequence of HWTX-X was obtained by overlaying two fragments from the 5' and 3' RACE. As shown in Fig. 3, the oligonucleotide sequence of the cDNA is 486 bp comprising the 5'-untranslated region, the open reading frame, and the 3'-untranslated region. The open reading frame encodes a 65-residue peptide corresponding to the precursor of HWTX-X, which contains a signal peptide of 20 residues, a prepropeptide of 17 residues, and a mature peptide of 28 residues. The prepropeptide of the HWTX-X precursor shows limited sequence identity with those of other reported precursors. Different from most \omega-conotoxins, HWTX-X has no extra amino acid Gly or Gly\textsubscr{16} or Arg\textsubscr{26} (HWTX-X numbering) at its C-terminus, known to allow "post-modification" of \alpha-amidation at the C-terminal residue (49), implying that the C-terminal residue of mature toxin is not amidated.

Biological Assays—HWTX-X elicited no toxic symptoms in either vertebrates or invertebrates during a period of 48 h post-injection, when it was assayed in vivo by direct injection into mice and cockroaches. \omega-Conotoxins (e.g. GVIA, MVIIA, and CVID) are known to block the twitch response to low frequency electrical nerve stimulation in rat vas deferens (3, 32). Because of the similarities between HWTX-X and these \omega-conotoxins, similar pharmacological experiments were carried out to test the effect of HWTX-X on the twitch response of vas deferens during 30 min, but 1 \mu M GVIA or MVIIA caused a rapid reduction of the twitch response, in agreement with previous studies (3, 32) (data not shown).

Effect of HWTX-X on Ca\textsuperscript{2+} Channels in Rat DRG Cells—It is widely accepted that rat DRG cells exhibit two categories of voltage-gated Ca\textsuperscript{2+} channels: low voltage-activated channels (T-type) and high voltage-activated (HVA) channels (N-, L-, P/Q-, and R-types), which can be discriminated by their voltage dependence and kinetics. Low voltage-activated currents can be elicited by a 100-ms step depolarization.
tion to −50 mV from a holding potential \( V_h \) of −90 mV, whereas only HVA currents are activated if the cell is depolarized from a \( V_h \) of −40 to 0 mV (33–36). Thus, rat DRG cells are appropriate for investigating the effect of HWTX-X on voltage-gated \( \text{Ca}^{2+} \) channels. As shown in Fig. 4A, 10 \( \mu \text{M} \) HWTX-X had no discernible effect on T-type \( \text{Ca}^{2+} \) channels, but it could eliminate 41.4 ± 4.1% of the HVA currents (Fig. 4B). From the current-voltage (I-V) curves of the HVA currents (Fig. 4C), it was found that HWTX-X caused no change on the initial activated voltage, the active voltage of peak inward current, and the reversal potential of the HVA currents.

The HVA currents in rat DRG cells were described as mainly N-type (sensitive to GVIA) and L-type (sensitive to nifedipine) (34). In this study, of the HVA currents, 41.7 ± 3.7% (N-type currents) could be blocked by a saturating concentration of 4 \( \mu \text{M} \) GVIA, whereas 40 ± 5.4% (L-type currents) could be reduced by 10 \( \mu \text{M} \) nifedipine and then the remaining currents (P/Q- and R-types) were abolished by 25 \( \mu \text{M} \) \( \text{Ni}^{2+} \). In the presence of 4 \( \mu \text{M} \) GVIA, 10 \( \mu \text{M} \) HWTX-X had no additional effect on the HVA currents, whereas the addition of nifedipine could inhibit another fraction of the currents that remained, and the currents resistant to these were finally eliminated by adding \( \text{NiCl}_2 \) (Fig. 4D). Analogously, after blockage by HWTX-X, GVIA could not cause further blockage (Fig. 4E). These results indicated that HWTX-X could specifically block the GVIA-sensitive, N-type \( \text{Ca}^{2+} \) currents in rat DRG cells.

When HWTX-X and MVIIA were compared, we found that after pretreatment with 10 \( \mu \text{M} \) HWTX-X, 3.2 \( \mu \text{M} \) MVIIA continued to block a small proportion of the remaining currents (P/Q- and R-types) were abolished by 25 \( \mu \text{M} \) \( \text{Ni}^{2+} \). In the presence of 4 \( \mu \text{M} \) GVIA, 10 \( \mu \text{M} \) HWTX-X had no additional effect on the HVA currents, whereas the addition of nifedipine could inhibit another fraction of the currents that remained, and the currents resistant to these were finally eliminated by adding \( \text{NiCl}_2 \) (Fig. 4D). Analogously, after blockage by HWTX-X, GVIA could not cause further blockage (Fig. 4E). These results indicated that HWTX-X could specifically block the GVIA-sensitive, N-type \( \text{Ca}^{2+} \) currents in rat DRG cells.
Based on the specificity of HWTX-X, GVIA was used to isolate the N-type currents in DRG cells. In each cell tested, 4 μM GVIA was finally added to produce maximal blockage of N-type currents, and then the proportion of blockage by HWTX-X was normalized. HWTX-X could block N-type currents in a dose-dependent manner, which yielded an IC\textsubscript{50} value of about 40 μM (Fig. 5A). Analysis of the dose-response data also revealed a 1:1 binding association of HWTX-X with the channels.

The rate of onset of 10 μM HWTX-X blockage was rapid (17.4 + 1.1 s), but it was relatively slower than those of GVIA and MVIIA (12.9 ± 0.3 s). The blockage by HWTX-X could be reversed by washing, with a recovery of 90% of the control currents within 2 min, whereas the currents blocked by MVIIA were recovered only to ~40% upon a 4-min wash. By contrast, there was little recovery from GVIA blockage of N-type currents (Fig. 5B). 4 μM GVIA could produce additional blockage of N-type currents after pretreatment with 100 nM HWTX-X, but the rate of blockage by GVIA was significantly slowed (τ\text{off} = 37 ± 3 s) (Fig. 5C). This raised the possibility that the slow blockage was because of the slow replacement of the reversible blocker by the irreversible toxin, suggesting therefore that the two toxins may be directly competing for overlapping (or partially overlapping) binding sites of N-type channels. The effect of HWTX-X on voltage-gated Na\textsuperscript{+} and K\textsuperscript{+} channels was also assessed in rat DRG cells according the method described by Liu et al. (37), which indicated that 10 μM HWTX-X had no effect on the two channels (data not shown).

Disulfide Determination and Structure Calculations of HWTX-X—Sequence-specific resonance assignments were performed according to the standard procedures established by Wüthrich (38). All of the backbone protons and more than 95% of the side chain protons were identified. The four Pro residues (Pro4, Pro5, Pro8, and Pro17) were assigned clearly by the strong sequential H\textsuperscript{α}-H\textsuperscript{α} cross-peaks for Xxx-Pro, which also indicated the presence of trans-peptide bonds for these residues.

As described above, the six Cys residues of HWTX-X are involved in three disulfide bridges. In the absence of chemical characterization, the disulfide linkage of HWTX-X was first speculated from its identical Cys...
residues motif with those of ω-conotoxins. Furthermore, these covalent links could also be identified from the His–His and His–His NOE contacts observed between the Cys residues involved in the linkage. The NOESY spectra (mixing time of 200 ms) of HWTX-X allowed us to detect several inter-cystinyl NOE cross-peaks (HA Cys$_{2}$–HB Cys$_{19}$, HB Cys$_{18}$–HA Cys$_{27}$, and HB Cys$_{18}$–HB Cys$_{27}$), and therefore to identify unambiguously the two disulfide bridges: Cys$_{2}$–Cys$_{19}$ and Cys$_{18}$–Cys$_{27}$ in which the Cys$_{18}$–Cys$_{27}$ disulfide bridge is formed by three disulfide bridges, Cys$_{2}$–Cys$_{19}$, Cys$_{18}$–Cys$_{22}$, and Cys$_{18}$–Cys$_{27}$, and therefore to identify unambiguously the two disulfide bridges: Cys$_{2}$–Cys$_{19}$ and Cys$_{18}$–Cys$_{27}$. The third disulfide bridge (Cys$_{2}$–Cys$_{19}$) could then be deduced indirectly as the only one possible. Accordingly, disulfide linkage was determined as the I–IV, II–V, and III–VI pattern, which is frequently found in a variety of toxic and inhibitory peptides from biologically diverse sources (39, 40).

The structure of HWTX-X was determined by using 261 intramolecular distance constraints, 10 dihedral constraints as well, as nine distance constraints derived from the three disulfide bridges. Altogether, the final experimental set corresponded to 10 restraints per residue on average. A family of 20 accepted structures with lower energies and better Ramachandran maps were selected to represent the three-dimensional solution structure of HWTX-X. The structures have no distance violations greater than 0.3 Å and no dihedral violations greater than 5.0°. They have favorable non-bonded contacts as evidenced by the low values of the mean Lennard-Jones potentials and good covalent geometry as indicated by the small deviations from ideal bond lengths and bond angles. Analysis of the structures in PROCHECK shows that 77.4% of non-Pro, non-Gly residues lie in the most favored regions of the Ramachandran plot with a further 20.8% in additionally allowed regions. The best fit superposition of the backbone atoms (N, C$_{α}$, and C) for the 20 converged structures of HWTX-X gives an average root mean square deviation with respect to mean structure values of 0.93 ± 0.25 Å for backbone atoms and 1.67 ± 0.28 Å for all heavy atoms. The N-terminal residue (Lys$_{1}$) and loop 2 region (Gly$_{11}$–Pro$_{17}$) show some apparent deviations among the obtained structures. For the remaining part of the structures (2–10 and 18–28), average root mean square deviation values were 0.57 ± 0.13 Å for backbone and 1.25 ± 0.23 Å for heavy atoms.

**Structure Description of HWTX-X**—A sausage representation of the backbone atoms of the 20 best converged structures of HWTX-X is shown in Fig. 6A. The three-dimensional structure of HWTX-X (Protein Data Bank code 1Y29) contains a compact disulfide-bonded core, from which four loops emerge (Leu$_{1}$–Pro$_{6}$, Tyr$_{10}$–Pro$_{17}$, Gly$_{20}$–Val$_{23}$, and Ser$_{25}$–Thr$_{29}$) as well as N and C termini. Fig. 6B shows the ribbon representation of the secondary structure in HWTX-X. The main element of the secondary structure is a short triple-stranded antiparallel β-sheet formed by strands Lys$_{5}$–Pro$_{6}$, Cys$_{12}$–Ser$_{13}$, and Lys$_{26}$–Cys$_{27}$, which are stabilized by the three disulfide bridges. Of the 28 residues of HWTX-X, only the side chains of the six Cys residues are buried, all the others are solvent accessible, and their conformations are constrained by steric interactions.

HWTX-X adopts an inhibitor cystine knot motif commonly observed in other toxic and inhibitory peptides (39, 40). The cystine knot in HWTX-X is formed by three disulfide bridges linked as Cys$_{2}$–Cys$_{19}$, Cys$_{18}$–Cys$_{22}$, and Cys$_{18}$–Cys$_{27}$, in which the Cys$_{18}$–Cys$_{27}$ disulfide bridge passes through a 12-residue ring formed by the intervening polypeptide backbone and the Cys$_{18}$–Cys$_{22}$ disulfide bridge. A comparison of the structures of HWTX-X and GVIA highlights a common fold adopted by both peptides (Fig. 6B) (41). The structural alignment by using the combinational extension (CE) method shows the root mean square deviation value between the structures of HWTX-X and GVIA as 2.1 Å. Similarly, they both contain a cystine knot and a triple-stranded, anti-parallel β-sheet. However, because HWTX-X has similar loop sizes with GVIA (Fig. 2), structural comparison between them also reveals some significant difference, most notably in loop 2. HWTX-X has a larger loop 2, and therefore the two additional residues in loop 2 make this loop structurally more undefined and Lys$_{1}$ protruding from the surface of HWTX-X in this loop (the solvent accessible surface of this residue is more than 68%).

**DISCUSSION**

In the present study, we have isolated a novel specific blocker of N-type Ca$^{2+}$ channels from the venom of *O. huwena*, which was named HWTX-X. HWTX-X contains 28 amino acid residues, which is the smallest peptide among the huwentoxins so far isolated; it is also similar in size to ω-conotoxins (24–29 residues). This peptide was chemically synthesized and folded to be indistinguishable from the native one. In analogy to ω-conotoxins, HWTX-X contains three disulfide bridges, and is basic. Inspection of the sequences shown in Fig. 2 reveals a con-
HWTX-X, A Specific Blocker of N-type Calcium Channels

FIGURE 6. A, a sausage representation of the backbone atoms of the 20 best converged structures of HWTX-X. B, Richardson-style diagrams of the backbone folding of HWTX-X and GVIA. The β-sheet is shown in red, and three disulfide bonds of each molecule are indicated in yellow. C, repartition of basic residues (in blue) on the structures of HWTX-X and GVIA. The critical aromatic residue is shown in purple. D, CPK representation of HWTX-X and GVIA shows seven putative residues in the binding surfaces of both peptides.

served cysteine framework and two conserved residues, Gly9 and Lys22/Arg26 (HWTX-X numbering), although HWTX-X shows low sequence homology (<32%) with ω-conotoxins other than SVIA. Compared with ω-conotoxins, HWTX-X has a larger loop 2, which demonstrates significant structural differences among those peptides. Cloning and sequencing of the mRNA transcripts from the venom duct of this spider confirmed that HWTX-X is expressed in this tissue. Similarly to other spider peptide toxins and ω-conotoxins, HWTX-X is expressed as a prepropeptide that is post-translationally processed to yield the mature toxin. In contrast to ω-conotoxins GVIA, MVIIA, and CVID, the C terminus of HWTX-X is not amidated.

Previous studies have shown that GVIA and MVIIA are potential blockers of N-type Ca2+ channels in various species and nervous tissues by physical occlusion of the pore of their receptors. Consequently, GVIA and MVIIA have become commonly used tools for isolation and identification of native N-type Ca2+ channels (4–6). Our studies in rat DRG cells demonstrate that HWTX-X can specifically block the HVA Ca2+ currents through GVIA-sensitive, N-type Ca2+ channels, whereas it has no detectable effect on the other Ca2+ channels, or on the voltage-gated Na+ and K+ channels. From the current-voltage (I–V) curves of the HVA currents, it has been found that HWTX-X can only suppress peak currents without affecting the kinetics of N-type Ca2+ channels, which distinguishes HWTX-X from ω-aga-IVA, ω-Aga-IVA, a peptide isolated from the venom of the funnel web spider Agelenopsis aperta, inhibits Ca2+ channels by altering the voltage dependence of gating (42, 43). In the present study, the dose-response data indicates that HWTX-X blocks N-type Ca2+ channels in a 1:1 manner. Pretreatment of HWTX-X also affects the time course of the subsequent blockage by GVIA, suggesting that HWTX-X should share overlapping (or partially overlapping) binding sites with GVIA. However, HWTX-X is distinct from GVIA or MVIIA in several aspects. MVIIA partially blocks HWTX-X-resistant Ca2+ currents, but GVIA does not, which indicates that HWTX-X have specificity similar to GVIA, but it should be more specific than MVIIA for N-type channels. In comparison, GVIA is a poorly reversible blocker and the blockage by MVIIA is only partially recovered, whereas the blockage by HWTX-X is nearly completely reversible. In addition, HWTX-X cannot affect the twitch response of rat vas deferens, whereas GVIA and MVIIA can. It has been reported that GVIA-sensitive, N-type Ca2+ channels mediated noradrenergic release in vas deferens at low frequency electrical stimulation (3). Thus, it appears that these N-type Ca2+ channels in vas deferens are resistant to HWTX-X, and HWTX-X would be a useful tool to discriminate these isoforms of N-type Ca2+ channels.

The structure and function relationships of GVIA have been widely analyzed by using Ala-scanning mutagenesis (32, 44–46). All those studies have shown that Tyr13 in loop 2 is the most critical residue for the binding to N-type Ca2+ channels. Also, several other residues including Lys2, Arg17, Tyr27, and Lys29 have been identified to have a secondary effect on potency. Additional residues with less importance are Hyp10, Thr11, Asn20, Arg25, and Tyr27. On the other hand, utilizing a chimeric combined with site-directed mutagenesis, Ellinor and co-workers (47) demonstrated that the large putative extracellular loop between IISS and IIIH5 was critically important for the block of N-type Ca2+ channels by GVIA. In particular, residues Gln1327, Glu1334, Glu1337, and Gln1339 of this region were identified as being important for blockage by GVIA, with Glu1337 having the largest effect (47). Furthermore, Feng et al. (48) found two additional residues, Gln1326 and Glu1332, to be important determinants for GVIA blockage. Therefore, it is proposed that the complex between N-type Ca2+ channels and GVIA involves the critical aromatic residue (Tyr13) as well as several basic residues that make hydrogen bonds and salt bridges with the corresponding residues located in the pore region of N-type Ca2+ channels (30).

HWTX-X shares the same pharmacological specificity and action mechanism in rat DRG cells as GVIA and therefore might be expected to present similar interacting surface profiles. The determination of the three-dimensional structure of HWTX-X would be helpful to elucidate this point. HWTX-X adopts a common structure fold to GVIA, but it is widely accepted that this overall three-dimensional scaffold generally provides little insight into bioactivities. The cystine knot simply provides the structure framework on which the bioactivity related residues are grafted. Therefore, the topological distribution of the key functional residues of GVIA, regardless of the three-dimensional scaffold, may be instructive for mapping the bioactive surface of HWTX-X. Molecular surface analysis of GVIA reveals that the interacting surface of GVIA encompasses the crucial aromatic residue (Tyr13) surrounded by some basic residues (Lys9, Arg17, Lys24, and Arg26) (Fig. 6C), as described by Bernard et al. (30). Such a functional motif is also found in HWTX-X, in which the aromatic residue is Tyr11, and the basic
residues are Lys1, Lys7, Lys15, and Lys24. Like Tyr13 of GVIA, residue Tyr10 of HWTX-X extrudes from the molecular surface (the solvent accessible surface of Tyr10 is about 52.4%, whereas that of Tyr13 of GVIA is about 59.4%) and is located in the center of the interacting surface (Fig. 6, C and D). However, HWTX-X and GVIA are different in some other respects, which highlights some important differences in their bioactive surfaces. These key residues are not in homologous positions when the amino acid sequences are aligned. For example, Tyr13 of GVIA is at the beginning of loop 2, whereas Tyr10 of HWTX-X is at the beginning of the same loop (Fig. 2), which results in reverse spatial orientation of those residues. In addition, HWTX-X lacks the two additional important binding residues Hyp10 and Thr11 that are close to Tyr13 in GVIA. It has been proposed that Pro8 and Asn25 could compensate for them, but they occupy different spatial positions (Fig. 6D). Therefore, similar functional motifs of HWTX-X and GVIA may indicate that they should interact with the same macrosite in N-type Ca2+ channels, their differences may involve the different selectivity of their binding surfaces, which highlights some important differences in their bioactivities. In summary, HWTX-X specifically blocks GVIA-sensitive, N-type Ca2+ channels in rat DRG cells. Its non-toxic effects, specificity, and compensating for them, but they occupy different spatial positions (Fig. 6D). Therefore, similar functional motifs of HWTX-X and GVIA may indicate that they should interact with the same macrosite in N-type Ca2+ channels, their differences may involve the different selectivity of their binding surfaces, which highlights some important differences in their bioactivities. In summary, HWTX-X specifically blocks GVIA-sensitive, N-type Ca2+ channels in rat DRG cells. Its non-toxic effects, specificity, and compensating for them, but they occupy different spatial positions (Fig. 6D). Therefore, similar functional motifs of HWTX-X and GVIA may indicate that they should interact with the same macrosite in N-type Ca2+ channels, their differences may involve the different selectivity of their binding surfaces, which highlights some important differences in their bioactivities. In summary, HWTX-X specifically blocks GVIA-sensitive, N-type Ca2+ channels in rat DRG cells. Its non-toxic effects, specificity, and

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REFERENCES

1. Catterall, W. A. (2000) Cell. Dev. Biol. 16, 521–555
2. Hofmann, F., Biehl, M., and Floercker, V. (1994) Annu. Rev. Neurosci. 17, 399–418
3. Olivera, B. M. (1994) Annu. Rev. Biophys. 23, 823–867
4. Oliveira, R. M., Gray, W. R., Zemisk, R., McIntosh, J. M., Varga, J., Rivier, J., Santos, V., and Cruz, L. J. (1985) Science 230, 1338–1343
5. Hertzig, A., Hernandez, J. F., Gagnon, J., Hong, T. T., Pharm, T. N., Nguyen, T. M., Le-Nguyen, D., and Chiche, L. (2001) Biochemistry 40, 7973–7983
6. Lew, M. J., Finn, J. P., Pallaghy, P. K., Murphy, R., Whorlow, S. L., Wright, C. E., Horton, R. S., and Angus, S. J. (1997) J. Biol. Chem. 272, 12014–12023
7. Lu, S. Y., Liang, S. P., and Gu, X. C. (1999) J. Biol. Chem. 18, 609–617
8. Diao, J., Lin, Y., Tang, J., and Liang, S. (2003) J. Magn. Res. 160, 66–74
9. Bernard, C., Corzo, G., Molabah, A., Nakajima, T., and Darbon, H. (2001) Biochemistry 40, 12795–12800
10. Ramilo, C., Zafarriola, G. C., Nadado, L., Hammerland, L. G., Yoshikami, D., Gray, W. R., Kristat, R., Ramachandran, J., Miljanich, G., Olivera, B. M., and Cruz, L. J. (1992) Biochemistry 31, 9919–9926
11. Lew, M. J., Finn, J. P., Pallaghy, P. K., Murphy, R., Whorlow, S. L., Wright, C. E., Horton, R. S., and Angus, S. J. (1997) J. Biol. Chem. 272, 12014–12023
12. Scroggs, R. S., and Fox, A. P. (1992) J. Physiol. 445, 639–658
13. Miller, R. J. (1987) Science 235, 46–52
14. Miller, R. J. (1992) J. Biol. Chem. 267, 1403–1406
15. Noworny, M. C., Fox, A. P., and Tsien, R. W. (1985) Nature 316, 440–443
16. Zai, D., Diao, J., Chen, Z., Hu, W., Xiao, Y., and Liang, S. (2003) Cell Mol. Life Sci. 60, 972–978
17. Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, John Wiley & Sons, Inc., New York
18. Pallaghy, P. K., Nielsen, K. J., Craik, D. J., and Horton, R. S. (1994) Protein Sci. 3, 1833–1839
19. Gelly, J. C., Gracy, J., Kaas, Q., Le-Nguyen, D., Heitz, A., and Chiche, L. (2004) Nucleic Acids Res. 32, 156–159
20. Mould, J., Yasuda, T., Schroeder, C. I., Beedle, A. M., Doering, C. J., Zamponi, G. W., Adams, D. J., and Lewis, R. J. (2004) J. Biol. Chem. 279, 34705–34714
21. Isabell, M. M., Virginia, J. V., Michael, E. A., and Bruce, P. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6628–6631
22. Sidach, S. S., and Mintz, I. M. (2000) J. Neurosci. 20, 7174–7182
23. Nielsen, K. J., Schroeder, T., and Lewis, R. (2000) J. Mol. Recognit. 13, 55–70
24. Kim, J. I., Takahashi, M., Ogura, A., Kohno, T., Kudo, Y., and Sato, K. (1994) J. Biol. Chem. 269, 23876–23878
25. Nadasdi, L., Yamashiro, D., Chung, D., Tarczyhornoch, K., Adrianiens, P., and Ramachandran, J. (1995) Biochemistry 34, 8076–8081
26. Ellinor, P. T., Zhang, J. F., Horner, W. A., and Tsien, R. W. (1994) Nature 372, 272–275
27. Feng, Z. P., Hamid, J., Doering, C., Bosey, G. M., Snutch, T. P., and Zamponi, G. W. (2001) J. Biol. Chem. 276, 15728–15735
28. Hannon, K., Ma, X., Eliaison, L., Czerwiec, F., Bueir, F., Bueir, B. C., Rorshman, P., and Stenflo, J. (2004) J. Biol. Chem. 279, 32463–32463