The First Gene-encoded Amphibian Neurotoxin**§

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Many gene-encoded neurotoxins with various functions have been discovered in fish, reptiles, and mammals. A novel 60-residue neurotoxin peptide (anntoxin) that inhibited tetrodotoxin-sensitive (TTX-S) voltage-gated sodium channel (VGSC) was purified and characterized from the skin secretions of the tree frog *Hyla annectans* (Jerdon). This is the first gene-encoded neurotoxin found in amphibians. The IC₅₀ of anntoxin for the TTX-S channel was about 3.4 μM. Anntoxin shares sequence homology with Kunitz-type toxins but contains only two of three highly conserved cysteine bridges, which are typically found in these small, basic neurotoxin modules, i.e. snake dendrotoxins. Anntoxin showed an inhibitory ability against tetrodospin with an inhibitory constant (Kᵢ) of 0.025 μM. Anntoxin was distributed in skin, brain, stomach, and liver with a concentration of 25, 7, 3, and 2 μg/g wet tissue, respectively. *H. annectans* lives on trees or other plants for its entire life cycle, and its skin contains the largest amount of anntoxin, which possibly helps defend against various aggressors or predators. A low dose of anntoxin was found to induce lethal toxicity for several potential predators, including the insect, snake, bird, and mouse. The tissue distribution and functional properties of the current toxin may provide insights into the ecological adaptation of tree-living amphibians.

Gene-encoded neurotoxins have been found in most vertebrates including fish, reptiles, and mammals. Many neurotoxins have been characterized such as stonustoxin from fish (*Synanceja horrida*) venoms (1), dendrotoxins from mamba snake (*Dendroaspis*) venoms (2), and *Ornithorhynchus* venom defensin-like peptide (OvDLP) from platypus venoms (3). However, there have been no protein or peptide toxins found in amphibians and birds, except some alkaloid toxins from poison dart frogs (*Dendrobatidae*), *Bufo marinus*, and salamanders (*Salamandra salamandra*) (4, 5). Amphibians, the first group of organisms forming a connecting link between land and water, have been forced to adapt and survive under a variety of conditions laden with pathogens and predators. Amphibian skin plays key roles in every day survival and the ability to exploit a wide range of habitats and ecological conditions. Although amphibian skin is exposed to biological or non-biological injuries such as microorganism infection, parasitization, predation, and physical harm including radiation and aseptic wound (6), amphibians have been endowed with a chemical defense system composed of pharmacological and antimicrobial gene-encoded peptides/proteins (7–9).

High levels of biochemical diversity make amphibian skins attractive subjects for chemical prospecting. Amphibian skin peptides, such as antimicrobial peptides (10–13), tachykinins (14–16), dermorphins (17, 18), cholecystokinin (19), and bradykinins (20–22), which play defensive roles, have been extensively studied in recent decades, although large numbers of pharmacological peptides with defensive functions remain to be identified (12).

Tree frogs spend much more time living on trees than other amphibians, so they may encounter more biotic and abiotic risk factors. A large number and variety of species can prey upon tree frogs, such as mammals, birds, reptiles, insects, spiders, and scorpions. Most tree frogs are green or brown in dorsal coloration that facilitate living in a forest canopy. In addition, there is an excellent chemical defense system in these frogs for maintaining survival. To investigate such chemical defense mechanisms, we purified and characterized a novel neurotoxin peptide from the skin secretions of the tree frog *Hyla annectans*.

**EXPERIMENTAL PROCEDURES**

*Collection of Frog Skin Secretions and Peptide Purification—* Adult *H. annectans* of both sexes (*n* = 150; weight range 3–5 g) were collected in the Yunnan Province of China. Their skins were subjected to a 6-volt electronic stimulation for 3–5 s to collect skin secretions. Skin secretions were dissolved with 0.1 M NaCl solution (containing protease inhibitor mixture, Sigma). All experiments were approved by the Kunming Institute of Zoology, Chinese Academy of Sciences. Peptide purifi-
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cation from the skin secretions was performed by Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration chromatography followed by C₄ reverse phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C₄, 30 × 0.46 cm) as illustrated in Fig. 1. All purifications were traced by trypsin-inhibitory testing. The purified neurotoxin peptide was named anntoxin.

Structure Analysis—The purity of the peptide was subjected to Tricine-SDS-PAGE analysis with a gel concentration of 17.5%, the method described by Schägger and von Jagow (23). The molecular mass was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) using an AXIMA CFR spectrometer (Kratos Analytical) in positive ion and linear mode. Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Assignment of the disulfide bridges was performed as follows. Native peptide (0.1 mg) was digested by endoproteinase Glu-C (34 μg). The hydrolytic fragments of the peptide were recovered by C₁₈ RP-HPLC chromatography and reduced in 20 μl of citrate buffer (1 m, pH 3.0) containing 6 μl guanidine-HCl and 0.05 M Tris (2-carboxyethyl) phosphine (TCEP) at 40 °C for 5 h. The products were purified again by C₁₈ RP-HPLC chromatography. The masses of all recovered fractions were determined by MALDI-TOF mass spectrometry and submitted to Edman degradation sequencing. Details are described under supplemental materials.

SMART cDNA Synthesis and cDNA Cloning—Total RNA was extracted using TRIzol (Invitrogen, Ltd.) from the skin of a single sample of amphibian. cDNA was synthesized by SMART™ techniques using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA) according to the manufacturer’s instruction. Two oligonucleotide primers, S₁ (5′-CA(G/A)/G)GA(T/C)TA(T/C)AG(A/G)TG(T/C)CA(A/G)(T/C)T(AG/ G)T/C)TC(A/G,T/C)-3′, in the sense direction, a specific primer designed according to the amino acid sequence and primer II A provided from the SMART™ PCR cDNA synthesis kit. The DNA polymerase was Advantage polymerase from Clontech. The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. Finally, PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed with an Applied Biosystems DNA sequencer, Model ABI PRISM 377.

Trypsin-inhibitory Testing—The inhibition effects of the peptide isolated upon hydrolysis of synthetic chromogenic substrates (S-2288, H-D-Phe-Pip-Arg-pNA, Kabi Vitrum, Stockholm, Sweden) by trypsin were assayed according to methods previously described (24).

Recombinant Expression and Purification—Escherichia coli BL21(DE3) and plasmid pET-32a (+) were used to express the recombinant anntoxin. A chemical cleavage site, -DDDDD-, is designed upstream to the anntoxin coding sequence, and these codons are in bold. The dipeptide -DP- introduced in the fusion protein is susceptible to formic acid (25), which releases anntoxin from the fusion protein. Details are described under supplemental materials.

Expression Profile Analysis of Tissues by RT-PCR, Western Blotting, and Enzyme-linked Immunosorbent Assays (ELISA)—Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to analyze gene expression of anntoxin in H. annectans using a pair of primers (forward: 5′-AGACTTCTGT-GGTITTTCTTGTTGT-3′, and reverse: 5′-CTCGAGGGTT-TTGAATCTATTGCCA-3′. Western blotting and ELISA analyses were performed to detect anntoxin expression in eight frog tissues: skin, brain, heart, stomach, liver, intestine, pancreas, and thigh muscle, using rabbit polyclonal antibodies against recombinant anntoxin. Details are described under supplemental materials.

NMR Experiments—Samples for NMR experiments contained 0.3 ml of 2 mM anntoxin in 90% H₂O, 10% D₂O at pH 6.5. For the detection of hydrogen bonds, this sample was lyophilized and redissolved in D₂O (99.99%). All NMR experiments were carried out on a Varian Unity Inova 600-MHz spectrometer equipped with three RF channels and a triple resonance z axis pulse-field gradient probe. The tertiary structure calculation was performed according to the standard ARIA/CNS protocol (26–29). Proton-proton distance restraints were derived primarily from the NOESY spectrum recorded with a mixing time of 150 ms. Final structures were analyzed using the program packages MOLMOL (30) and PROCHECK (31). Details are described under supplemental materials.

Preparation of Rabbit Polyclonal Antibodies against Recombinant Anntoxin—Purified recombinant anntoxin was used as antigen to immunize male New Zealand White rabbits in our laboratory. The polyclonal antibodies (IgG) were purified from harvested rabbit sera by double ammonium sulfate precipitations (35% final concentration) and then dialyzed against phosphate-buffered saline. The IgG of a negative control was purified from pre-immunized rabbit sera.

Acute Toxicity—The acute toxicities of anntoxin against several potential aggressors or predators including insect, snake, bird, and mouse were tested. For all the tested animals, the same volume of buffers was used as a blank control. Bovine serum albumin with the same volume and concentration was administered as a protein control. The tested animals were observed for 24 h after injection. Survival times were recorded, and the LD₅₀ value was calculated according to a previous report (32). All animal studies were reviewed and approved by the animal care and use committee of Kunming Institute of Zoology, Chinese Academy of Sciences. Details are described under supplemental materials.

Patch Clamp Recording on Rat Dorsal Root Ganglion Neurons (DRG)—Rat DRG neurons were acutely dissociated and maintained in a short-term primary culture according to the procedures adapted from Xiao et al. (33). Potassium, sodium, and calcium currents were recorded from experimental cells using the whole-cell patch clamp technique by an Axon 700B patch clamp amplifier (AXON, American). The P/4 protocol

The abbreviations used are: RP-HPLC, reverse phase high performance liquid chromatography; RMSD, root mean square deviation; TTX-S, tetrodotoxin-sensitive; TTX-R, tetrodotoxin-resistant; VGSC, voltage-gated sodium channel; DRG, dorsal root ganglion; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; ELISA, enzyme-linked immunosorbent assay.
was used to subtract linear capacitive and leakage currents. Experimental data were acquired and analyzed using the program Clampfit10.0 (AXON) and Sigmaplot (Sigma). Details are described under supplemental materials.

Oocyte Manipulation and Electrophysiological Recording—Capped cRNAs encoding potassium ion channels (Kv1.1, Kv1.2, Kv1.3, Kv2.1, Kv4.2, Kv4.3) were synthesized after linearizing the plasmids and performing the transcription by a standard protocol (34). Using the linearized plasmids as templates, cRNAs were synthesized in vitro using the large-scale T7, SP6, or T3 mMESSAGE mMACHINE transcription kit (Ambion). These cRNAs were expressed in Xenopus laevis oocytes. Whole-cell currents from oocytes were recorded using the two-microelectrode voltage-clamp (TURBO TEC-03X, NPI Electronic, Germany). Details are described under supplemental materials.

RESULTS

Purification of Native Antitoxin—Considering that many neurotoxins have trypsin-inhibitory activity (35), we used trypsin inhibition as a marker for identifying the possible neurotoxins from all purified fractions. As illustrated in Fig. 1A, the skin secretions of H. annectans have been fractionated into several peaks by Sephadex G-50 gel filtration. The trypsin-inhibitory activity was concentrated in peak III, which was purified further by C8 RP-HPLC. A sharp peptide peak (III-3) at 215 nm was eluted at acetonitrile percentage of around 40% (Fig. 1B). Tricine-SDS-PAGE and MALDI-TOF mass spectrometry analysis revealed that this peak was a purified peptide (Fig. 1C and supplemental Fig. S1, A and B), which was named antitoxin. MALDI-TOF mass spectrometry analysis gave it a mass of 6724.92 (supplemental Fig. S1A). Native antitoxin could inhibit the hydrolysis of synthetic chromogenic substrates by trypsin. The $K_i$ value was determined to be 0.023 μM under the assay conditions.

Primary Structure—Edman degradation sequencing gave antitoxin a sequence of AQDYRCQLSRNYGKGSFTNYYDKATSSCTFR-YRGSGGNRFKTLDECATCVTAE. This sequence contains four half-cysteines. Analysis using the ExPASy MW/pl tool showed that antitoxin with all reduced cysteines has a predicted molecular weight of 6728.31, which is 3.4 units larger than the observed mass of 6724.92 (supplemental Fig. S1A), suggesting that these cysteines indeed form disulfide bridges. To confirm the intramolecular disulfide bridges, native antitoxin was hydrolyzed by endoprotease Glu-C, and those hydrolytic fragments were reduced and sequenced by Edman degradation.
as described under “Experimental Procedures.” Sequencing results (supplemental Fig. S2) indicated that two disulfide bridges were in the sequence of anntoxin, Cys6-Cys56 and Cys31-Cys52 (supplemental Fig. S2). NMR results as described below also confirmed the disulfide bridge profile.

**cDNA Cloning**—Several cDNA clones encoding precursors of anntoxin were cloned from a skin cDNA library of *H. annectans*. The open reading frame of cDNA encodes a polypeptide composed of 81 amino acids, including the mature anntoxin sequence (Fig. 2A, GenBankTM accession FJ598043). The amino acid sequences deduced from the cDNA sequences completely match the amino acid sequences determined by Edman degradation. The cloning further confirms the presence of gene-encoded anntoxin in this frog. With a Blast search, the sequence of mature anntoxin showed an identity of 44% and a similarity of 53% with the snake neurotoxin, dendrotoxin delta-DaTX, or venom basic protease inhibitor K identified from green mamba venoms (2) as illustrated in Fig. 2B. The Blast search also found the presence of the conserved typsin inhibitor domain and conserved interactive sites (14KGSGST²⁰) with trypsin in the anntoxin sequence (Fig. 2B).

**Recombinant Expression of Anntoxin**—To confirm the structure and function of anntoxin and to get enough purified anntoxin for bioassays, the recombinant vector construct was transformed into the *Escherichia coli* strain BL21 for anntoxin expression. As illustrated in Fig. 4A, the expression of recombinant anntoxin was intensively induced by isopropyl-1-thio-
β-n-galactopyranoside. The fusion protein was released by formic acid, and then purified by Sephadex G-50 and C₁₈ RP-HPLC. The purified recombinant anntoxin has the same behavior on Tricine-SDS-PAGE as native anntoxin (supplemental Fig. S1, A–D). The MALDI-TOF mass spectrometry analysis gave it a mass of 6822.01 that matched well with the predicted molecular weight of 6821.43. Its sequence was further confirmed by Edman degradation sequencing. Recombinant anntoxin is bigger than native anntoxin by 97.1 units, resulting from a proline extension at the N-terminus of recombinant anntoxin. The recombinant anntoxin had similar trypsin-inhibitory activity as the native anntoxin, with a *Kᵢ* value of 0.025 μM.

**Solution Structure**—Proton resonances of almost all residues of anntoxin are identified in the TOCSY and NOESY spectra except G43 (supplemental Table S1, BMRB ID: 16094). Backbone atom superposition of the 10 lowest energy structures of anntoxin in solution; B, ribbon representation of the mean structure of anntoxin; C, line representation of the mean structure of anntoxin with cysteines marked in red; D, backbone alignment of anntoxin with conkunitzin-S1. Anntoxin is shown in green and conkunitzin-S1 in blue.

**FIGURE 2.** A, cDNA sequence encoding anntoxin and the deduced amino acid sequence. The mature anntoxin is boxed. The stop codon is indicated by a bar (—). The poly(A) signal sequence is in italics. B, sequence comparison of anntoxin (AT) with dendrotoxin delta-DaTX (DT) and conkunitzin-S1 (CT). The sequences of dendrotoxin delta-DaTX and conkunitzin-S1 are cited from Refs. 2 and 23, respectively. Identical amino acid residues are indicated by a star (*). Disulfide bridges are connected by bold lines. The same amino acid residues as the first sequence are shaded. The percentage value indicates the identity between anntoxin and CT/DT.

**FIGURE 3.** Solution structure of anntoxin by NMR. A, backbone superimposition of the 10 lowest energy structures of anntoxin in solution; B, ribbon representation of the mean structure of anntoxin; C, line representation of the mean structure of anntoxin with cysteines marked in red; D, backbone alignment of anntoxin with conkunitzin-S1. Anntoxin is shown in green and conkunitzin-S1 in blue.
contributions of the backbone dihedral angles (\( \phi, \varphi \)) within the whole structure (residues 1–60) of the ensembles are 64.4% of the residues in the most favored regions, 25.4% in additionally allowed regions, 6.55% in generously allowed regions. The structure of anntoxin resembles the typical Kunitz-type fold, which consists of a twisted \( \beta \)-hairpin (residues Thr\(^{89}\)-Phe\(^{34}\)), an \( \alpha \)-helix (residues Leu\(^{49}\)-Ala\(^{59}\)), and a short \( \beta \) helix (residues Tyr\(^{14}\)-Cys\(^{6}\)) in the N terminus (Fig. 3B). The disulfide bridges of Cys\(^{6}\)-Cys\(^{56}\) and Cys\(^{31}\)-Cys\(^{52}\) (Fig. 3C) are confirmed by their direct NOE contacts as well as long range NOEs of the adjacent residues. To compare the structures of anntoxin and other Kunitz-type proteins, the structure of anntoxin was aligned with that of conkunitzin-S1 (PDB ID: 2CA7), which is also a Kunitz-type peptide with two disulfide bridges (Fig. 3D) (36). These two structures possess similar secondary structures and fold. The RMSD for the two structures is 1.05 Å, indicating anntoxin adopts a typical Kunitz fold.

_Tissue Expression of Anntoxin and Its mRNA—_Frog tissues including skin, brain, heart, stomach, liver, intestine, pancreas, and thigh muscle were used to check the tissue expression of anntoxin and its mRNA. By RT-PCR analysis, it was found that only three tissues including skin, brain, and liver have anntoxin mRNA (Fig. 4A). By Western blotting using the antibody against recombinant anntoxin, the peptide anntoxin was found in four tissues including skin, brain, stomach, and liver (Fig. 4B). Anntoxin was found in stomach although no mRNA was cloned by RT-PCR. The reason could be either that the frog stomach might have trace anntoxin mRNA that cannot be checked by RT-PCR or the anntoxin was transferred from other tissues to stomach. The amounts of anntoxin in these tissues were determined by ELISA. Compared with other tissues, skin was the tissue containing the highest concentration of anntoxin (around 25 \( \mu \)g/g WT). There are 7, 3, and 2 \( \mu \)g/g WT anntoxin concentrations in brain, stomach, and liver, respectively (Fig. 4C).

_Acute Toxicities—_Recombinant anntoxin showed lethal toxicities against tested animals like insects, reptiles, birds, and mammals. Administered intraperitoneally, the toxic dose (LD\(_{50}\)) against _L. exigua_ Hubner, _E. plumbea_, _C. coturnix_, and Kunming mice is 0.05, 0.45, 2.5, and 3 mg/kg body weight, respectively. Anntoxin could rapidly exert its toxicity against these animals. Insects (_L. exigua_ Hubner) administered the toxin intensively wriggled 5–10 s after injection and lost mobility after 1–3 min of injection. Snakes (_E. plumbea_) had a comparatively slow response for the toxin administration, and the toxicosis symptom of wriggling was observed after 2–3 h of injection. The toxicosis symptoms of the common quails (_C. coturnix_) were respiratory distress, convolution, leg paralysis, and shambling/tottering after 2–5 min of injection. The Kunming mice injected with the toxin showed symptoms of respiratory distress, rotating, and convulsions after 2–5 min of injection. All these results indicated that anntoxin is a highly lethal toxin.

The Effects of Anntoxin on Voltage-gated Channels—The biological activity of the peptide on voltage-gated channels was performed using a whole-cell patch clamp recording technique. Sodium currents were elicited from adult rat dorsal root ganglion neurons by a depolarization to \(-10\) mV from a holding potential of \(-80\) mV. As seen in Fig. 5A, 1 \( \mu \)M anntoxin inhibited tetrodotoxin-sensitive (TTX-S) sodium current amplitude only by 23 ± 5%, but when toxin concentration in the extracellular solution increased 10-fold, almost 75 ± 10% of TTX-S sodium current amplitude was depressed with a time constant (\( \tau \)) of 62.5 s (Fig. 5D). Because both TTX-S and tetrodotoxin resistance (TTX-R) sodium channels are expressed in small DRG neurons, we added 200 nM TTX into extracellular solution to separate TTX-R currents from mixture currents. However, the following application of 10 \( \mu \)M toxin exhibited no effect on TTX-R currents (Fig. 5B). The IC\(_{50}\) of anntoxin for TTX-S channels estimated from the concentration effect curve was about 3.4 \( \mu \)M (Fig. 5C).

The current-voltage (Fig. 5E) curve of TTX-S sodium channels indicated that anntoxin did not change the threshold of

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**FIGURE 4. Tissue expression profile of anntoxin in _H. annectans_.** _A_, semi-quantitative RT-PCR analysis of the expression of anntoxin in frog tissues. RT-PCR was performed for anntoxin with the cycle parameters shown and actin as an internal control, using total RNA from adult frog tissues as indicated. The PCR was replicated three times. _B_, Western blotting of adult frog tissues as indicated using the antibody against recombinant anntoxin, using the antibody against actin as an internal control. The immunoblot was replicated three times. _C_, amounts of anntoxin in different frog tissues were determined by ELISA. The ELISA was replicated three times.
activation or the activation voltage of inward peak currents, and there was also no shift in the membrane reversal potential, implying that it did not change the ion selectivity of channels.

As seen in Fig. 6A, calcium current amplitude is depressed only by 7 ± 5% when exposed to 10 μM annotoxin. Outward potassium currents were elicited from adult rat dorsal root ganglion neurons by a depolarization to 30 mV from a holding potential of −80 mV. 10 μM annotoxin reduced the control potassium current amplitude in rat DRG neurons by 14 ± 8% (Fig. 6B). Using the two-microelectrode voltage clamp technique, we also observed the actions of the annotoxin on six potassium channel isoforms (Kv1.1, Kv1.2, Kv1.3, and Kv2.1) that we tested were not significantly affected by annotoxin at the same concentration (Fig. 6, C–F). Another two potassium channel isoforms (Kv4.2 and 4.3) were not affected by annotoxin at all (data not shown). Results indicated that the annotoxin might have little effect on outward potassium channels.

**DISCUSSION**

Annotoxin, a novel neurotoxin peptide, was purified and characterized from skin secretions of the tree frog, *H. annectans*. It is composed of 60 amino acid residues including 4 cysteines. This is the first gene-encoded neurotoxin ever found in amphibians. The two disulfide bridges in annotoxin, Cys6-Cys56 and Cys31-Cys52 (Fig. 2B), are assigned and confirmed by sequence analysis of hydrolytic fragments by endoproteinase Glu-C (supplemental Fig. S2) and NMR experiments (Fig. 3). Annotoxin shares sequence homology with Kunitz-type protein toxins, i.e. snake dendrotoxins, although annotoxin lacks one highly conserved cysteine bridge (Fig. 2B). Just like other Kunitz-type protein toxins, annotoxin shows a typical Kunitz-type fold, which consists of a twisted β-hairpin (residues Thr20-Phe34), an α-helix (residues Leu49-Ala59), and a short 310 helix (residues Tyr4-Cys6) in the N terminus (Fig. 3). The trypsin-inhibitory function of annotoxin further supports the model of a Kunitz-type fold.

Similar to other neurotoxins, annotoxin showed lethal toxicities against potential aggressors or predators including insect, snake, bird, and mouse at a low dose. Most of the tested animals showed obvious intoxication symptoms a short time after administration of annotoxin. Annotoxin was mainly expressed in the frog skin (25 μg/g WT), while only minor expression was found in brain (7 μg/g WT), stomach (3 μg/g WT), and liver (2 μg/g WT) (Fig. 4). Amphibian naked skins are directly exposed to environmental factors, and thus they have to act on the first line against various aggressions and injuries. Most amphibian skins are devoid of good physical protection, but they have a perfect chemical defense system. The chemical system is made up of many bioactive substances eliciting pharmacological effects like cardiotoxic, myotoxic, neurotoxic, and antimicrobial activities (4–10). All these properties clearly repel potential predators or pathogens. Annotoxin is an
important component of the skin chemical defense system of the tree frog, *H. annectans*. Anntoxin’s strong and fast-acting toxicity against potential predators and its skin distribution with a high concentration make it an excellent chemical component to act as the first-line defense and enable the forest-living frog to evolve an ecological adaptation.

Potassium channels are the largest and most diverse class of voltage-gated ion channels. As of now, nearly 100 genes encoding potassium channel have been characterized (37, 38). Considering the structural similarity of anntoxin with snake potassium channel neurotoxins and conkunitzin-S1 (36), a 60-residue potassium channel neurotoxin from the venom of the cone snail *Conus striatus* (Fig. 2), we hypothesized that anntoxin may have some effect on the potassium channel. As illustrated in Fig. 6, anntoxin had little effect on the potassium current amplitude. Six potassium channel isoforms including Kv1.1, Kv1.2, Kv1.3, Kv2.1, Kv4.2, and Kv4.3, were tested without being significantly affected by anntoxin. In addition, anntoxin had no effect on the calcium channel (Fig. 6A).

Results of our experiments have proven that anntoxin inhibits the neuronal TTX-S voltage-gated sodium channel (VGSC), with an IC_{50} value of 3.4 μM in adult rat dorsal root ganglion neurons, and no significant effect on the TTX-R VGSC (Fig. 5). VGSCs play important roles in electrical signaling in almost all kinds of excitable tissues. They are responsible for the generation of action potentials and nervous influx conduction in sensory nerves (39, 40). Up to date, two different types of VGSC, TTX-S and TTX-R VGSCs have been found (40). Many neurotoxins such as ciguatoxin (41), scorpion toxins (42), μ-conotoxins (43–45), and spider toxin (46, 47) have shown interaction with VGSCs. All the neurotoxins from scorpion, conus, and spider contain at least three disulfide bridges. However, the anntoxin described in the current report only possesses two disulfide bridges. It is the first neurotoxin interacting with VGSC ever found containing other than three disulfide bridges. This unique structure of anntoxin may be a powerful tool for the study of the interaction between VGSCs and neurotoxins.

Although conkunitzin-S1, which shares the same disulfide bridge motif as anntoxin, has been demonstrated to inhibit shaker potassium channels (Kv1) (36), our study results show that its analogue anntoxin exhibited no significant effect on potassium current. On the other hand, anntoxin could markedly inhibit the TTX-S VGSC. Therefore, divergent amino acid residues between conkunitzin-S1 and anntoxin might be responsible for the different ion channel interaction behavior.

In summary, the anntoxin in the current report is the first gene-encoded amphibian neurotoxin. This neurotoxin with its special disulfide bridge motif should aid in the investigation of multiple molecular forms of VGSCs. Further work needs be done to explore the interaction of anntoxin with VGSCs.

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