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Postsynaptic short-chain neurotoxins from *Pseudonaja textilis*

cDNA cloning, expression and protein characterization

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Two lethal proteins, which specifically bind to the nAChR from *Torpedo californica*, were isolated from the venom of *Pseudonaja textilis*, the common brown snake from Australia. The isolated proteins have masses of 6236 and 6345 Da and are structurally related to short-chain neurotoxins from other elapids. Six cDNAs encoding isoforms of related neurotoxins were cloned using the RT-PCR of the venom gland mRNAs. The sequences of the corresponding proteins consist of 57–58 amino acid residues and display several unique features when compared with all known short-chain neurotoxins. Accordingly, they grouped separately in phylogenetic analysis. The six cDNAs were expressed in *Escherichia coli* and the recombinant proteins were characterized. They have similar masses and display similar toxicities and binding constants to the nAChR as the native toxins isolated from the venom. Thus, a new group of short-chain postsynaptic neurotoxins from the venom of an Australian elapid has been characterized.

Keywords: cDNA; molecular cloning; postsynaptic; *Pseudonaja textilis*; short neurotoxin.

More than 100 α-neurotoxins have been isolated from various snake venoms and their amino acid sequences have been determined. They have the ability to block nerve transmission by binding specifically to the nicotinic acetylcholine receptors on the postsynaptic membranes of skeletal muscles and/or of neurons [1]. Based on the sequences, they can be classified into short-chain and long-chain neurotoxins. Short-chain neurotoxins consist of 60–62 amino acid residues, including four disulfide bonds, whereas long-chain neurotoxins contain 66–79 amino acid residues with five disulfide bridges [2]. These toxins share a common three finger-loop structure [2], although a diversity in their primary structures can be observed.

While a reasonable number of studies has been documented for presynaptic (β) neurotoxins [3], relatively few studies have been carried out on the postsynaptic (α) neurotoxins in the venom of Australian elapids. To date, only six long-chain and four short-chain α-neurotoxins have been reported. The long-chain neurotoxins are Aa b, Aa e and Aa d from *Acanthophis antarcticus* [4–6], Notechis III-4 from *Notechis scutatus* [7], Pa ID from *Pseudechis australis* [8] and Pseudonajatoxin b from *Pseudonaja textilis* [9]. Except for Pa ID, which is not lethal, all the other long-chain neurotoxins from Australian elapids are lethal and act as potent postsynaptic inhibitors of neurotransmission. The four short-chain neurotoxins are: Aa c from *Acanthophis antarcticus* [10], Pa a from *P. australis* [11] and Taipan toxins 1 and 2 from *Oxyuranus scutellatus* [12]. Aa c from *Acanthophis antarcticus* is a lethal toxin homologous to other elapid short-chain neutotoxins, particularly from the sea snakes. Pa a is a typical short-chain neurotoxin, which produces peripheral paralysis. Both Aa c and Pa a contain an extra cysteine residue at the N-terminus which may have tertiary structural implications. Taipan toxins 1 and 2, which differ by only one amino acid residue, inhibit the binding of α-bungarotoxin to nicotinic acetylcholine receptors in skeletal muscles but not to central neuronal nicotinic receptors.

P. textilis, a common brown snake, inhabits from dry areas to watercourse swamps, and is commonly found in the farmlands of eastern Australia. It possesses a highly toxic venom with LD_{50} of 0.040 $\mu g \cdot g^{-1}$ mouse. Thus far, the sequences of one β-neurotoxin textilotoxin [13] and one long-chain α-neurotoxin Pseudonajatoxin b [9] from this snake, have been reported. Pseudonajatoxin b shows considerable homology (51–76%) with other long-chain α-neurotoxins. However, unlike its counterparts, it remains highly lethal (LD_{50} of 0.015 $\mu g \cdot g^{-1}$ mouse) [9]. To our knowledge, there have been no studies carried out on short-chain α-neurotoxins of *P. textilis*. Cloning of cDNAs and genes encoding snake α-neurotoxins have also been extensively carried out on sea snakes [14–16] and on many other land snakes (R. C. Chu & C. C Yang (1996) GenBank accession numbers U42582, U58519, U58520, U58521, U77490, U77491 and U77492; [17]) except those found on the Australian continent. In this work, cDNAs encoding six isoforms of short-chain α-neurotoxins from an Australian elapid have been cloned and expressed in *Escherichia coli* for the first time.

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Abbreviations: ESI-MS, electron spray ionization-mass spectrometry; GST, glutathione-*S*-transferase; IPTG, isopropyl thio-β-D-galactoside; pI, isoelectric point.

Note: the novel nucleotide sequence data published here have been submitted to the EMBL sequence data bank and are available under accession numbers AF082975, AF082976, AF082977, AF082979, AF082980, AF082981.

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MATERIALS AND METHODS

Venom and venom glands

An adult snake (*P. textilis*) kept undisturbed and unfed for 2 weeks was milked and the venom lyophilyzed and stored at

−20 °C. The venom glands were immediately frozen in liquid nitrogen and kept at −85 °C.

Purification and N-terminal amino acid sequencing of short-chain α-neurotoxins

Lyophilyzed crude venom (78 mg) was reconstituted in 2 mL water and subjected to gel filtration using a P10 (BioRad) column. Fractions obtained were further purified on RP-HPLC (SMART system, Pharmacia) using a Sephasil C18 μBore column. The buffer systems used were 0.1% trifluoroacetic acid (buffer A) and 80% acetonitrile in 0.1% trifluoroacetic acid (buffer B). The RP-HPLC purified proteins were subjected to N-terminal amino acid sequencing using a Procise-HT (Model 494) Protein Sequencer attached to a 140C PTH analyzer (Applied Biosystems) after analysis by electron spray ionization mass spectrometry (ESI-MS, Applied Biosystems Inc.). Protein concentrations were determined using the Bradford method [18].

Isolation of total RNA and RT-PCR

Total RNA was isolated from the venom glands using the guanidine isothiocyanate method [19] and the integrity of the total RNA was analyzed by denaturing formaldehyde agarose electrophoresis [20]. Oligonucleotide primers synthesized by Oswel DNA service (UK) based on the sequence at the 5′ and 3′ UTR regions of the previously cloned cDNAs [16,21] from a spitting cobra, *Naja naja sputatrix* have been used. The sense and antisense primers were: X133 (5′-TCCA-gAAAAagATCgCAAgATg-3′) and X132 (5′-gAATTTAgA-CATTATCAgTTg-3′), respectively.

Total RNA (3 μg) was reverse transcribed using 10 units of MuMLV reverse transcriptase, 40 ng of antisense primer, 2 μL of reverse transcription buffer (100 mM Tris/HCl, pH 8.4; 250 mM KCl; 12.5 mM $MgCl_2$ and 0.5 mg·mL^{-1} BSA) in a total volume of 10 μL at 42 °C for 1 h. All the reverse transcribed products (10 μL) were used for the PCR. PCR was carried out for 30 cycles with each cycle consisting of a denaturing step (94 °C for 1 min), an annealing step (50 °C for 1 min) and an elongation step (72 °C for 2 min), followed by final extension step of 10 min at 72 °C, using a Perkin-Elmer Cetus thermal cycler (Model 480). The reaction mixture contained 250 μmol each of dNTPs, 10 μmol each of sense and antisense primers in a final 50 μL reaction buffer (50 mM KCl, 10 mM Tris/HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.1 mg·mL^{-1} gelatin) and 1 unit of *Taq* DNA polymerase [22].

Cloning and sequencing of cDNAs

The PCR products were cloned into pT7Blue (R) vector (Novagen) using the procedures described by the supplier. The ligated products were then transformed into the *E. coli*, DH5α: *sup*E44 Δ*lac*U169 (Φ80*lac*ZΔm15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 [23] and the recombinants were selected on an Luria–Bertani/Amp (50 μg·mL^{-1}) plate supplemented with isopropyl thio-β-D-galactoside (IPTG) and X-Gal according to the method described by Sambrook *et al.* [23]. The plasmids isolated from the recombinant clones were then subjected to Sanger dideoxy DNA sequencing [24], using M13/pUC forward and reverse primers [22] on an automated DNA sequencer (Applied Biosystems, Model 373A). The DNA sequences and deduced amino acid sequences were analyzed using the CLUSTAL W program.

Expression of cDNAs encoding short-chain α-neurotoxins

Primers containing *Bam*HI (sense) and *Hin*dIII (antisense) restriction sites were used to amplify the structural gene from the cDNA by PCR. The sense and antisence primers were

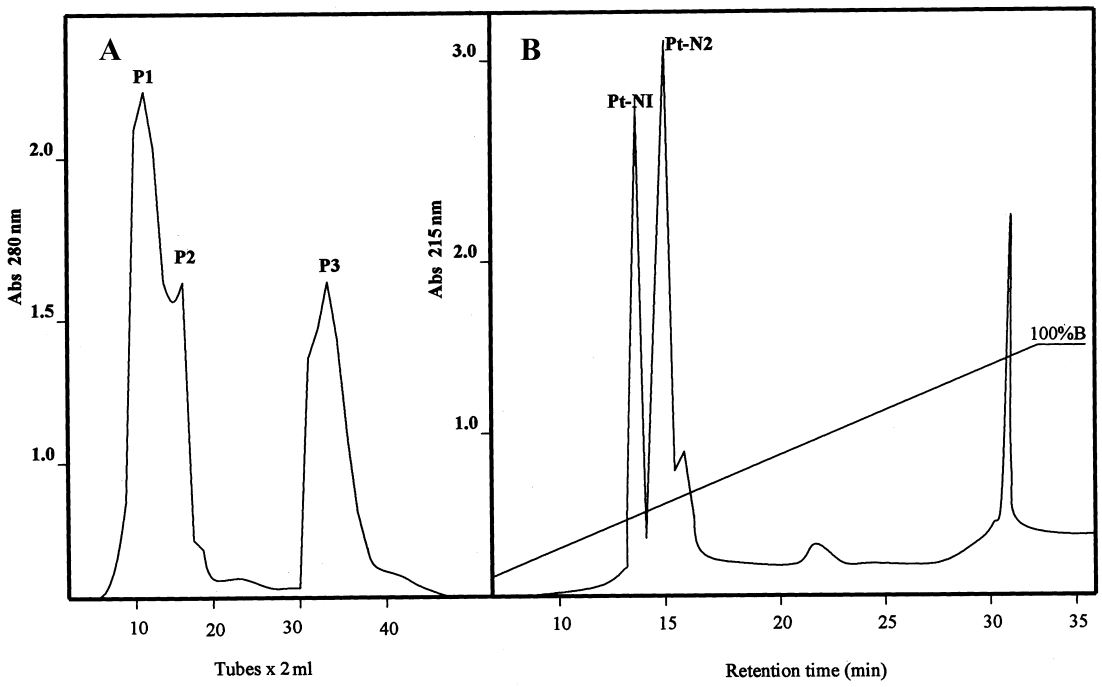

Fig. 1. Purification of Pt-N_1 and Pt-N_2 from venom by chromatography. (A) Gel filtration on Biogel P10 using 78 mg of *P. textilis* crude venom. P1–P3 represent three peaks obtained. (B) RP-HPLC on fraction number 12 of Peak 2 (P2) from gel filtration. Pt-N1 and Pt-N2 represent the native short-chain α-neurotoxins in *P. textilis*.

X470 (5′-gACCgCggATCCATgCTAACATgT-3′) and X372 (5′-AAgCTTCTACTTgTTgCACAg-3′), respectively. The resulting fragments were subcloned into pGEX-KG expression vector (Pharmacia) between *Bam*HI and *Hin*dIII sites. Recombinant plasmids were sequenced to confirm the in-frame fusion of each of the neurotoxin structural gene to the glutathione S-transferase (GST) sequence, containing the thrombin endoproteinase site.

The expression of the cloned genes was induced by IPTG (0.2 mM) at 37 °C for 5 h and the fusion proteins were analyzed by 12% Tris/Tricine SDS/PAGE [25]. The fusion proteins were passed through the glutathione–agarose column and digested while being bound to the column with thrombin [26]. The recombinant neurotoxins were then eluted by 50 mM Tris/HCl (pH 7.4).

LD_{50} and acetylcholine receptor binding assays

Adult Swiss albino mice (20 ± 1 g) were injected intravenously as follows: 0.1 mL of saline as control and at least four

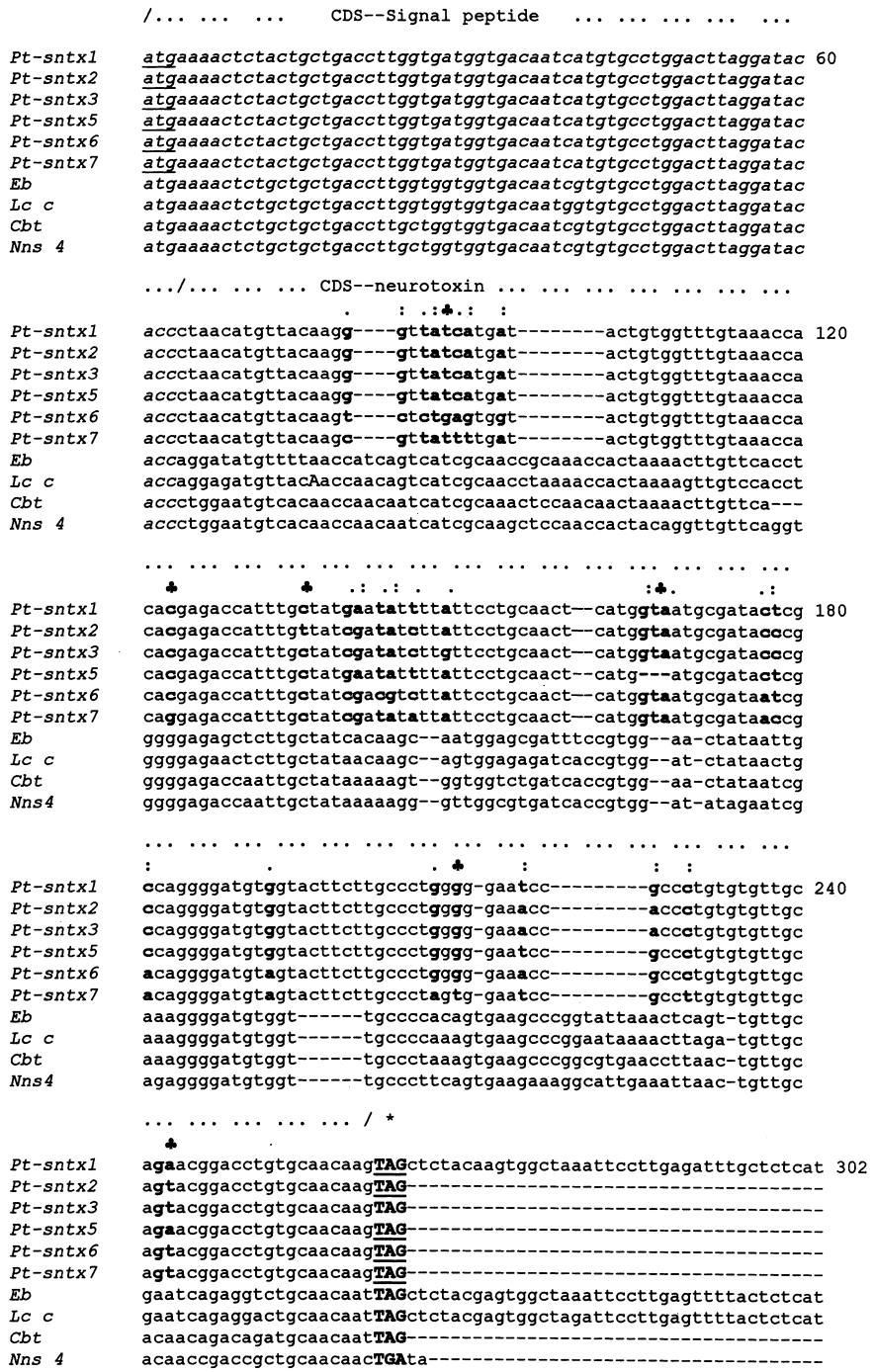

Fig. 2. Nucleotide sequences of cDNAs encoding short-chain α-neurotoxins. Pt, *Pseudonaja textilis*; Eb, *Laticauda semifasciata*; Cbt, *Naja naja atra*; Nns, *Naja naja sputatrix*. The 3′-ends of primers used in RT-PCR are underlined. CDS (the coding region) for signal peptides and neurotoxins are shown. The variant nucleotides for base 1, 2 and 3 in the codons (Pt-sntxs) are indicated by ·, :, ♣, respectively. * Stop codon.

different doses of toxin. The mice were observed for up to 48 h, and the LD_{50} (intravenous) values were calculated according to the Spearman–Karber method [27]. Experiments were carried out in accordance with the guidelines laid down by the NIH (USA) regarding the care and use of animals for experimental procedures.

Both native and recombinant neurotoxins were tested for their ability to compete with [^{125}I]α-bungarotoxin for binding sites on the AchRs from *T. californica* prepared according to the method described by Ishikawa *et al*. [28]. A 2.5 μg sample of *Torpedo* membrane suspension was incubated with 5 nM [^{125}I]α-bungarotoxin at room temperature (20–25 °C) and a range of concentrations of purified toxins in a total volume of 200 μL. After 1 h, the reaction was quenched on ice. The membranes were recovered by centrifugation, washed with 1 mL of buffer containing 0.1% BSA and dried before subjecting to radioactive monitoring in a Packard, COBRA Auto Gamma Counter (Packard Instruments Co. Inc.). The results were analyzed and plotted on Slidewrite Plus 2 (AdvanceGraphic Software Inc; Carlsbad, USA).

RESULTS

Purification and analysis of venom neurotoxins

Fractionation of the crude venom using a P10 column gave three peaks, P1 to P3 (Fig. 1A). Fraction 12, corresponding to peak 2 (P2) and containing the low molecular mass proteins (6–7 kDa), was selected for further study as it was expected to contain the short-chain neurotoxins. Upon RP-HPLC, this fraction resolved into two homogenous peaks containing the short-chain α-neurotoxins, Pt-N1 and Pt-N2 (Fig. 1B). The presence of these neurotoxins was confirmed by N-terminal amino acid sequencing. The sequences obtained were as follows: Pt-N1, LTXYKGYRDTV and Pt-N2, LTXYKGY-HDTVVXKP. ESI-MS analysis showed that the purified proteins are homogenous and with molecular masses of 6236 and 6345 Da for Pt-N1 and Pt-N2, respectively.

cDNA cloning and sequencing

Total RNA prepared from the venom glands of a snake was used in RT-PCR. Two independent RT-PCR reactions using the primers X133 and X132 on the total RNA gave ≈ 300 bp PCR products. Fifty-five putative clones were obtained upon subcloning of these cDNAs. In another experiment using an adaptor primer (AP1)-ligated cDNA as a template and the primers X133 and AP1, 13 more clones were obtained. All 68 clones were subjected to DNA sequencing on both strands of each cDNA and the results were analyzed using SeqEd program from Applied Biosystems Inc. Six different types of cDNAs (Pt-sntx 1 to Pt-sntx 3 and Pt-sntx 5 to Pt-sntx 7; Fig. 2) representing a set of novel short-chain α-neurotoxins from *P. textilis* were obtained. These isoforms contained an identical leader sequence and a variable mature neurotoxin sequence (Fig. 3). They exhibited high sequence similarity to many short-chain α-neurotoxins from other elapids (Fig. 3).

The average frequency of finding the cDNAs encoding Pt-sntx 1 and 2 were 91.17 and 2.94%, respectively. The others, Pt-sntx 3, 5, 6 and 7 were found at a lower frequency of 1.47% each. The fidelity of *Taq* 1 polymerase was tested by amplifying a fragment (1.2 kb) of an unrelated gene, the

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                /.. signal peptide ../  /...loop I .. /      /.....loop II...   /  / loop III  /
                                           ***           *     ****     *  **   *       * ***   *
               -20.......-10.......-1 1.......10........20........30........40........50........60.....
Pt-sntx1        MKTLLLTLVMVTIMCLDLGYT LTCYKGYHD----TVVCKPHETICYEYFIPATHGNAILARGCGTSCP---GGIRPVCCRTDLCNK
Pt-sntx2        MKTLLLTLVMVTIMCLDLGYT LTCYKGYHD----TVVCKPHETICYRYLIPATHGNAIPARGCGTSCP---GGNHPVCCSTDLCNK
Pt-sntx3        MKTLLLTLVMVTIMCLDLGYT LTCYKGYHD----TVVCKPHETICYRYLVPATHGNAIPARGCGTSCP---GGNHPVCCSTDLCNK
Pt-sntx5        MKTLLLTLVMVTIMCLDLGYT LTCYKGYHD----TVVCKPHETICYEYFIPATH-DAILARGCGTSCP---GGIRPVCCRTDLCNK
Pt-sntx6        MKTLLLTLVMVTIMCLDLGYT LTCYKSLSG----TVVCKPHETICYRRLIPATHGNAIIDRGCSTSCP---GGNRPVCCSTDLCNK
Pt-sntx7        MKTLLLTLVMVTIMCLDLGYT LTCYKRYFD----TVVCKPQETICYEYIIPATHGNAITYRGCSTSCP---SGIRLVCCSTDLCNK
Nmm III                               LNCHNQMSAQPPTTTRCSRWETNCYKKRWRDHR-GYKTERGCG--CPTVKKGIQLHCCTSDNCNN
Nk c-6                                LECHNQQSIQTPTTTGCSGGETNCYKKRWRDHR-GYRTERGCG--CPSVKNGIEINCCTTDRCNN
Nns 1           MKTLLLTLLVVTIVCLDLGYT LECHNQQSSETPTTTGCSGGETNCYKKRWRDHR-GYRIERGCG--CPSVKKGIEINCCTTDRCNN
Nns 4           MKTLLLTLLVVTIVCLDLGYT LECHNQQSSQAPTTTGCSGGETNCYKKGWRDHR-GYRIERGCG--CPSVKKGIEINCCTTDRCNN
Nnv δ                                 LECHNQQSSQPPTTKTCPG-ETNCYKKRWRDHR-GSITERGCG--CPSVKKGIEINCCTTDKCNN
Nha α                                 LECHNQQSSQPPTTKTCPG-ETNCYKKRWRDHR-GSITERGCG--CPSVKKGIEINCCTTDRCNN
Nm D                                  MECHNQQSSQPPTTKTCPG-ETNCYKKQWSDHR-GTIIERGCG--CPSVKKGVKINCCTTDKCNR
Nhh 6                                 MICHNQQSSQPPTIKTCPG-ETNCYKKQWRDHR-GTIIERGCG--CPSVKKGVGIYCCKTDKCNN
Tx α                                  LECHNQQSSQPPTTKTCPG-ETNCYKKVWRDHR-GTIIERGCG--CPTVKPGIKLNCCTTDKCNN
Nnp                                   LECHNQQSSQAPTTKTCSG-ETNCYKKWWSDHR-GTIIERGCG--CPKVKPGVKLNCCRTDRCNN
Nno II                                LECHNQQSSQPPTTKTCSG-ETNCYKKWWSDHR-GTIIERGCG--CPKVKPGVNLNCCRTDRCNN
Ea                                    RICFNHQSSQPQTTKTCSPGESSCYNKQWSDFR-GTIIERGCG--CPTVKPGIKLSCCESEVCNN
Lc d                                  RRCFNQQSSQPKTTKSCPPGENSCYNKQWRDHR-GSITERGCG--CPKVKPGIKLRCCESEDCNN
Taipan tx2                            MTCYNQQSSEAKTTTTCSGGVSSCYKKTWSDIR-GTIIERGCG--CPSVKKGIERICCRTDKCNN
Taipan tx1                            MTCYNQQSSEAKTTTTCSGGVSSCYKKTWSDGR-GTIIERGCG--CPSVKKGIERICCRTDKCNN
Aa c                                  MQCCNQQSSQPKTTTTCPGGVSSCYKKTWRDHR-GTIIERGCG--CPRVKPGIRLICCKTDECNN
Pa a                                  MTCCNQQSSQPKTTTICAGGESSCYKKTWSDHR-GSRTERGCG--CPHVKPGIKLTCCKTDECNN
Al d            MKTLLLTLVVVTIVCLDLGYT LTCCNQQSSQPKTTTDCAD--DSCYKKTWKDHR-GTRIERGCG--CPQVKPGIKLECCKTNECNN
Al b            MKTLLLTLVVVTIVCLDLGYT LTCCNQQSSQPKTTTDCAD--NSCYKMTWRDHR-GTRIERGCG--CPQVKPGIKLECCKTNECNN
Lh                                    MTCCNQQSSQPKTTTNCAE--SSCYKKTWSDHR-GTRIERGCG--CPQVKPGIKLECCHTNECNN
Ap a                                  MTCCNQQSSQPKTTTNCAG--NSCYKKTWSDHR-GTIIERGCG--CPQVKSGIKLECCHTNECNN
As a                                  MTCCNQQSSQPKTTTNCAG--NSCYKKTWSDHR-GTIIERGCG--CPQVKSGIKLECCHTNECNN
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Fig. 3. Comparison of amino acid sequences of short-chain α-neurotoxins. The short-chain α-neurotoxins from *P. textilis* are compared with those from other snakes using CLUSTAL W program. The region of the loops of short-chain neurotoxins are shown at the top of the figure. The variant amino acid residues of the *P. textilis* neurotoxins are highlighted and asterisked. PT, *Pseudonaja textilis* (this study); Nmm III, *Naja mossambica mossambica* [29]; Nk c-6, *Naja kaouthia* [30]; Nns 1 and Nns 4, *Naja naja sputatrix* [17]; Nnv δ, *Naja nivea* [31]; Nha α, *Naja haje annulifera* [32]; Nm D, *Naja melanoleuca* [33]; Nhh 6, *Naja haje haje* [34]; Tx α, *Naja nigricollis* [35]; Nnp, *Naja naja philippinensis* [36]; Nno II, *Naja naja oxiana* [37]; Ea, *Laticauda semifasciata* [38]; Lc d, *Laticauda colubrina* [39]; Taipan tx 1 and Taipan tx 2, *Oxyuranus scutellatus scutellatus* [12]; Aa c, *Acanthophis antarcticus* [10]; Pa a, *Pseudechis australis* [11]; Al d and Al b, *Aipysurus laevis* [40]; Lh, *Lapemis hardwickii* [41]; Ap a, *Acalyptophis peronii* [42]; As a, *Astrotia stokesii* [43].

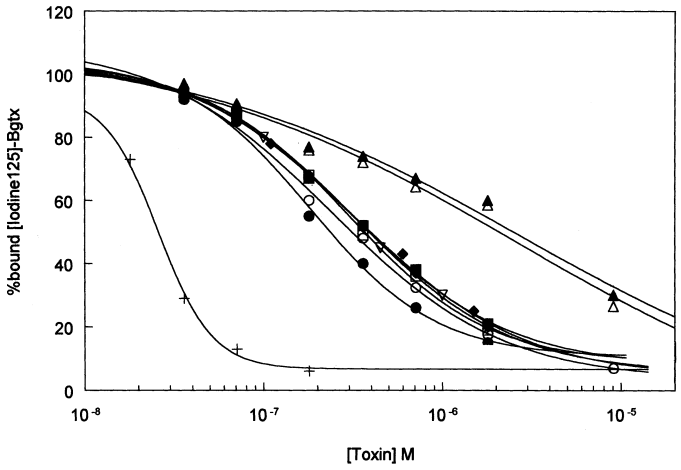

Fig. 4. Competitive binding assay on [^{125}I]α-bungarotoxin and native or recombinant neurotoxins of *P. textilis* for binding to nAChR of *Torpedo*. +, Ea; Δ, Pt-N1; ○, Pt-N2; ◆, Pt-sntx1; ∇, Pt-sntx2; □, Pt-sntx3; ▲, Pt-sntx5; ●, Pt-sntx6; ■, Pt-sntx7.

human debrisoquine hydroxylase ($P450_{6\beta}$) using the same RT-PCR conditions. The sequence obtained for this $P450_{6\beta}$ cDNA remained consistent with that of the expected sequence even though we repeated the PCR consecutively six times.

Expression and purification of short–chain α-neurotoxins

The pGEX vector system was used for expression of neurotoxins. The resulting recombinant plasmids produced GST–sntx fusion proteins upon expression in *E. coli*.

The fusion proteins were first separated from *E. coli* proteins by affinity chromatography using a glutathione–agarose column. After removing all the contaminating proteins from the resin-bound fusion proteins, the recombinant neurotoxins were cleaved from GST by digesting with thrombin (while the fusion protein remains bound to the glutathione–agarose column). The toxins eluted using 50 mM Tris/HCl (pH 7.4) were collected and used for further analysis. The purity of recombinant proteins were analyzed by SDS/PAGE and the proteins were found to cross-react with antibodies raised against the crude venom of *P. textilis*. The recombinant proteins were further purified by RP-HPLC before use in binding assays.

Properties of native and recombinant neurotoxins

LD_{50} values for Pt-N1 and Pt-N2 in Swiss albino mice were 0.84 and 0.80 $\mu g \cdot g^{-1}$, respectively. The binding activity studies of these neurotoxins on AChR isolated from *T. californica* showed IC_{50} for Pt-N1 and Pt-N2 as 2.6×10^{-6} and 2.3×10^{-7} M, respectively (Table 1). These results demonstrate

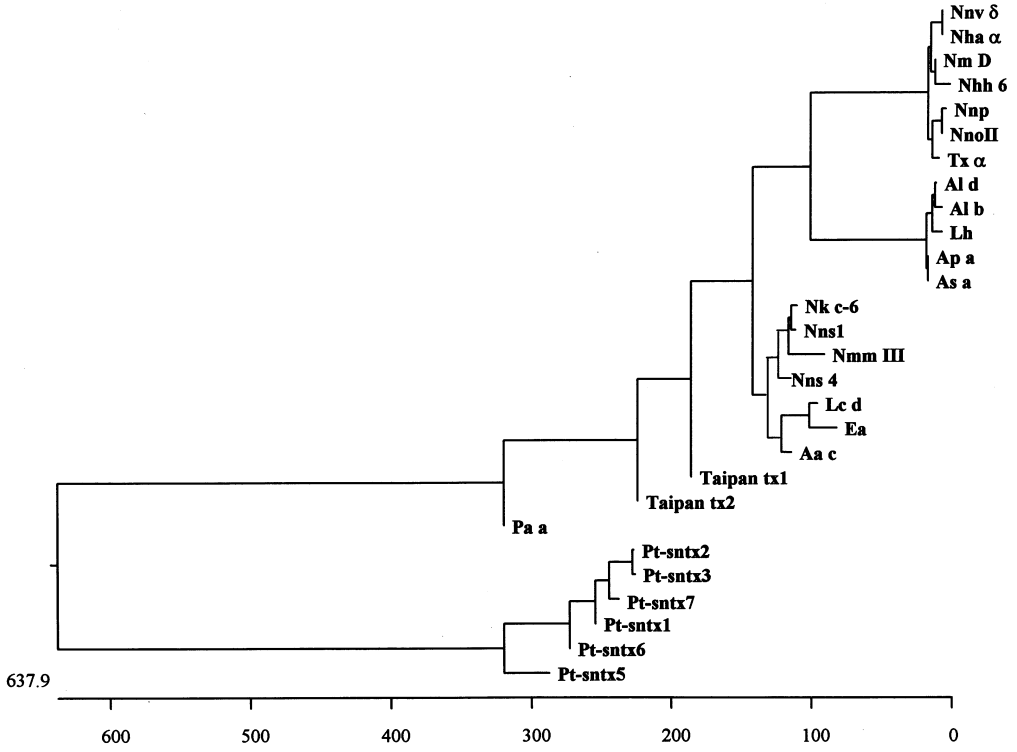

Fig. 5. Phylogenetic analysis of short-chain α-neurotoxins. A cladogram constructed from phylogenetic analysis using MegAlign from DNASTAR is shown. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences. Units indicate the number of substitution event. The abbreviations used for the toxins are the same as in Fig. 3.

Table 1. Properties of short-chain αneurotoxins of *P. textilis*. For Pt-N1 and Pt-N2, molecular weight is based on ESI-MS; for Pt-sntxs, molecular mass and pI were calculated from their sequences. IC_{50} for both native and recombinant neurotoxins are actual values obtained in this study. ND, not determined.

Proteins	*m* (Da)	pI	IC_{50} (M)
Pt-N1	6236	ND	2.6×10^{-6}
Pt-N2	6345	ND	2.3×10^{-7}
Pt-sntx1	6351	7.82	3.1×10^{-7}
Pt-sntx2	6240	7.83	3.0×10^{-7}
Pt-sntx3	6227	7.83	3.0×10^{-7}
Pt-sntx5	6240	6.92	3.1×10^{-6}
Pt-sntx6	6215	8.28	1.8×10^{-7}
Pt-sntx7	6504	7.81	3.0×10^{-7}

that Pt-N1 and Pt-N2 are lethal α-neurotoxins with the capacity to compete with the binding of [^{125}I]α-bungarotoxin on AChRs from *Torpedo* (Fig. 4). The recombinant proteins were found to exhibit similar features as the native proteins (Table 1). Their LD_{50} values were found to be 1 $\mu g \cdot g^{-1}$ mouse and their IC_{50} ranged between 1.8×10^{-7} and 3.1×10^{-6} M.

Comparative analysis and evolutionary relationship of neurotoxins

Figure 3 shows the comparison of amino acid sequences of 28 selected short-chain neurotoxins including six neurotoxins of *P. textilis*. Pt-sntxs from *P. textilis* share the same conserved residues including all the cysteines, Thr14, Glu21, Tyr25, Arg40, Gly41, Gly43, Pro47, Gly52, Thr60, Asp61 and Asn64, but do not have the conserved residues, which are found in most short neurotoxins from other elapids, such as Asn5, Gln6, Gln7, Ser8, Ser9, Gln10, Thr13, Thr24, Lys26, Lys27, Arg33, Glu39, Val49 and Lys50.

A phylogenetic tree mapped for the selected short-chain neurotoxins is shown in Fig. 5. There are two primary divergent groups. Neurotoxins of *P. textilis* form one cluster while the other neurotoxins from Australian elapids, sea snakes as well as Asian cobras form the other divergent group.

DISCUSSION

The cDNAs encoding short-chain neurotoxins of *P. textilis* show that nucleotides 1–63 (Fig. 2) encode a signal peptide consisting of 21 amino acids with a large number of hydrophobic residues including six leucines and two valines. Nucleotides 64–237 of Pt-sntx 1, 2, 3, 6 and 7 encode the five neurotoxin (mature) proteins with 58 amino acid residues per polypeptide, while nucleotides 64–234 of Pt-sntx 5 encode a neurotoxin with 57 amino acid residues. This neurotoxin seems to have lost the Gly34. In addition, Pt-sntx 5 shows Asp35 instead of Asn35 found in the other Pt-sntxs (Fig. 3).

Comparison of the nucleotide sequences of cDNAs encoding short-chain neurotoxins of *P. textilis* shows that the cDNAs of Pt-sntx 2, 3, 5, 6 and 7 are ≈ 95.4, 96.0, 98.2, 89.7 and 92.0% similar to that of Pt-sntx 1 (Fig. 2). The mutations in these six isoforms of neurotoxins are caused by point mutations (29 in total) with a majority of the mutations (80.0%) being concentrated on first and second bases of the codons. This suggests that the neurotoxins have evolved via directional mutations (accelerated evolution) as seen in the case of phospholipase A_2 [44], cardiotoxins [45] and neurotoxins [46] instead of random mutations. Davidson and Dennis [47] pointed out that because digestion of food remains the primary function of the venom, isoforms of toxins could nave a direct correlation to the type of prey that the snakes feed on. Daltry *et al*. [48] have recently proposed that the geographical variation in the venom composition of the pit viper, *Calloselasma rhodostma* is closely associated with its prey. Thus it is possible that such selection pressure may have favored the multiplicity of isoforms of short-chain neurotoxins in *P. textilis*.

Figure 3 shows the complete amino acid sequence of each of the short-chain neurotoxins from *P. textilis* and other selected short-chain neurotoxins. The signal peptide being a classical leader sequence [49] should presumably be involved in the secretion of neurotoxins. The identical leader sequences of *P. textilis* also contain a cysteine residue at position −7, which is known to influence the rate of folding of the neurotoxin [50,51]. The leader sequences of *P. textilis* neurotoxins possess methionine residues (at positions −12 and −8) instead of valines, which are commonly found in the neurotoxins of land snakes (*N. n. sputatrix*) and sea snakes (*Aipysurus laevis*). However, the Val at position −13 in *P. textilis* leader sequence seems to remain as valine in sea snakes but changed to Leu in land snakes. The significance of these substitutions are not known.

Comparison of Pt-sntxs with other short-chain neurotoxin sequences (Fig. 3) shows that the Pt-sntxs form the shortest neurotoxins identified thus far. However, they possess the eight cysteines as in the conventional short-chain neurotoxins and show 40% identity with them.

The *in vitro* and *in vivo* functional studies of Pt-N1 and Pt-N2, which are similar to Pt-sntx5 and Pt-sntx1, respectively, based on the molecular masses, N-terminal sequences and IC_{50} values, show that these are pharmacologically active neurotoxins capable of exerting muscle paralysis, spasms and increased respiration, high binding affinity to AChR from muscles and thus inhibiting the physiological function of the receptor by blocking the binding of acetylcholine. However, both IC_{50} and LD_{50} values were lower than those of typical short-chain neurotoxins, which have dissociation constants in the range 10^{-10} to 10^{-11} M and typical median lethal dose (LD_{50}) values for mice between 50 and 150 $\mu g \cdot kg^{-1}$ [52]. The functional differences could be attributed to the differences in the primary structure and the differences in the conformation of the proteins. Further structural analysis such as CD spectra, NMR and X-ray crystallography will throw more light on these aspects. The striking differences in structures of short-chain neurotoxins of *P. textilis* from those of other short-chain neurotoxins are as follows. First, amino acid deletions can be observed in the first (four amino acids) and third (three amino acids) loop of the neurotoxins. In addition, an insertion of two amino acid residues Thr44 and Ser45 can be observed between loops II and III. At least one proline residue has been lost from the loop I of sntxs. The insertion within Cys42 Gly43 Cys46 between loops II and III separate Gly43 and Cys46, which always remain adjacent to each other in all other short-chain neurotoxins (Fig. 3). A similar insertion involving two Ala residues at this position, in addition to the replacement of Gly by Thr can be observed in long-chain neurotoxins. These two insertions occur in the vicinity of the global head (core) formed by four disulfide bridges of the molecule, which is thought to be occupied by invariant residues. In contrast, the insertion occurred on the top of the globular head, which is shown to be occupied by least-conserved residues [52]. Secondly, Pt-sntxs lack Trp29, which is present in all potent postsynaptic

toxins of cobra and sea snakes. It has been claimed that this residue together with Lys27, Asp31 and Arg33 are essential for the toxicity of erabutoxin [53]. Thirdly, the Pt-sntxs have prolines (Pro30, Pro47 for all Pt-sntxs, Pro38 for only Pt-sntx 2 and Pt-sntx 3, Pro55 for all except Pt-sntx 7) in loops II and III. Another proline (Pro19) can be found at the top of the global head between loops I and II. The other short-chain neurotoxins, however, have the prolines (Pro11 or Pro12 and Pro47) particularly in loops I and III. Pro47 in Eb appears to be an important residue determining the conformation of the closed loop III [54]. As a result of more prolines and differences in distribution of prolines along the peptides, Pt-sntxs might be rendered less flexible, especially in loop II, and this might affect their capability of binding to AChR. However, mutations in Pro11 and Gln12 in Ea produce virtually no effect on the affinity to nAChR [55]. The combination of the above features could contribute to the observed lower binding affinity of Pt-sntxs to AChRs.

Of the six Pt-sntxs, Pt-sntx 2 and 3 are identical except for the substitution Ile29 in Pt-sntx2 for Val29 in Pt-sntx3. These result from first base mutation of the codon in mRNAs. Pt-sntx 5 could have been a product of alternative splicing of mRNA from the Pt-sntx 1 gene as it is shorter by one amino acid residue, Gly34, than Pt-sntx 1. Despite having the same number of amino acid residues as Pt-sntx 1, 6 and 7 possess more amino acid substitutions to make them more different to Pt-sntx 1.

Phylogenetic analysis shows that Pt-sntxs form a special cluster quite different to other snakes, including those found in Australia, which is consistent with the statement by Housset and Fontecilla-Camps [56] that the differences in the core residue sequences may be interpreted as representing evolutionary distance, if the assumption that the core residues play mostly structural roles is correct. It seems that Pt-sntxs and Pa a diverged from an ancestral gene at a very early stage in evolution. Pa a could have evolved into taipan toxins and short-chain neurotoxins found in sea snakes and land snakes of Asia and Africa.

The significance of the presence of short-chain neurotoxins in the venom of *P. textilis* with lower pharmacological effect, in addition to that of Pseudonajatoxin b with relatively high lethality [9] may be related to the prey available in the habitat of the snakes. It is possible that each of these short-chain neurotoxins may exert different physiological effects depending on the prey. Therefore short-chain neurotoxins in *P. textilis* might be more lethal to small lizards and frogs, while Pseudonajatoxin b may be highly lethal to mammals, such as *Mus musculus*, which the snake is known to feed on.

In this study we cloned and expressed a unique set of short-chain neurotoxins from *P. textilis*, one of the Australian elapids. They have the ability to compete with [^{125}I]α-bungarotoxin for the *Torpedo* muscle AChR. The variability seen among the short-chain neurotoxins, appears to have been brought about by point mutations. Further studies on structure–function relationships will provide more insight into their physiological roles *in vivo*.

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