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Two Novel α-Neurotoxins Isolated from Taiwan Cobra: Sequence Characterization and Phylogenetic Comparison of Homologous Neurotoxins

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Two novel postsynaptic neurotoxins (α-neurotoxins) isolated and purified from the Taiwan cobra venom (*Naja naja atra*) possess distinct primary sequences and different neurotoxicities as compared with the most abundant and lethal component in the venom, i.e., cobrotoxin characterized before from the same venom. The complete sequences of two neurotoxin analogues were determined by N-terminal Edman degradation and comparison of amino acid compositions of proteolytic toxin fragments with other homologous toxins of known sequences. The short-chain neurotoxin consists of 61 amino acid residues with eight conserved cysteine residues and is found to show 78% sequence identity with cobrotoxin. The other toxin, consisting of 65 residues with ten cysteines, belongs to the family of long-chain neurotoxins. It is the first long-chain α-neurotoxin reported from the Taiwan cobra. The lethal toxicities of these two novel neurotoxins were much lower than cobrotoxin, albeit with close structural homology among the three toxins in terms of their primary sequences and tertiary structure predicted by homology modeling. Multiple sequence alignment and comparison coupled with construction of a phylogenetic tree for various α-neurotoxins of *Naja* and closely related genuses have established that all nicotinic α-neurotoxins present in the snake family of *Elapidae* are closely related to each other, presumably derived from an ancestral polypeptide by gene duplication and subsequent multiple mutational substitutions.

KEY WORDS: Snake venom; postsynaptic neurotoxins; α-cobratoxin; multiple sequence alignment; sequence comparison; phylogenetic tree.

1. INTRODUCTION

There have been many reports on the characterization of varied types of toxins from the *Elapidae* family of snakes, notably in the cobras of *Naja* genus (Lee, 1979; Karlsson, 1979; Dufton and Hider, 1983). Among these toxins, cardiotoxins, neurotoxins, and phospholipases A_2 are the three major classes of polypeptides involved in the toxicity and pharmacology of bites by these snakes (Dufton and Hider, 1988). All these toxin molecules are chemically and thermally very stable.

In contrast to another prominent group of structurally similar cardiotoxins with no defined cellular targets and very diverse pharmacological functions, neurotoxins (α-cobratoxins) from elapid species possess well-established acetylcholine receptors and modes of action at the molecular level (Changeux, 1981; Ruan *et al.*, 1990, 1991). More than 100 sequences of neurotoxins have been determined from varied snake families. When many homologous sequences are known for a particular class of proteins, comparative analysis of the data can prove to be most informative for their evolutionary relatedness.

Previously we reported that cobra cardiotoxin and its various isoforms (Chiou *et al.*, 1993), like

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other naturally occurring bioactive amphiphilic polypeptides such as mastoparan (Raynor *et al.*, 1991) and melittin (Katoh *et al.*, 1982), are specific and strong inhibitors of protein kinase C (PKC) (Nishizuka, 1988). Interestingly receptor-specific α-cobratoxin (cobrotoxin) of the same cobra venom (Yang *et al.*, 1969) showed no effect on PKC. A systematic structure comparison of these cobra toxins by the secondary-structure predictions together with homology model building (Chiou *et al.*, 1995) based on the primary sequences and the established X-ray and NMR structures of homologous toxins has revealed a grossly similar structure shared by these snake toxins, a three-loop tertiary structure with eight critically important cysteines situated at the base to form a hydrophobic core and three flexible external loops extending outward. In order to gain some insight into the structure–function correlation of various cardiotoxins and neurotoxins with similar tertiary structure, it was deemed essential to isolate and characterize more toxin isoforms or variants for the defined structural comparison to reveal minor structural differences among these analogous toxins which may account for their functional diversity at the molecular level. In the present study we have identified two novel neurotoxic components distinct from the well-known major α-type cobratoxin, i.e., cobrotoxin characterized from Taiwan cobra. Construction of a phylogenetic tree based on multiple-sequence alignment and pairwise comparison of sequence divergence for varied neurotoxins (NTXs) reported for the *Naja* genus corroborate the supposition that these snake neurotoxins form a superfamily with a common ancestry.

2. MATERIALS AND METHODS

2.1. Isolation and Purification of Venom Toxins

The lyophilized venom powder was obtained from Sigma Chemical Company (St. Louis, MO). Various toxins were isolated by cation-exchange chromatography on an open column (50 × 2.5 cm I.D.) packed with TSK CM-650 (M) as described before (Chiou *et al.*, 1993). Dissolved venom powder in 0.025 M ammonium acetate, *p*H 6.0, starting buffer (20–50 mg/ml) was applied to TSK CM-650 column equilibrated with the same buffer, followed by elution in a gradient of 0.7–1.0 M ammonium acetate, *p*H 5.9 buffer. A reverse-phase HPLC (RP-HPLC) was also carried out on a Hitachi' liquid chromatograph with a model L-6200 pump and a variable UV monitor. The column (4.6 × 250 mm, Vydac RP-C_8, 5.0 μm bead) was used to purify and desalt the dried toxin fractions isolated from the above ion-exchange chromatography.

2.2. SDS–Polyacrylamide Gel Electrophoresis

Due to difficulty in the electrophoresis of most snake toxins with small molecular sizes, the purity and subunit molecualr weights of the isolated toxins were checked by 10% Tricine SDS–polyacrylamide slab gel as described (Schagger and von Jagow, 1987).

2.3. Amino Acid Analysis

The amino acid compositions were determined with a Beckman 6300 amino acid analyzer using a single-column system based on a conventional ion-exchange chromatography system. The special facile procedure for the preparation of protein hydrolyzates using heat-resistant Pyrex tubes and high temperature (150°C, 1.5 hr) for amino acid analysis was essentially according to the previous report (Chiou and Wang, 1988).

2.4. Protein Sequence Analysis

The N-terminal sequences of the isolated and desalted peak fractions from the reverse-phase HPLC column were carried out by automated Edman degradation with a pulsed-liquid phase protein sequencer (Model 477A, Applied Biosystems, Foster City, CA). The samples each containing about 1–5 nmol of protein were dissolved in 100 μl of 0.1% trifluoroacetic acid (TFA) and 5 μl each was taken for sequence determinations. For the determination of cysteine residues along the sequences of various NTXs, the purified toxins were reduced with β-mercaptoethanol (MSH, fivefold over toxins) and then reacted with iodoacetic acid (fivefold over MSH). The resulting reduced and carboxylmethylated (RCM-) proteins were then subjected to automated Edman degradation to obtain the N-terminal sequences of intact toxins and confirm the location of cysteine residues along the toxin chains. The RCM-proteins were also digested with Glu-C and Arg-C endoproteinases, and the digests

were separated on RP-C_{18} HPLC (Bio-Rad Bio-Sil ODS-5S C_{18} column, 4×250 mm). For some highly purified NTXs the complete 60–65 residues can be determined in a single nonstop run of successive Edman degradation. By comparing the amino acid compositions and primary sequences determined by the sequencer, the unambiguous sequence assignment of each NTX can be made.

2.5. Sequence Comparison of Various α-Cobratoxins and Homology Search

In the comparison and analysis of amino acid sequences from various α-cobratoxins of the elapid snake family, a multiple-sequence alignment software program (DNASTAR Inc., Madison, WI) was used for the estimation of sequence homology based on percent sequence identity.

2.6. Construction of a Phylogenetic Tree for α-Cobratoxins of *Elapidae*

A LaserGene software package for the Apple Macintosh computer from DNASTAR was used for the estimation of sequence homology based on percent similarity and divergence among different protein sequences. Percent divergence is calculated by comparing sequence pairs in relation to the phylogenetic tree. On the other hand, the percent similarity is estimated by comparing sequences directly without accounting for phylogenetic relationships. A phylogenetic or evolutionary tree was then constructed using the algorithm of Hein (1990) in the MegAlign programs of the package. It is a multiple-sequence alignment program that builds trees as it aligns protein sequences using a combination of distance matrix and approximate parsimony methods. This method constructs multiple alignment by imposing restrictions based on evolutionary relatedness of the aligned sequences, which is useful to align highly evolved gene families that have clear evolutionary relationships such as snake α-cobratoxins.

3. RESULTS AND DISCUSSION

Understanding the mechanism for the evolution of functionally or structurally related proteins from different species remains a general biological problem. Despite the recent rapid development of facile methodology for cDNA amplification and sequence analysis, most snake toxin sequences were derived from conventional protein sequence determination. More than 100 small postsynaptic neurotoxins have been isolated, including short neurotoxins (60–62 amino acids and four disulfide bridges), and long neurotoxins (65–74 amino acids and five disulfide bridges), all sequences sharing significant sequence homology (Karlsson, 1979). The venom of Taiwan cobra (*Naja naja atra*) is extremely lethal when compared with those of crotalid and viperid snakes. One major short neurotoxin (named cobrotoxin) consisting of 62 amino acid residues has previously been characterized as the major principal neurotoxic component from this cobra (Yang, 1965). In the present study two novel neurotoxin isoforms have been isolated from the crude venom of Taiwan cobra which are distinct from the major cobrotoxin.

3.1. Isolation and Characterization of Cobra Neurotoxins

Figure 1 shows an improved elution pattern of the crude venom on a TSK CM-650 cation-exchange column. More than ten fractions were separated by a single run of ion-exchange chromatography using stepwise and gradient elutions in 0.025–1.0 M ammonium acetate, *p*H 5.9 buffer. Three fractions were shown to possess neurotoxicity and identified as P5, P12-1, and a predominant cobrotoxin reported previously (Yang, 1965; Yang *et al.*, 1969). Since P5 and P12-1 have not been reported before, they were further purified on reverse-phase HPLC based on their subtle differences of surface hydrophobicity (Wu *et al.*, 1982; Bougis *et al.*, 1986). In Fig. 2 we compare the retention times on reverse-phase HPLC for three neurotoxic components. It is of interest to note that a purified fraction from P5, designated as P5-1, was shown to have the same retention time as the major cobrotoxin, indicative of their being closely related neurotoxin isoforms. All these three HPLC-purified components were also shown to be pure and homogeneous as judged by showing single bands in SDS-gel electrophoresis (Fig. 3). In Fig. 3 we compare the other two major toxin components of cobra venom, i.e. phospholipase A_2 and cardiotoxin, with these three α-type cobratoxins. It is evident that by our purification protocol of combining ion-exchange and reverse-phase chro-

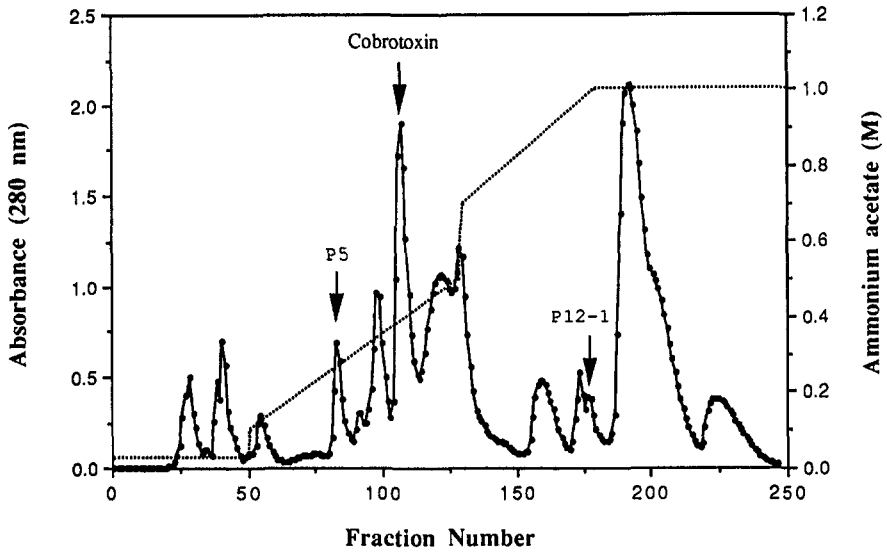

Fig. 1. Chromatography of crude venom of *Naja naja atra* on a TSK CM-650 column. The lyophilized venom (400 mg) was dissolved in 10 ml of 0.025 M ammonium acetate buffer (*p*H 6.0) and applied to the column (2.5 × 50 cm) equilibrated with the same buffer. After the column had been washed with 300 ml of the initial buffer, the proteins adsorbed were eluted with a two-stage linear gradient (0.1–0.5 M and 0.7–1.0 M ammonium acetate buffer) as indicated in the figure. The column eluates (6 ml/tube/7.5 min) were monitored for absorbance at 280 nm. The arrows indicate the fractions collected for further purification on a reverse-phase HPLC (RP-HPLC).

matographies, all three major components could be separated clearly without cross-contamination, good enough for further structural and functional studies. The molecular weights for these three neurotoxins were estimated to be about 7000–7500.

3.2. Amino Acid and Sequence Analysis of Purified Neurotoxins

The amino acid compositions of three purified neurotoxins (NTXs) isolated from Taiwan cobra are shown in Table I. They consist of 60–65 residues with eight or ten cysteines, similar to two types of neurotoxins reported in the literature. The common characteristics of amino acid compositions of purified NTX 5-1 and cobrotoxin lie in their lack of Ala, Met, and Phe, in contrast to NTX 12-1, which possesses one residue of Ala and Phe each. We have completely sequenced these neurotoxin fractions by straightforward Edman degradation. The pure samples as indicated in clear-cut amino acid compositions (Table I) and single bands shown in SDS-PAGE (Fig. 3) made the sequencing analysis of the whole 60–65 amino acid residues in nonstop single runs of successive Edman degradation possible. The additional steps taken in confirming the structures are (i) preparation of reduced and carboxymethylated (RCM-) toxins for the detection of PTH-cysteine residues, since the recovery of this PTH-amino acid is usually poor for the unmodified proteins, (ii) microsequencing analysis of the blotted bands from SDS-PAGE gel, and (iii) the comparison of amino acid contents for the proteolytic fragments of each NTX by digestion with Glu-C and Arg-C endoproteinases. The complete sequences of NTX 5-1 and NTX 12-1 as determined by these relatively simple procedures are shown in Figs. 4A and 4B for sequence comparison with homologous NTXs from other elapid snakes. The sequence of cobrotoxin was checked and found to be identical to that reported previously (Yang *et al.*, 1969).

3.3. Structural Comparison of Homologous Neurotoxins of Elapid Snakes

Multiple sequence alignments were used for the comparative analysis of the determined amino acid sequences of NTX 5-1 (Fig. 4A) and NTX 12-1 (Fig. 4B) and those homologous sequences reported for other α-type cobratoxins. It was found that these two neurotoxins of Taiwan cobra show about 70–97% sequence identity to their respective short- and long-chain NTXs of other species. It is of interest to note that short- and long-chain NTXs show less than 20% sequence identity (e.g., NTX

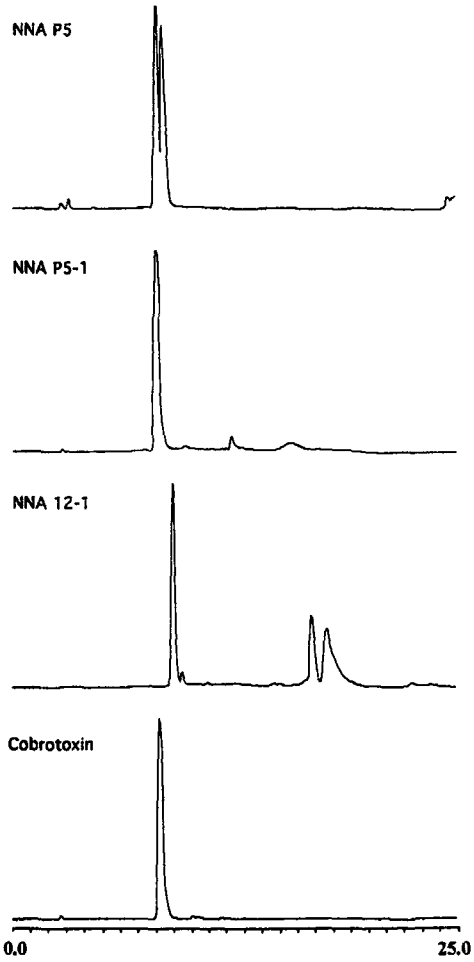

Fig. 2. Purification of neurotoxin fractions (NTXs) on a reverse-phase HPLC (RP-HPLC). The peak fraction of NTXs collected in Fig. 1 was lyophilized and chromatographed on a RP-HPLC column (C_8, 0.46 × 25 cm). Solvent A: 0.1% TFA in water/acetonitrile (90/10). Solvent B: 0.1% TFA in water/acetonitrile (10/90). The separation was carried out in a linear gradient of 25–40% solvent B in solvent A for 25 min; the elution was run at 1.0 ml/min and the eluates monitored at 280 nm.

5-1 and NTX 12-1 show only 13.1% sequence similarity). In general, due to the presence of one extra pair of disulfide bonds in long-chain NTXs, it is difficult to have an optimal alignment between these two classes of NTXs for homology comparison. This certainly underlines the unusual evolutionary relationship between these two classes of NTXs and deserves a further study on their tertiary structures.

In Figs. 4A and 4B we show the optimal alignment by introducing a minimum number of gaps along the entire lengths of toxin sequences.

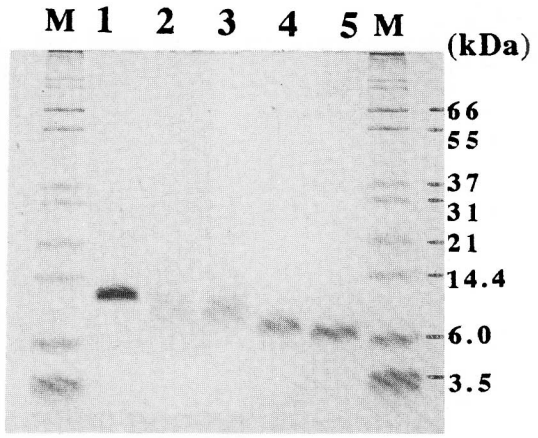

Fig. 3. Gel electrophoresis of purified fractions from RP-HPLC under denaturing conditions (10% Tricine-SDS-PAGE) in the presence of 5 mM dithiothreitol. Lane M, standard proteins used as molecular mass markers (in kDa): bovine serum albumin (66), glutamic dehydrogenase (55), lactate dehydrogenase (37), carbonic anhydrase (31), soybean trypsin inhibitor (21), lysozyme (14.4), aprotinin (6.0), and insulin B chain (3.5). Lanes 1–5 correspond to the purified proteins from RP-HPLC for (1) phospholipase A_2, (2) cobrotoxin, (3) NTX 5-1, (4) NTX 12-1, and (5) cardiotoxin of crude venom, respectively (Fig. 1). The gels were stained with Coomassie blue.

Table I. Amino Acid Composition of Neurotoxin Isoforms and Cobrotoxin from Formosan Cobra[a]

Amino acid	NTX 5-1	NTX 12-1	Cobrotoxin
1/2 Cys	7.2(8)	9.1(10)	8
Asx	8.2(8)	6.2(6)	8
Thr	7.6(8)	3.8(4)	8
Ser	3.7(4)	1.0(1)	4
Glx	6.3(6)	5.3(5)	7
Pro	3.4(3)	3.0(3)	2
Gly	5.4(5)	3.0(3)	7
Ala	0.0(0)	1	0
Val	2.0(2)	2.9(3)	1
Met	0.0(0)	0.0(0)	0
Ile	1.7(2)	3.7(4)	2
Leu	2	5.4(5)	1
Tyr	1.0(1)	2.9(3)	2
Phe	0.0(0)	1.0(1)	0
His	2.0(2)	2.0(2)	2
Lys	4.9(5)	9.4(9)	3
Arg	3.4(3)	5.3(5)	6
Trp	N.D.	N.D.	1
Total	59	65	62
R.T. (min)	7.983	8.940	8.168

[a] Amino acid composition expressed as the number of residues per molecule of protein using leucine (NTX 5-1) and alanine (NTX 12-1) as the references to calculate the other amino acids; and those of cobrotoxin are from Yang *et al.* (1969). Values represent the mean of duplicate determinations. The hydrolysis condition is 150°C and 90 min using 6 N HCl.

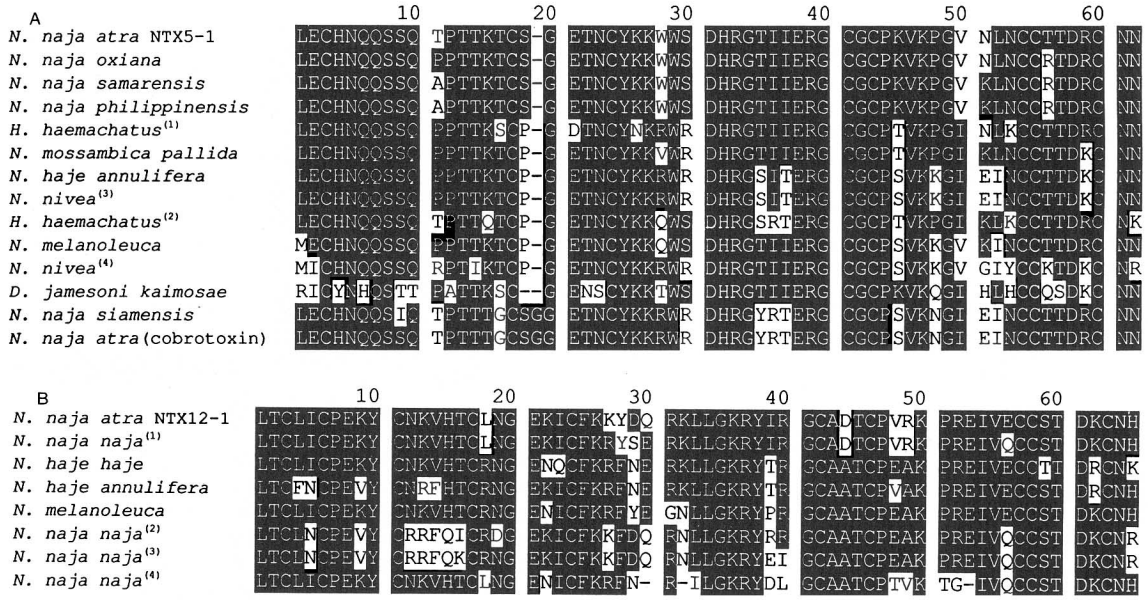

Fig. 4. Multiple sequence alignment and sequence comparison of short (A) and long (B) chain neurotoxin sequences. The identical amino-acid residues among various sequences were expressed in white letters with black-background blocks. All sequences except NTX 5-1 and NTX 12-1 were taken from the Protein Data Bank of the EMBL Data Library. The gaps were introduced for optimal alignment and maximum homology among the sequences. Superscript numerals in parentheses indicate multiple neurotoxin isoforms used for sequences (A) (1) short neurotoxin 1 (H.h-1), (2) short neurotoxin 2 (H.h-2), (3) short neurotoxin 2 (N.n-2), (4) short neurotoxin 1 (N.n-1), NTX 5-1 (N.n.a.-2), Cobrotoxin (N.n.a-1); (B) (1) neurotoxin 6 (N.n.n.-6), (2) neurotoxin 7 (N.n.n-7), (3) neurotoxin 8 (N.n.n-8), (4) neurotoxin 5 (N.n.n-5), NTX 12-1 (N.n.a-3). All other toxins are abbreviated according to snake species.

Without exception all short- and long-chain NTXs compared show long stretches of good matches and their cysteine residues all lie at relatively identical positions. The sequence variation in each class of NTX seems to be fairly conservative, indicating a preservation of a common structure in each class of NTX during species diversification. However, the high divergence in protein sequences between short- and long-chain NTXs appears to be the result of comparing paralogous products of different toxin genes in the elapid family of snakes.

In the pairwise sequence comparison of NTX 5-1 and NTX 12-1 with those published NTXs in the protein data banks using the software package from DNASTAR, sequence homology ranging from 75% to 97% was found among those NTXs found in cobras of some *Naja* genus, with homology of >90% found for some closely related species such as Taiwan and Thailand cobras. We have compared the general distribution of surface-charge groups in these structurally related toxins using the program analysis of surface hydrophilicity in these sequences based on the Kyte–Doolittle hydropathy scale (data not shown). All members of the short-chain NTXs show similar hydrophathy patterns, which are different from those of long-chain NTXs. Apparently the polypeptide folding should be more similar among each member of the same class than that from different class of NTXs.

3.4. Sequence Divergence and Construction of a Phylogenetic Tree

In our systematic pairwise sequence comparison of crystallin genes and their deduced protein sequences from varied species of the animal kingdom, higher sequence homology is generally found between cDNA sequences than protein sequences (Chiou, 1988; Lu *et al.*, 1996). It seems to be more sensitive to detect sequence divergence based on protein sequences than cDNA sequences when comparing highly homologous protein families. We have therefore constructed a phylogenetic tree based on all short- and long-chain NTX sequences shown in Fig. 4A and 4B using the algorithm of Hein (1990), which is a multiple-sequence alignment program that builds trees as it aligns phylogenetically related protein sequences using a combination of distance matrix and approximate parsimony methods. It has been

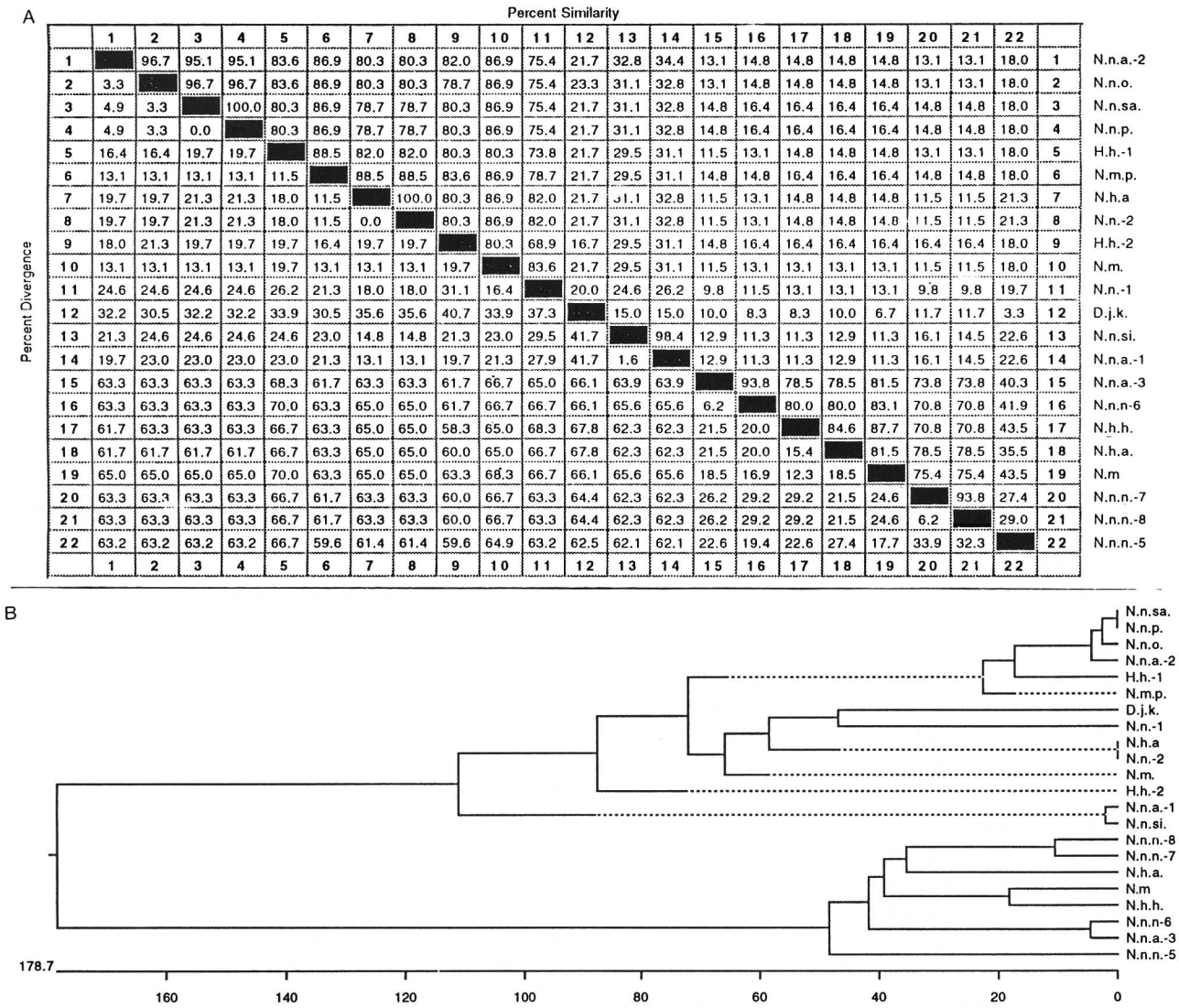

Fig. 5. (A) Pairwise comparison of amino acid sequence similarity and divergence and (B) construction of phylogenetic tree of 22 short- and long-chain NTXs of various species from Fig. 4. Analysis of sequence data was carried out with a LaserGene software package for the Apple Macintosh computer (DNASTAR Inc., Madison, WI). Percent divergence is calculated by comparing sequence pairs in relation to the relative positions in the phylogenetic tree. On the other hand, the percent similarity is estimated by comparing percent sequence identity directly without accounting for phylogenetic relationships. A phylogenetic tree was then constructed based on the percent divergence between protein sequences using a combination of distance matrix and approximate parsimony methods in the phylogeny generation program of Hein (1990). This algorithm carries out multiple alignment by imposing restrictions based on evolutionary relatedness of the aligned sequences. The length of each pair of branches represents the sequence distance between aligned pairs. The scale beneath the tree measures the distance between sequences (in millions of years). The dotted lines indicate that the sequence distance is not proportional to the scale.

shown to be useful to align highly evolved gene families and their corresponding protein sequences that have clear evolutionary relationships as in the case of the γ-crystallin family (Lu *et al.*, 1996). The results (Fig. 5) indicate that the phylogenetic tree based on the sequence divergence among these toxin sequences indeed exemplifies the close relatedness between various toxin members of short-chain or long-chain NTX class. It is also of interest to find that two classes of neurotoxins indeed are located at different branching points of the tree (Fig. 5B). This leads to the supposition that short- and long-chain NTXs diverged from the common ancestral neurotoxin molecule and the

evolutionary rate (percent divergence) for short-chain NTX seems to be much higher than that for long-chain NTX. It would be useful for the cDNA for each member of the NTXs to be available in order to provide a phylogenetic tree based on DNA divergence to shed some light on the evolution of neurotoxins at the gene level.

4. CONCLUSION AND PERSPECTIVES

The search for a structural or molecular basis to account for the different pharmacological actions of cardiotoxins and neurotoxins has prompted us to isolate and characterize various minor and novel cardiotoxins (Hung *et al.*, 1993) and neurotoxins (this report). These two major classes of toxins share a common three-dimensional folding pattern as revealed by X-ray crystallography: a core consisting of a series of short loops and four disulfide bridges. A novel long-chain neurotoxin isoform isolated and reported for the first time from Taiwan cobra should prove to be a useful tool for examining the structure/function relationship for short- and long-chain neurotoxins. Preliminary study by homology modeling and computer graphics of neurotoxins has revealed some defined and major differences between these two types of α-cobratoxins (manuscript in preparation). Multiple-sequence alignment and comparison coupled with construction of a phylogenetic tree for various α-neurotoxins of *Naja* and closely related genuses have corroborated the results of computer modeling in establishing that all nicotinic α-neurotoxins present in the snake family of *Elapidae* are closely related to each other in terms of primary and tertiary structures, presumably derived from an ancestral polypeptide by gene duplication and subsequent multiple mutational substitutions.

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