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Characterization of α-neurotoxin and phospholipase A_2 activities from *Micrurus* venoms

Determination of the amino acid sequence and receptor-binding ability of the major α-neurotoxin from *Micrurus nigrocinctus nigrocinctus*

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New World elapids are coral snakes that belong to the genus *Micrurus*, and for which the venom biochemistry is mostly unknown. Analysis has been difficult because the coral snakes produce small quantities of venom. Clinical observations following bites show mainly neurotoxic effects. Experimentally, cardiotoxic, haemolytic and myotoxic activities are also reported. An experimental approach, using reverse-phase high-performance liquid chromatography and specific assays for α-neurotoxin and phospholipase A_2 activities, was conducted on milligram quantities of venoms from three *Micrurus* species from Costa Rica; *M. nigrocinctus nigrocinctus*, *M. alleni yatesi* and *M. multifasciatus*. Neurotoxicity was determined by competition binding experiments with the *Torpedo marmorata* acetylcholine receptor. Phospholipase A_2 activity was measured by fluorimetry using a pyrene lipid substrate. In this way, we purified and characterized seven α-neurotoxins, five phospholipases A_2 and four toxin homologs. The amino acid sequence of the major α-neurotoxin from *M. nigrocinctus nigrocinctus* venom was fully determined and compared to Old Word representatives. Distance matrix data were generated to set up phylogeny relationships among elapid short-chain α-neurotoxins, which proved to be in accordance with the taxonomic classification and geographical distribution of snake species.

Keywords: *Micrurus*; α-neurotoxin; phospholipase A_2; venom.

The elapid representatives of the New World are coral snakes. They comprise a group of about 120 species and subspecies (genera *Micrurus*, *Leptomicrurus* and *Micruroides*) distributed from Southern United States to Argentina (Mc Dowell, 1987; Campbell and Lamar, 1989). Clinical observations following bites show mainly neurotoxic effects, and in severe cases of envenomation, death due to respiratory arrest (Weis and Mc Isaac, 1971; Ramsey et al., 1972; Vital Brazil et al., 1976, 1977; Moussatche and Melendez, 1979). Experimental studies suggest an extended spectrum of activities in *Micrurus* venoms. Neurophysiological changes similar to those induced by α-neurotoxins have been observed, as well as presynaptic effects (Parmentier and Carpenter, 1976; Vital Brazil and Fontana, 1983, 1984; Goularte et al., 1995). Cardiotoxic, haemolytic and myotoxic activities have been reported also (Gutiérrez et al., 1980, 1983, 1986, 1992; Tan and Ponnudurai, 1992). The major obstacle to study *Micrurus* venoms is the small quantity of material that can be collected (Bolanõs, 1972). Thus, the biochemistry and the pharmacology of components isolated from coral snake venoms are not well documented. After the first attempts to purify venom proteins (Ramsey et al., 1971), only two phospholipases A_2 have been characterized from *Micrurus fulvius microgalbineus* and *M. nigrocinctus nigrocinctus* venoms (Possani et al., 1979; Arroyo et al., 1987). More recently, a comparative chromatography study on Brazilian coral snake venoms has been conducted (Da Silva et al., 1991). From an immunological point of view, *Micrurus* and other elapid venoms contain some cross-reacting components (Minton, 1979; Alape-Girón et al., 1994). Cross-neutralization among some *Micrurus* venoms has been described (Bolaños et al., 1975, 1978; Bolaños and Cerdas, 1980), although the venom of *Micrurus multifasciatus* and *Micrurus surinamensis* seem to be unique in their antigenic characteristics (Bolaños et al., 1978).

Considering the lack of knowledge of *Micrurus* venom biochemistry, in this work we applied several micro-methods to analyze minute quantities of venoms from the three species: *M. nigrocinctus nigrocinctus* (Coral macho), *M. alleni yatesi* and *M. multifasciatus* (Gargantilla). Several α-neurotoxins, phospholipases A_2 and toxin homologs were characterized. The complete amino acid sequence of a *Micrurus* α-neurotoxin was determined and compared by phylogeny to Old World representatives. This is the first report, to our knowledge, of an amino acid sequence of an α-neurotoxin for American elapids.

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Abbreviation. LD_{50}, 50% lethal dose.

MATERIALS AND METHODS

Venoms and reagents. *M. nigrocinctus nigrocinctus*, *M. alleni yatesi* and *M. multifasciatus* venoms were from the Clodomiro Picado Institute, University of Costa-Rica. The *M. nigroc-*

inctus nigrocinctus venom was a pool of 30 mg obtained from adults specimens. The *M. multifasciatus* and *M. alleni yatesi* venoms were samples of 10 mg each obtained from one adult specimen/species. After being collected, venoms were clarified by centrifugation, lyophilized and kept at −70°C. Rehydrated venoms were first dialyzed against water at 4°C using Spectrapore (Spectrum Medical Industries) with a pore size limit of 3000 Da, then lyophilized. HPLC-grade formic acid, analytical-grade chemicals and reagents were from Merck. HPLC-grade acetonitrile was from Touzard et Matignon. Sequencing-grade trifluoroacetic acid was from Sigma. Enzymes were from the following sources: *Staphylococcus aureus* V_8 from Miles Laboratories, and carboxypeptidase A from Sigma. Water was drawn from a MilliRO/MilliQ system (Millipore).

High-performance liquid chromatography. Venom fractionation by reverse-phase HPLC was carried out on a 10 mm×250 mm column prepacked with 5 µm ultrasphere-octyl (Beckman Instruments) as previously described (Bougis et al., 1986). The solvents were: A, 0.15 M ammonium formate, pH 2.70, conductivity 12 mS; B, acetonitrile. The flow rate was 5 ml/min. The sample loaded was 1.5 mg of dialyzed venom. The gradient of solvents was 8−35% B in A, for 95 min. Peptide purification was performed on a 4.6 mm×250 mm column packed with 5 µm ultrasphere-ODS (Beckman). The solvent were: A, 0.1% trifluoroacetic acid; B, acetonitrile made 0.05% trifluoroacetic acid. The flow rate was 1 ml/min. The gradient of solvents was 5−39% B in A, for 45 min. 0.5-ml fractions were collected. The absorbance was monitored at both 214 nm and 280 nm.

Protein chemistry. 15% PAGE was performed at pH 4.5 (Riesfeld et al., 1962). About 2 nmol protein was loaded onto a polyacrylamide gel poured in 200 µl micro-pipette (Scientific Manufacturing Industries). 12.5% SDS/PAGE was based on methods by Laemmli (1970) and Swank and Munkres (1971), using molecular-mass markers of 2500−17 000 Da. Amino acid analyses were performed on a Beckman 6300 auto analyzer after protein samples of 0.5−1.0 nmol had been hydrolyzed at 110°C under vacuum for 20 h or 70 h in the presence of 6 M HCl. Tryptophan was estimated after *p*-toluene sulfonic acid hydrolysis. Reduction and *S*-carboxymethylation of *M. nigrocinctus nigrocinctus* I were performed with 200 nmol native protein in 660 µl propanol-1 and 0.5 M $NaHCO_3$, pH 8.3 (by vol.). After addition of 200 µl 5% tributylphosphine in propanol-1, the reaction was extended for 4 h at 25°C with agitation in the dark under a nitrogen atmosphere. Carboxymethylation was obtained according to the method of Crestfeld et al. (1963). The sample was then separated by chromatography on a 1 cm×20 cm column prepacked with 200−400 mesh Biogel P2 (Bio-Rad Laboratories) in 30% CH_3COOH. *S. aureus* V8 (10%, by mass) cleavage of 40 nmol reduced and *S*-carboxymethylated *M. nigrocinctus nigrocinctus* I was performed at 37°C during 48 h in 5 mM NH_4HCO_3, pH 7.9, with 50% of the enzyme added first, then again after 24 h. Peptides produced from the cleavage were purified by reverse-phase HPLC, as described above. Protein and peptide sequencing were performed on 1−2 nmol samples using a Beckman 890 M sequenator. Characterization of phenylthiohydantoin−amino-acids was obtained by HPLC according to the method of Bosc-Bierne et al. (1981). Carboxypeptidase A (2%, by mass) hydrolysis was carried out at 37°C in 0.2 M 4-ethylmorpholine, pH 8.5. Sample aliquots were removed after 1, 5, 15, 30, 60, 180, 480 and 1360 min, acidified with an equal volume of acetic acid, lyophilized and analyzed for their amino acid content.

Screening for α-neurotoxin activity. A STGV-Millititer 96-well filtration plate (0.22 µm pore size, Millipore) receptor assay using ^{125}I-toxin I from *Naja mossambica mossambica* and *Tor-*

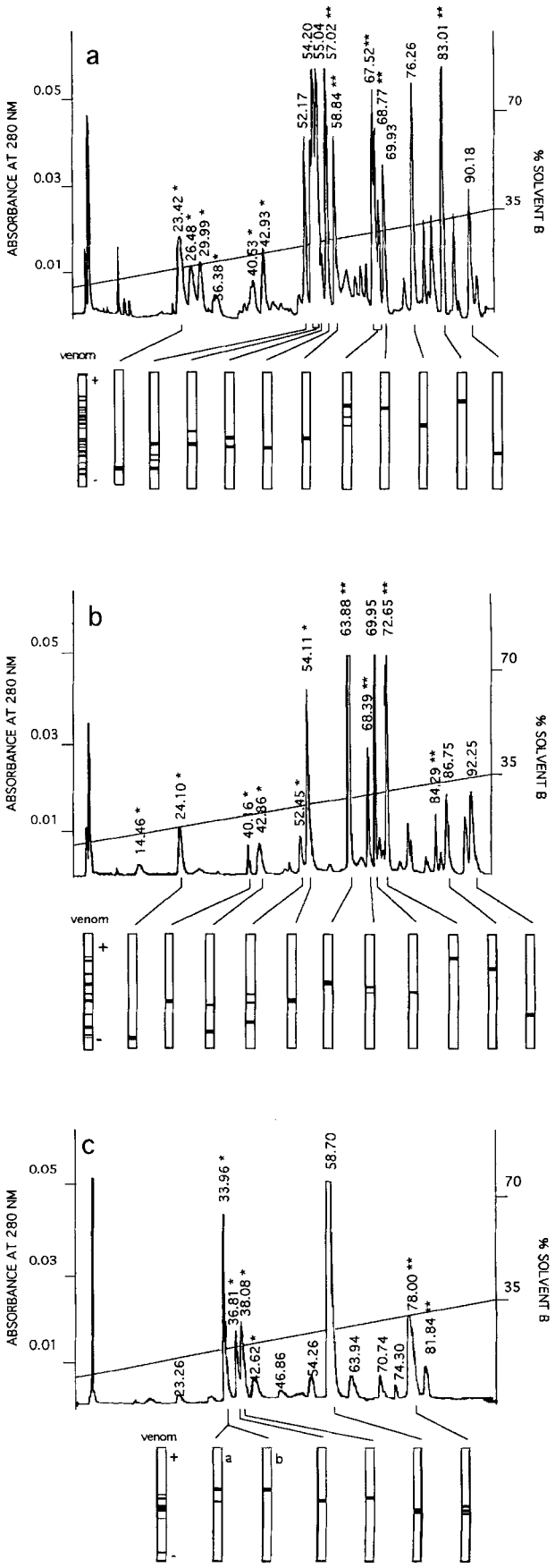

Fig. 1. Reverse-phase HPLC of (a) *M. nigrocinctus nigrocinctus* (b) *M. alleni yatesi* and (c) *M. multifasciatus* venoms. Fractions are identified by the retention time (min). PAGE data are shown below the chromatograms. *, fractions with α-neurotoxic activity; **, fractions with phospholipase A_2 activity.

Table 1. Characteristic data of the fractions issued from reverse-phase HPLC of the *Micrurus* venoms (see Fig. 1). n.d., not determined.

Micrurus venom	Retention time	Total absorbance at 280 nm	Intracerebro-ventricular LD_{50}	Intraperitoneal LD_{50}	Phospholipase A2 activity
	min	%	µg/mouse		µmol · min^{-1} · mg^{-1}
M. nigrocinctus nigrocinctus				6.7	
	23.42	2.5	1.30	1.30	
	52.17	4.0			
	54.20	14.7			
	55.04	7.2			
	57.02	7.5	3.00	>20	0.13
	58.84	4.0	2.35	>20	0.10
	67.52	14.1			
	68.77				
	69.93	4.5			
	76.26	5.7			
	83.01	14.4	0.45	>20	3.07
	90.18	4.0			
M. alleni yatesi				12.0	
	24.10	3.2			
	40.16	1.0			
	42.86	2.6			
	52.45	1.8			
	54.11	10.9	0.10	1.50	
	63.88	37.6	0.01	>20	1.31
	68.39	3.9			
	69.95	6.8			
	72.65	12.7	0.25	>20	11.05
	86.75	4.1			
	92.25	5.4			
M. multifasciatus				23.0	
	33.96 a	5.0			
	33.96 b	5.0		8.0	
	36.81	4.1			
	38.08	5.5			
	58.70	52.6	0.04	>20	
	78.00	10.9			n.d.

pedo marmorata electric organ membrane fragments was utilised for processing each HPLC fraction, as previously described (Bougis et al., 1986). The binding competition experiment was carried out using the same experimental procedure (Marchot et al., 1988).

Screening for phospholipase A_2 activity. Phospholipase A_2 activity was measured using a fluorescent pyrene lipid substrate, as previously described (Bougis et al., 1986).

***In vivo* assay.** NMRI white mice (Evic-Ceba) of 20 g were used to perform lethality tests. Injection was made by the intraperitoneal, intravenous or intracerebroventricular routes (Haley and Mc Cormik, 1957). The 50% lethal dose (LD_{50}) was determined using the formulation of Behrens and Karber (1935).

Sequence data analysis. The search for sequence similarities and sequence data analyses was made using BISANCE, a French service for access to biomolecular sequence databases (Dessen et al., 1990). FASTA was used to search for similarities within the NBRF-PIR protein sequence library with *ktup* of 2. CLUSTAL W (1.4) used to generate multiple sequence alignment and distance matrix data, i.e. pairwise alignment parameters, gap open penalty of 10, gap extension penalty of 0.1, protein weight matrix PAM350; multiple alignment parameters, gap opening penalty 10, gap extension penalty 0.05. From the phylogeny inference package (PHYLIP, version 3.5 C), FITCH and DRAWTREE were run on distance matrix data to generate an unrooted tree. A rooted phenogram was obtained by running KITSCH and DRAWGRAM. In both cases, the input order of species was randomized 30 times and the power was set to 2.

RESULTS AND DISCUSSION

The majority of Old World elapid venoms includes α-neurotoxins, cardiotoxins (or cytotoxins) and phospholipases A_2. With the development of HPLC, micromethods in protein chemistry and sensitive biological assays, considerable progress has been made concerning the purification and the characterization of animal toxins. Such techniques are particularly advantageous for the investigation of *Micrurus* venoms. Adult specimens of some *Micrurus* species give an average of 7 mg dried venom/milking.

HPLC of venoms. Venom polypeptides, such as toxins, have a compact spatial structure, which allows their charge and hydrophobic character to be expressed mainly on the surface of the molecule. Hence, α-neurotoxins elute mostly at the beginning of the acetonitrile gradient, followed by phospholipases A_2. Alternatively, cardiotoxins, which are essentially basic and hydrophobic, are generally eluted at the end of the acetonitrile gradient. As previously determined, the following reverse-phase HPLC experimental conditions gave the best resolution of polypeptidic

Table 2. Amino acid composition and molecular masses of seven α-neurotoxins isolated from *M. nigrocinctus nigrocinctus* (Mnn), *M. alleni yatesi* (May), and *M. multifasciatus* (Mm) venoms. Underlined numbers refer to the differences compared to elapid short-chain and long-chain α-neurotoxins. n.d., not determined. The numbers refer to the retention time of the HPLC fraction (see Fig. 1).

Amino acid	Short-chain α-neurotoxin	Mnn 23.42 (Mnn I)	May 24.10	May 46.10	Mm 33.96b	Mm 36.81	Mm 38.08	Long-chain α-neurotoxin	May 54.11
Aspartic acid	5–9	4 (3.96)	4 (4.13)	5 (5.12)	4 (4.00)	5 (5.04)	5 (4.90)	6–10	11 (11.19)
Threonine	5–9	5 (4.81)	6 (5.99)	5 (4.71)	4 (3.95)	6 (5.76)	8 (7.84)	5–9	5 (4.79)
Serine	6–10	6 (5.87)	4 (3.72)	8 (7.82)	7 (6.81)	4 (4.08)	4 (3.79)	3–6	3 (2.90)
Glutamic acid	5–8	6 (6.12)	6 (6.19)	4 (4.20)	6 (6.17)	5 (5.11)	7 (7.22)	1–6	3 (2.13)
Proline	2–5	4 (4.02)	4 (4.03)	2 (1.81)	2 (2.08)	2 (1.97)	2 (2.16)	6–8	2 (2.09)
Glycine	5–7	5 (5.09)	5 (5.39)	6 (6.05)	8 (8.17)	7 (7.00)	6 (5.94)	4–6	5 (5.00)
Alanine	0–1	2 (2.12)	2 (2.25)	3 (2.76)	2 (1.98)	1 (1.08)	1 (0.91)	2–5	4 (4.10)
1/2 Cystine	8	8 (6.25)	8 (6.48)	8 (6.39)	8 (7.02)	8 (7.11)	8 (6.70)	10	10 (8.60)
Valine	1–2	1 (0.93)	2 (2.06)	1 (0.87)	3 (3.01)	2 (1.81)	3 (2.81)	2–8	3 (2.92)
Methionine	0–1	1 (0.87)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.84)	1 (0.92)	0–2	1 (0.80)
Isoleucine	1–5	5 (5.11)	2 (1.84)	3 (2.89)	1 (0.83)	4 (4.07)	3 (2.90)	3–5	2 (2.02)
Leucine	0–2	0 (0.00)	3 (2.75)	1 (0.87)	2 (2.01)	1 (1.11)	1 (1.08)	1–5	3 (3.05)
Tyrosine	1–3	1 (0.91)	1 (0.87)	1 (0.74)	2 (1.86)	3 (2.78)	2 (1.98)	1–3	2 (1.90)
Phenylalanine	0–1	0 (0.00)	2 (1.90)	2 (1.71)	1 (1.09)	1 (1.14)	0 (0.00)	1–3	4 (4.08)
Histidine	1–3	3 (3.14)	0 (0.00)	0 (0.00)	2 (2.10)	1 (1.17)	1 (1.03)	0–1	2 (2.10)
Lysine	3–7	5 (5.12)	10 (9.95)	5 (4.73)	4 (4.03)	5 (5.08)	4 (3.72)	4–9	10 (10.12)
Arginine	3–6	3 (3.07)	1 (0.91)	4 (4.09)	1 (1.09)	3 (2.87)	3 (2.85)	3–6	3 (3.03)
Tryptophan	1–2	1 (0.89)	1 (1.23)	1 (0.95)	2 (1.89)	1 (0.86)	1 (1.04)	1–3	2 (1.88)
Total	60–62	60	61	59	59	60	60	71–74	74
M_r (calculated)		6562	6560	6247	6268	6622	6508		9665
M_r (SDS/PAGE)		6500	n.d.	n.d.	6600	n.d.	n.d.		9600

toxins: elution solvent A, 0.15 M ammonium formate, pH 2.70; conductivity 12 mS; elution solvent B, acetonitrile; stationary silica phase bound with medium acyl chain (C8) (Bougis et al., 1986). We used the same HPLC experimental conditions to fractionate *Micrurus* venoms. To eliminate the nontoxic low molecular-mass components, venom was extracted first with water, then dialyzed using a pore size limit of 3000 Da. At 280 nm, the overall yield in absorbance was 95%. All proteins were eluted before 35% acetonitrile. *M. nigrocinctus nigrocinctus* venom had the most complex chromatogram (Fig. 1a, Table 1). This may be due to genetic variability because *M. nigrocinctus nigrocinctus* venom was a pool of many individuals. In contrast, *M. alleni yatesi* and *M. multifasciatus* venoms, both issued from one individual, were simpler (Fig. 1b and 1c, Table 1). This observation was also made studying scorpion venoms (Martin et al., 1987). The main HPLC fractions were analyzed by native PAGE and assayed for phospholipase A_2 activity, binding competition with ^{125}I-toxin I from *Naja mossambica mossambica* to acetylcholine receptor from *Torpedo marmorata*, lethality in mice and amino acid composition. Accordingly, 16 proteins were purified to homogeneity and characterized. If larger quantity of venoms were available for separation by HPLC, more proteins could have been characterized.

α-neurotoxins. 16 HPLC fractions displayed α-neurotoxin activity (Fig. 1a–c). Table 2 shows the amino acid composition of seven α-neurotoxins, corresponding to fractions for which a unique band was observed by native PAGE. Six of them were short-chain α-neurotoxins (59–61 amino acid residues, eight Cys residues), and one (*M. alleni yatesi* 54.11) was a long-chain α-neurotoxin (74 amino acid residues, 10 Cys residues). As mentioned above, all were eluted at the beginning of the acetonitrile gradient. According to both native PAGE analyses and amino acid compositions, *M. alleni yatesi* 24.10 was the most basic neurotoxin, and *M. multifasciatus* 33.96b was the most acid. Lethality in mice occured at 1–8 μg/mouse (Table 1). However, *M. alleni yatesi* 54.11 was tenfold more lethal when injected into the brain rather than in the peritoneal cavity; this is not common for α-neurotoxins. The amino acid composition of *M. alleni yatesi* 54.11 was also unusual, with a high number of Lys and Asp esidues, and a low number of Pro residues. ^{125}I-Toxin I from *N. mossambica mossambica* and *M. nigrocinctus nigrocinctus* 23.42, the major α-neurotoxin from *M. nigrocinctus nigrocinctus* venom, and subsequently denoted *M. nigrocinctus nigrocinctus* I, were assayed in binding competition experiments. The competition occured within two orders of magnitude of the *M. nigrocinctus nigrocinctus* I concentration and gave 50% of the maximum inhibition of ^{125}I-toxin I binding at 0.2 nM ($K_{0.5}$). A K_d of 19 pM was calculated according to the equation: $K_{0.5} = K_d\,(1+[^{125}\text{I-toxin I}]/K_d^*])$. K_d^* (7 pM) was the dissociation constant for ^{125}I-toxin (Marchot et al., 1988). This value of K_d is in the concentration range expected for α-neurotoxin binding to acetylcholine receptors at the neuromuscular junction (Changeux et al., 1970).

Amino acid sequence of *M. nigrocinctus nigrocinctus* I and short-chain α-neurotoxin phylogeny relationships. The first and complete α-neurotoxin amino acid sequence determination from *Micrurus* species is presented here (Fig. 2). Direct sequencing on reduced and S-carboxymethylated *M. nigrocinctus nigrocinctus* I permitted the identification of 25 positions out of 27. At position 19, the Glu residue allowed the use of *S. aureus* V8 protease to generate peptides. Peptides P 37–60 and P 20–36 were HPLC purified and sequenced in full. Carboxypeptidase A hydrolysis of *M. nigrocinctus nigrocinctus* I gave the three C-terminal amino acids (positions 58–60). Herein, the complete amino acid sequence of *M. nigrocinctus nigrocinctus* I had 60 amino acid residues. FASTA was used to search for homologous

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RCM-Mnn I      MICHNQQSSQPPTIKTCSEG-CYKK-W                              CNA
P 20-36                          GQCYKKTWRDHRGTISE
P 37-60                                                 RGCGCPTVKPGIHISCCASDKC--
               MICHNQQSSQPPTIKTCSEGQCYKKTWRDHRGTISERGCGCPTVKPGIHISCCASDKCNA
                .    .    .    .    .    .    .
               1    10   20   30   40   50   60
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Fig. 2. Determination of the amino acid sequence of *M. nigrocinctus nigrocinctus* I (Mnn I). RCM-Mnn I, reduced and *S*-carboxymethylated Mnn I; P 20–36, peptide 20–36; P 37–60, peptide 37–60. Underlined residues were obtained from carboxypeptidase A digestion of RCM-Mnn I.

NBRF-PIR FASTA CLUSTAL W (1.4) multiple sequence alignment
initn

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                    1        10        20        30        40        50        60
                    .         .         .         .         .         .         .
Mnn I               MICHNQQSSQ PPTIKTCS-- EGQCYKKTWR DHRGTISERG CGCPTVKPGI HISCCASDKC NA

N1NJ1B      268     LE........ ...T...PG. .TN....V.. ......I... .......... KLN..TT... .N
N1NJ2E      266     .......... .......PG. .TN....R.. ......I... ....S..K.V G.Y..KTN.. .R
N1EP1J      265     R..Y.H..TT .A.T.S.G.. .NS......S ......I... ....K..Q.. .LH..Q.... .N
N1EP1V      264     R..Y.H..TT .A.T.S.G.. .NS......S ......I... ....K..R.V .LH..Q.... .N
N1NJ1C      262     .......... R......PG. .TN....R.. ......I... ....S..K.V G.Y..KT... .R
N1EP1D      258     R..Y.H..TT RA.T.S.E.. .NS....Y.. ......I... ....K....V G.H..Q.... .Y
B39327      257     .E........ ...TTH..GG .TN..E.R.H ......I... .........V KLN..TT... .N
N1NJ1R      252     LE........ ...T....G. .TN....W.S ......I... ....K....V NLN..RT.R. .N
N1NJ1W      252     .E........ ...T...PG. .TN....Q.S ......I... ....S..K.V K.N..TT.R. .N
N1NJ1M      246     LE.......E ...TTR..GG .TN....R.. ....YRT... .......K.. ELN..TT.R. .N
N1NJ1P      245     LE........ A..T....G. .TN....W.S ......I... ....K....V KLN..RT.R. .N
N1NJ1S      245     LE........ A..T....G. .TN....W.S ......I... ....K....V KLN..RT.R. .N
N1NJ1F      244     LE........ T..TTG..GG .TN....R.. ....YRT... ....S..N.. E.N..TT.R. .N
N1RI1       242     LE........ ...T.S.PG. DTN..N.R.. ......I... .......... NLK..TT.R. .N
JK0221      241     LE......I. T..TTG..GG .TN....R.. ....YRT... ....S..N.. E.N..TT.R. .N
N1RI2       241     LE........ T..TQ..PG. .TN....Q.S ....SRT... .......... KLK..TT.R. .K
N1LT1E      240     R..F.H.... .Q.T....PG .SS..N.Q.S .F....I... .......... KL...E.EV. .N[1]
F25866      237     RR.F...... .Q.N.S.PPG .NS..R.Q.. ......I... .......... KLR..Q.ED. .N
B25866      236     RR.Y...... .K.T.S.PPG .NS..N.Q.. ....S.T... .......... KLR..E.ED. .N
E25866      236     RR.F...... .Q.N.S.PPG .NS..R.Q.. ......I... .........V KLR..Q.ED. .N
N1AW1       236     .Q.C...... .K.TT..PGG VSS....... ......I... ....R..... RLI..KT.E. .N
N1NJ3M      234     LN....M.A. ...TTR..RW .TN....R.. ....YKT... .......K.. QLH..T..N. .N
N1AY1       232     LT.C...... .K.TTD.AD. -NS......Q .....RI... ....Q..... KLE..KTNE. .N
S02484      230     .T.C...... .K.TTN.AG. -NS......S ......I... ....E..S.. KLE..HTNE. .N
N1EY1H      230     .T.C...... .K.TTN.A.. -SS....... .F...RI... ....Q..... KLE..HTNE. .N
A27580      229     .T.C...... .K.TTN.AG. -NS......S ......I... ....Q..S.. KLE..HTNE. .N
N1AT1       229     .T.C...... .K.TTN.AG. -NS......S ......I... ....Q..S.. KLE..HTNE. .N
N1NJ3E      216     ...YK...L. F.ITTV.PG. .KN....Q.S G.....I... ....S..K.. E.N..TT... .R
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[1] Erabutoxin a

Fig. 3. Sequence alignment between *M. nigrocinctus nigrocinctus* I (Mnn I) and other elapid short-chain α-neurotoxins using CLUSTAL. NBRF-PIR library accession number and FASTA initn score are indicted on the left side. Dots are identities with Mnn I amino acid sequence.

sequences in the NBRF-PIR library (release 43). Only one (rank 139) of the 150 sequences with the highest similarity score did not fit with the elapid toxin family. The highest score observed, with 77% identity in a 60-amino-acid overlap, aligned with the α-neurotoxin I from *Naja mossambica mossambica*. From the 49 top ranking sequences, all being short-chain α-neurotoxins, 29 were selected (1/snake species) and subjected to a blocked multiple alignment using CLUSTAL. A consensus of 62 positions was generated (Fig. 3). Two gaps were introduced at positions 20 and 21 within the *M. nigrocinctus nigrocinctus* I sequence (positions refer to the consensus sequence). Cytotoxins, cardiotoxins, fasciculins and other members of the elapid toxin family have a structural unit in common with α-neurotoxins. The structural unit for α-neurotoxins is a dense core rich in disulfide bridges from which emerge three long loops (loop I is from the N-terminal) displayed as the central fingers of a hand (Low et al., 1976). For all of them, several amino acid residues are conserved and believed to be relevant to the structural integrity of the molecule. Among them are height Cys residues at positions 3, 17, 24, 41, 43, 54, 55 and 60, Tyr at position 25, Gly at position 40, Pro at position 44, and Asp at position 61. Based on recent mutagenesis studies on erabutoxin a, the functional site is spread over the three toxin loops, mostly on the concave face (Pillet et al., 1993; Trémeau et al., 1995). The site comprises three polar clusters, Lys and Gln at positions 27 and 38, respectively, and Arg and Asp at positions 33 and 31, respectively, in loop II, and Gln at positions 7 and 10 in loop I, which all interact with the central hydrophobic cluster made of Trp and Ile at positions 29 and 36, respectively, in loop II. The site also encompasses Ser at position 8, with its hydroxyl group forming a hydrogen bond with the NH group of Ile at position 37, and Lys at position 47, which is the only loop-III residue that seems to be involved. All of these amino acid residues were well conserved in the *M. nigrocinctus nigrocinctus* I sequence. The amino acids not yet found in short-chain α-neurotoxins are Gly-Gln at positions 22–23, Ser at position 37 and Ala at positions 56 and 62.

Since the *M. nigrocinctus nigrocinctus* I sequence was the first determined for the American elapids, estimating the phylogeny relationships was of interest. FITCH was run on distance

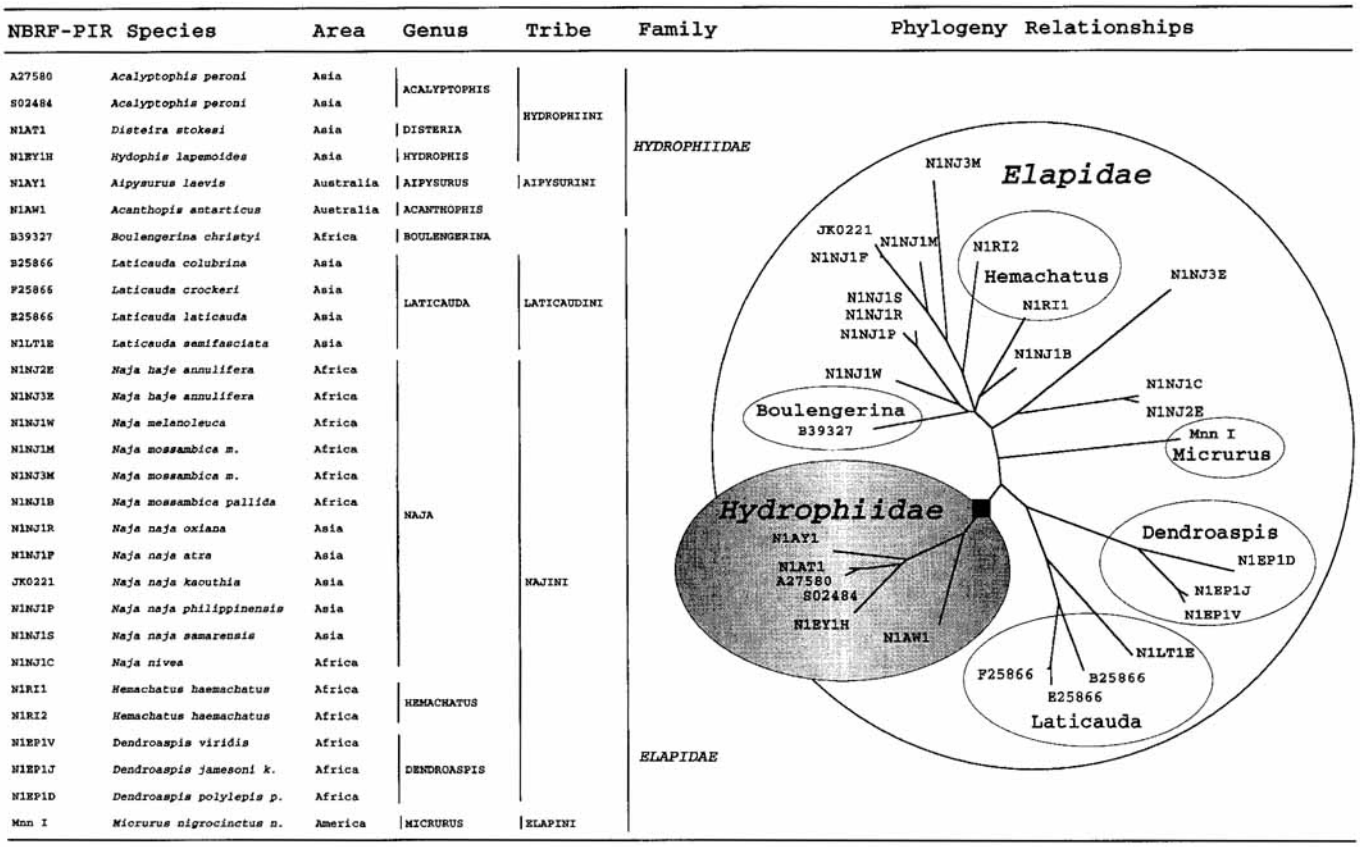

Fig. 4. Phylogeny relationships among short-chain α-neurotoxins. Taxonomic data are from Harding and Welch (1980). Input distance matrix data correspond to the sequence alignment of Fig. 6. The filled square locates the root of the phenogram generated when KITSCH is run on the same distance matrix data.

matrix data output issued from the previous multiple alignment (Fig. 3) to generate unrooted phylogenies (sum of squares of 4.87). The species involved, their geographical distribution and taxonomic classification (Harding and Welch, 1980) and the unrooted tree constructed are shown (Fig. 4). All species were proteroglyphe snakes from the *Elapidae* and *Hydropiidae* families. The tree depicted a branching pattern for the sequences that overall corresponded closely to the snake taxonomic classification. In this pattern, *M. nigrocinctus nigrocinctus* I may well be located in a monophyletic group. The determination of the amino acid sequences of other α-neurotoxins from *Micrurus* venoms, such as *M. alleni yatesi* 24.10, 46.10, 54.11 and *M. multifasciatus* 33.96b, will be of interest to define this group better. Since the tree was unrooted, it was not possible to determine the direction of the genetic events, i.e. the ancestral sequence. Yet, there was a possibility to assume an evolutionary clock using KITSCH on the same distance matrix data (sum of squares of 13.15). The resulting phenogram (not shown) had an identical phylogeny pattern and a root located on the segment joining *Hydrophiidae* and *Elapidae* (see the filled square in Fig. 4). This was consistent with the taxonomic classification and the geographical distribution of the snake species involved.

Phospholipases A_2. 11 HPLC fractions displayed phospholipase A_2 activity, but only five appeared homogenous by PAGE (Fig. 1a–c, Table 1).

Compared to an average amino acid composition, there seemed to be a low number of Pro (*M. nigrocinctus nigrocinctus* 83.01, 58.84, and *M. alleni yatesi* 63.88), Tyr (*M. alleni yatesi* 63.88 and 72.65) and Lys residues (*M. nigrocinctus nigrocinctus* 83.01 and *M. alleni yatesi* 63.88; Table 3). *M. nigrocinctus nigrocinctus* 57.02 was the most basic phospholipase A_2 if compared to *M. alleni yatesi* 72.65, which was the most acid phospholipase A_2. The five phospholipases A_2 displayed no toxicity when injected intraperitonealy or intravenously at up to 20 μg/mouse. No bleeding or sputum were observed. However, when they were injected intracerebroventricularly into the brain at doses ranging over 10–3000 ng/mouse, toxicity developed, with *M. alleni yatesi* 63.88 being the most toxic (Table 1). It was noticeable that the specific enzymic activity was not directly proportional to lethality, as was the case for *M. alleni yatesi* 63.88. Myotoxicity has been already observed for *M. nigrocinctus nigrocinctus* 83.01 (Arroyo et al., 1987).

Toxin homologs. Snake venoms are known to contain toxin homologs (on a structural basis), for which no peripheral lethality is observed and, *a fortiori*, no biological function has been recognized (Joubert et al., 1975, 1978). We isolated to homogeneity four toxin homologs, which on the basis of the amino acid composition were not related to α-neurotoxin or phospholipase A_2 (Table 4). In the peripheral nervous system, *M. multifasciatus* 58.70 had no lethal effect up to 20 μg/mouse, whereas in the central nervous system lethality was promoted at a very low dose (Table 1). From *Bungarus multicinctus* venom, Dryer and Chiappinelli (1983) isolated κ-bungarotoxin, which is responsible for the blockade of the neuronal nicotinic acetylcholine receptor, without any action at the neuromuscular junction. Comparison of amino acid compositions of *M. multifasciatus* 58.70 and κ-bungarotoxin showed some identities, specially for Ser, Lys, and Trp residues. For the three other toxin homologs, *M.*

Table 3. Amino acid composition and molecular masses of five phospholipases A_2 isolated from *M. nigrocinctus nigrocinctus* (Mnn) and *M. alleni yatesi* (May) venoms. Underlined numbers refer to the differences compared to elapid phospholipases A_2. The numbers refer to the retention times of the HPLC fractions (see Fig. 1a and b).

Amino acid	Mnn 57.02	Mnn 58.84	Mnn 83.01	May 63.88	May 72.65	Phospholipase A_2
Aspartic acid	17 (17.05)	15 (14.98)	17 (16.86)	20 (20.20)	20 (20.08)	15–23
Threonine	8 (7.84)	8 (7.71)	6 (5.78)	8 (7.71)	12 (11.54)	3– 9
Serine	5 (5.05)	6 (5.83)	6 (6.14)	6 (6.01)	8 (7.91)	3– 9
Glutamic acid	5 (5.10)	7 (7.07)	8 (7.71)	6 (6.11)	12 (12.17)	5–13
Proline	3 (3.01)	2 (2.01)	2 (2.05)	2 (2.01)	4 (4.04)	3– 6
Glycine	7 (7.07)	7 (7.00)	8 (7.78)	8 (8.05)	8 (7.97)	6–11
Alanine	6 (5.89)	5 (5.13)	7 (7.20)	6 (6.10)	8 (8.02)	8–11
1/2 Cystine	14 (12.87)	12 (10.77)	12 (10.24)	12 (10.47)	14 (13.01)	12–14
Valine	5 (5.01)	4 (3.86)	4 (4.04)	3 (2.87)	4 (3.98)	2– 5
Methionine	2 (1.82)	2 (1.91)	2 (2.05)	1 (0.86)	2 (1.94)	1– 3
Isoleucine	2 (2.05)	2 (2.00)	3 (2.79)	2 (1.92)	4 (4.01)	2– 9
Leucine	7 (7.11)	6 (6.03)	6 (5.82)	7 (7.14)	8 (7.89)	3– 8
Tyrosine	7 (6.91)	6 (5.81)	6 (5.82)	2 (1.92)	2 (1.95)	7–11
Phenylalanine	5 (5.12)	4 (4.18)	5 (5.25)	4 (4.06)	4 (4.09)	2– 4
Histidine	4 (4.09)	3 (3.14)	4 (4.08)	2 (2.17)	4 (4.02)	1– 4
Lysine	15 (15.18)	11 (10.89)	7 (6.86)	11 (10.88)	8 (8.17)	5–11
Arginine	4 (4.11)	4 (4.07)	4 (3.92)	3 (3.11)	4 (3.88)	2– 9
Tryptophan	3 (2.88)	3 (2.91)	3 (2.80)	2 (2.12)	4 (3.81)	0– 3
Total	119	107	110	105	130	120[a]
M_r (calculated)	13 723	12 472	12 704	11 597	14 475	
M_r (SDS/PAGE)	14 000	12 000	12 000	11 000	15 000	

[a] Average number.

Table 4. Amino acid composition and molecular masses of four toxin homologs, not related to α-neurotoxin nor phospholipase A_2, isolated from *M. nigrocinctus nigrocinctus* (Mnn), *M. alleni yatesi* (May) and *M. multifasciatus* (Mm) venoms, and comparison with short-chain and long-chain toxin homologs, cardiotoxins, and κ-bungarotoxin. The numbers refer to the retention times of the HPLC fractions (see Fig. 1).

Amino acid	Short-chain homolog	Cardio-toxin	Mnn 90.18	May 69.95	May 92.25	Long-chain homolog	κ-bungaro-toxin	Mm 58.70
Aspartic acid	2–9	5– 8	4 (4.12)	5 (5.09)	6 (5.88)	4–6	7	6 (6.09)
Threonine	4–8	2– 4	3 (2.98)	5 (4.76)	3 (2.88)	2–8	6	6 (5.84)
Serine	2–5	2– 3	6 (5.67)	3 (2.71)	4 (3.81)	0–3	6	6 (6.01)
Glutamic acid	2–7	0– 3	5 (5.12)	5 (5.08)	4 (4.11)	5–7	6	7 (7.12)
Proline	2–5	4– 6	3 (3.02)	2 (1.75)	4 (4.11)	0–4	5	4 (4.02)
Glycine	2–6	2– 3	5 (5.14)	7 (7.09)	4 (4.20)	3–4	3	4 (4.17)
Alanine	0–4	1– 3	2 (2.00)	6 (6.08)	1 (1.02)	1–3	2	2 (1.98)
1/2 Cystine	8	8	8 (7.23)	8 (7.44)	8 (7.43)	10	10	10 (8.25)
Valine	0–4	2– 6	2 (2.12)	2 (2.03)	1 (0.89)	1–4	2	1 (0.91)
Methionine	0–2	2– 4	0 (0.00)	1 (0.91)	0 (0.00)	0–2	0	0 (0.00)
Isoleucine	0–6	1– 4	3 (2.78)	1 (0.98)	5 (4.88)	2–4	4	2 (1.92)
Leucine	1–7	4– 6	2 (2.05)	4 (3.98)	3 (3.00)	3–4	4	3 (3.11)
Tyrosine	2–4	2– 4	1 (0.93)	1 (0.87)	1 (0.79)	1–3	1	1 (0.92)
Phenylalanine	0–3	0– 2	2 (2.14)	1 (1.01)	3 (3.07)	1–4	3	2 (2.09)
Histidine	1–2	0– 1	1 (1.11)	1 (1.02)	0 (0.00)	1–2	1	2 (2.12)
Lysine	3–7	6–10	5 (5.01)	4 (4.21)	10 (10.17)	5–7	2	2 (2.03)
Arginine	2–5	1– 3	1 (1.12)	1 (1.04)	2 (2.15)	4–9	4	5 (4.97)
Tryptophan	0–1	0– 1	2 (1.76)	1 (0.93)	2 (1.69)	0–3	0	1 (0.90)
Total	57–65	60	55	58	61	62–64	66	64
M_r (calculated)			5995	6074	6766			8144
M_r (SDS/PAGE)			6750	6500	6900			8300

nigrocinctus nigrocinctus 90.18, *M. alleni yatesi* 69.95 and 92.25, a slight similarity was observed with the cardiotoxin amino acid composition (Table 4). Unfortunately, no laboratory assay is available for such minute quantities of cardiotoxins.

The results obtained here constitute a contribution to the knowledge of the venoms of American elapids. Such comparative study of *Micrurus* venoms should permit a better understanding of the biological and immunological relationships of these venoms and might contribute to the design of more effective antivenoms. In addition, *Micrurus* venoms may be a source of toxins valuable for understanding basic cellular process.

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