Characterization of multiple nicotinic acetylcholine receptor-binding proteins and phospholipases A_2 from the venom of the coral snake *Micrurus nigrocinctus nigrocinctus*

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Abstract **The presence of multiple nicotinic acetylcholine receptor (AchR)-binding proteins and phospholipases A_2 was detected in the venom of a member of the Elapinae subfamily, *Micrurus nigrocinctus nigrocinctus*. Multi-step chromatographies were used to isolate four AchR-binding proteins (Mnn-9, Mnn-4, Mnn-3C and Mnn-1A) and five basic PLA_2s (nigroxins A, B, C1, C2 and C3). The *Micrurus* AchR-binding proteins are antigenically and structurally related to short- and long-chain α-neurotoxins from *Naja*. The nigroxins are antigenically similar and constitute a new antigenic subclass of PLA_2s. Nigroxins A and B are class I PLA_2s, structurally more related to enzymes from Bungarinae than to those from Hydrophinae/Laticaudinae. These data contribute to clarify the relationships between *Micrurus* venom proteins and other elapid toxins and may be useful to improve the neutralizing efficiency of antivenoms.**

Key words: Phospholipase A_2; Neurotoxin; HPLC; Monoclonal antibody; Amino acid sequence; *Micrurus*

1. Introduction

Snake venoms are complex mixtures of proteins with pharmacological properties of medical interest [1]. Elapids are classified into six subfamilies distributed worldwide: Hydrophiinae (sea snakes and Australian elapids), Bungarinae (cobras, kraits and mambas), Laticaudinae, Calliophiinae, Maticorinae (Asian coral snakes) and Elapinae (American coral snakes). There are studies on venom proteins from the three former subfamilies, while little is known about proteins from Calliophiinae, Maticorinae and Elapinae. Up to 70 proteins with similar molecular weights have been identified in a single elapid venom [2]. However, the best-characterized elapid toxins are phospholipases A_2 (PLA_2s), α-neurotoxins and cardiotoxins [3,4].

Micrurus snakes are the most abundant elapids in America and *M. nigrocinctus* is medically the most important coral snake of Central America [5]. Its venom induces paralysis, myotoxicity and electrophysiological changes that suggest the presence of post- and presynaptically acting toxins [6,7]. We previously found that *M. n. nigrocinctus* venom binds to the nicotinic acetylcholine receptor (AchR) and that antivenom inhibits the binding of heterologous short- and long-chain α-neurotoxins to AchR [8]. We also produced monoclonal antibodies (MAbs) against *M. n. nigrocinctus* venom proteins and isolated, by immunoaffinity with MAb 9A, nigroxin, a myotoxic fraction with PLA_2 activity [5]. We have now isolated four *M. n. nigrocinctus* AchR-binding proteins, resolved nigroxin into five closely related isozymes and studied their antigenic and structural relationships with other elapid neurotoxins.

2. Materials and methods

2.1. Venom, toxins and antibodies

M. n. nigrocinctus venom was from Instituto Clodomiro Picado [5]; *Naja naja atra* α-cobrotoxin from Ventoxin (Frederick, MD); *Naja naja oxiana* neurotoxins I and II and *Bungarus multicinctus* α- and β-bungarotoxins from Sigma; and *Notechis scutatus* notexin kindly provided by Dr. Bertil Persson (Uppsala University). Nigroxin was immunoaffinity-purified with MAb 9A [5]. *M. n. nigrocinctus* venom proteins were separated on a C4 column (250 × 10 mm, BioRad) with a Waters 600E instrument using linear gradients of acetonitrile in 0.1% trifluoroacetic acid (TFA) and flow rates of 2 ml/min. Samples dissolved in phosphate-buffered saline (PBS) were acidified to pH 2.5 with TFA before injection. Ion-exchange chromatography was carried out with a Mono S HR 16/10 column and a Pharmacia FPLC instrument using linear gradients of NaCl and flow rates of 3 ml/min. Samples were dissolved in a 50 mM HEPES buffer, pH 8.0. Homogeneity of isolated proteins was verified by reverse-phase HPLC, capillary electrophoresis [9] and N-terminal sequence analysis.

Mouse antisera to *Naja naja atra* α-cobrotoxin and *N. n. oxiana* neurotoxins I and II were generated as described [10,11]. Antisera against notexin and β-bungarotoxin were prepared in rabbits by s.c. injection of native toxins (1 μg) emulsified in Freund's complete adjuvant (Difco, Detroit, MI). Subsequent boosters with increasing amounts of native toxins (1 to 24 μg) were given weekly, three in Freund's complete adjuvant and eight in Freund's incomplete adjuvant. Rabbit antisera to taipoxin, textilotoxin and mulgotoxin were from Venom Supplies (Tanunda, Australia). Horse *M. nigrocinctus* antivenom was produced as described [12]. Ascites fluids containing MAbs were produced in pristane-primed BALB/c mice [5,11].

2.2. Assays for AchR-binding, PLA_2 activity and immunoreactivity

AchR purified from *Torpedo californica* (Pacific Biomarine, Venice, CA) was used for binding assays [8]. Competitive binding assays were done by incubation of the AchR (5 μg) with varying amounts of *Naja n. oxiana* neurotoxin II for 20 min at room temperature and the mixture added to wells coated with *M. n. nigrocinctus* venom proteins. The PLA_2 activity was measured [13] and enzyme-linked immunosorbent assays (ELISAs) were performed as described [8]. For the capture ELISA, microtiter wells were coated overnight with

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M. nigrocinctus antivenom diluted in carbonate buffer, pH 9.6. The remaining binding sites were blocked with PBS containing 2% serum albumin (blocking solution) for 15 min at 37°C. After aspiration, nigroxins (0.5 μg/well) diluted in blocking solution, were added and incubated for 1 h at 37°C, before addition of antisera, conjugate and substrate [8]. Absorbances were read after 60 min at 405 nm in a MR500 Dynatech instrument. Titration curves were obtained and the titer defined as the negative logarithm of the dilution yielding 0.3 absorbance units.

2.3. Sequence analysis and comparisons

Nigroxins A and B, in 0.4 M Tris-HCl, pH 8.1 buffer, containing 6 M guanidine-HCl and 2 mM EDTA, were reduced with 300 mM dithiothreitol for 2 h at 37°C and carboxymethylated with iodo[^{14}C]acetate for 2 h at 37°C. After removal of reagents by gel filtration chromatography on PD-10 (Pharmacia), samples were submitted to sequence analysis with Applied Biosystems 470A or MilliGen/Biosearch 6600 instruments. AchR-binding proteins were analysed without carboxymethylation. Homology searches were done in the National Cancer Institute mainframe data base (Frederick, MD) using the GCG sequence analysis software package.

3. Results

3.1. Fractionation of M. n. nigrocinctus venom proteins

Venom proteins were fractionated by reverse-phase HPLC (Fig. 1). Fractions 9–11 and 19–20 exhibited AchR-binding activity that was dose-dependently inhibited by *N. n. oxiana* neurotoxin II, indicating the presence of multiple AchR-binding proteins. Fractions 9–11 were also recognized by antibodies against *N. n. oxiana* neurotoxin II and *N. n. atra* α-cobrotoxin, suggesting that they contained short-chain α-neurotoxin(s). Twelve HPLC fractions had PLA_2 activity and six of them were also recognized by antibodies to notexin (Fig. 1). These results indicate that *M. n. nigrocinctus* venom has an extensive multiplicity of PLA_2s that differ in hydrophobicity, including several isozymes antigenically related to notexin. Six MAbs against *M. n. nigrocinctus* venom PLA_2s [5] reacted differently with fractions having PLA_2 activity: MAb 9A recognized fractions 28–30, indicating that nigroxin contains the least hydrophobic PLA_2 isozymes of the venom. MAb 7H reacted as MAb 9A, whereas MAbs 4, 21, 27 and 28, also recognized some of the most hydrophobic PLA_2s, indicating the existence of epitopes shared between isozymes with very different hydrophobicities.

3.2. Analysis of nigroxin

Immunoaffinity-purified nigroxin was separated into five peaks by Mono S (Fig. 2). Material corresponding to these peaks contained PLA_2 isozymes, designated nigroxins A, B, C1, C2 and C3, that were homogeneous by capillary electrophoresis. Upon reverse-phase HPLC, nigroxins C2 and C3 coeluted later than isozymes A, B and C1, indicating that the two former proteins are more hydrophobic than the latter three. Sequence analysis (Table 1) showed nigroxins A and B to differ only at positions 3–5 in their N-terminal segments, which are 64–71% identical with those of *N. n. atra* PLA_2 and *N. melanoleuca* DE III [3].

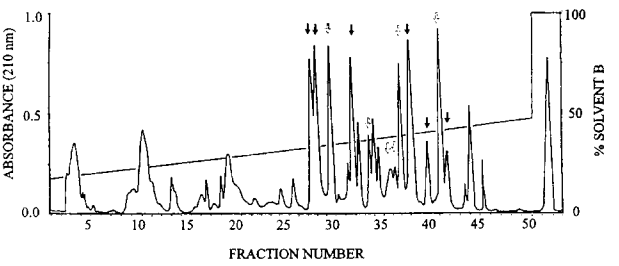

Fig. 1. Elution profile of crude *M. n. nigrocinctus* venom (4 mg) on reverse-phase HPLC, obtained with a linear gradient of CH_3CN; solvent A: 0.09% aqueous TFA; solvent B: A + 70% CH_3CN; gradient: 19–48% B in 48 min. Fractions having phospholipase A_2 activity are noted by arrows (black and white). Fractions recognized by antibodies to *N. scutatus* notexin in an ELISA are indicated by white arrows.

Antisera to *M. nigrocinctus* venom and various elapid PLA_2s were titrated in an ELISA against nigroxins. The antivenom and antisera to textilotoxin and nigroxin A had equivalent titers for all nigroxins. In contrast, antiserum to mulgotoxin had higher titers with nigroxins C2 and C3 than with the other isozymes. Antisera to β-bungarotoxin and taipoxin were not reactive with any of the nigroxins and antiserum to notexin recognized only isozymes C2 and C3 when the nigroxins were adsorbed onto microtiter wells. However, all nigroxins were recognized if captured by an antivenom to *M. nigrocinctus* (Fig. 3). Rabbit antiserum to nigroxin A cross-reacted in an ELISA with notexin, confirming the existence of common epitope(s) among these proteins.

Six MAbs to *M. n. nigrocinctus* PLA_2s [5] reacted similarly with nigroxins A and B (Table 2); MAbs 4, 7H and 9A had higher titers for these isozymes than for nigroxins C1, C2 and C3, whereas MAb 27 had similar titers for nigroxins A, B and C3, but only weakly recognized isozymes C1 and C2. Thus, the five nigroxin isozymes share several epitopes, but nigroxins A and B are antigenically the most closely related.

3.3. Analysis of AchR-binding proteins

Fraction 9 (Fig. 1) was rechromatographed on C4 and eluted with a linear gradient of acetonitrile, 14–21% in 30 min. The material corresponding to the major peak was applied to Mono S and eluted with a linear gradient of NaCl, 0.04–0.24 M in 20

Table 1
The N-terminal sequences of the two basic phospholipases A_2 and four AchR-binding proteins isolated from *M. n. nigrocinctus* venom

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Basic phospholipases																														
Nigroxin A	N	L	I	D	F	K	N	M	I	K	C	T	N	T	R	H	W	V	S	F	T	N	Y	G	C	Y	C	G	G	Y
Nigroxin B	N	L	Y	Q	L	K	N	M	I	K	C	T	N	T	R	H	W	V	S	F	T	N	Y	G	C	Y	C	G	G	Y
AchR-binding proteins																														
Mnn-9	M	I	X	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	X	S	E	G			Q	X	Y	K	K	T		
Mnn-4	L	K	X	Y				S	S		R	T	E	T	M	T	X	P	E	G	K	D	K	X	E	K	Y	A	V	
Mnn-3C	K	K	X	L	T	K	Y	S	A	G	L	Q	T	S	Q	T	X	P	A	G	Q	K	I	X	F		K	K	W	
Mnn-1A	S	K	X	R		I	G	K	D		G	F	Y	S	V	T	X	T	E	K	E	N	L	X	F	T	Q	F		

Empty spaces indicate deletions assigned to maximize homology with known toxins, X indicates unassigned residue.

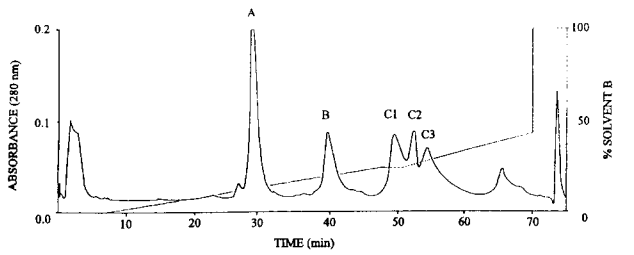

Fig. 2. Elution profile of nigroxin on Mono S FPLC with a two-step linear gradient, 0–0.12 M NaCl in 36 min, isocratic elution for 4 min, 0.12–0.22 M NaCl in 20 min.

min. Only one protein, Mnn-9, was eluted at 0,07 M NaCl. Its N-terminal segment (Table 1) is 81% identical with the short-chain α-neurotoxins *N. n. oxiana* neurotoxin II and *N. melanoleuca* toxin d [14].

Fractions 10 and 11 (Fig. 1) were applied to Mono S and eluted with a linear gradient of NaCl, 0.04–0.34 M in 30 min. Two major proteins with AchR-binding activity, Mnn-4 and Mnn-3C, were eluted at 0.08 and 0.22 M NaCl, respectively. Both were homogeneous by reverse-phase HPLC. The N-terminal segment of Mnn-4 (Table 1) is 35% identical with those of *N. n. oxiana* neurotoxin II and *N. n. atra* α-cobrotoxin, while the N-terminal part of Mnn-3C is 42% identical with that of the long-chain α-neurotoxin 3.9.4 from *N. melanoleuca* [14]. Mnn-3C was also recognized in an ELISA by polyclonal antibodies to the long-chain α-neurotoxin *N. n. oxiana* neurotoxin I, but not by five MAbs to this toxin [11].

Fraction 19 (Fig. 1) was applied to Mono S and eluted with a linear gradient of NaCl, 0.04–0.34 M in 30 min. Two major proteins eluted at 0.05 and 0.08 M NaCl, but only the first eluted one, designated Mnn-1A, had AchR-binding activity. Mnn-1A was homogeneous by reverse-phase HPLC and its N-terminal sequence (Table 1) is 32% identical with that of *N. melanoleuca* toxin 3.9.4 [14].

In summary, four *M. n. nigrocinctus* AchR-binding proteins were isolated and found to be structurally and antigenically similar to *Naja* α-neurotoxins, Mnn-9 and Mnn-4 to short-chain toxins and Mnn-3C and Mnn-1A to long-chain toxins.

4. Discussion

Presynaptically active PLA_2s and postsynaptic neurotoxins have been characterized earlier from venoms of Hydrophiinae, Laticaudinae and Bungarinae, but not from Elapinae. We now demonstrate that *M. n. nigrocinctus* venom, of the Elapinae subfamily, contains multiple AchR-binding proteins and PLA_2s related to toxins from Bungarinae. The findings also suggest that the neurotoxic effect of this venom is not due to a single major component, but results from the combined action of several toxins.

On reverse-phase HPLC, PLA_2s eluted later than the AchR-binding proteins, which is in agreement with the known interfacial affinity of PLA_2s for phospholipids [15]. There were more and higher peaks containing PLA_2 activity, vs. AChR-binding activity. Furthermore, an extensive multiplicity of antigenically similar *M. n. nigrocinctus* PLA_2 isozymes was demonstrated. Taken together with a previously observed predominance of 14–15 kDa components [5], the results suggest that PLA_2s are major components of *M. n. nigrocinctus* venom. In contrast, α-neurotoxins are major components of *Bungarus* and some *Naja* venoms [16,17].

The antiserum to native notexin did not recognize β-bungarotoxin, but in contrast, it reacted with several *M. n. nigrocinctus* PLA_2s. This result is remarkable, because antisera to notexin, the most toxic monomeric venom PLA_2, have a narrow range of cross-reactivity with heterologous PLA_2s [18]. Consistent with the existence of several notexin-related *M. n. nigrocinctus* PLA_2s, we previously observed that notexin is recognized by *M. nigrocinctus* antivenoms [5].

Elapid PLA_2s are classified antigenically into two subclasses, one includes enzymes from cobras and Australian elapids while the second includes only β-bungarotoxin [19]. Nigroxins are recognized by antibodies to textilotoxin and notexin and, therefore, are antigenically most related to the first subclass. However, all previously known members of this subclass are recognized by antisera to β-bungarotoxin and taipoxin [19], whereas nigroxins are not. Therefore, nigroxins constitute a new antigenic subclass of elapid PLA_2s, which share common epitopes with textilotoxin, mulgotoxin and notexin. Isoforms C2 and C3 are antigenically the ones most related to the latter two neurotoxic PLA_2s. The epitope(s) of nigroxins A, B and C1, shared with notexin, apparently become(s) altered or masked by direct adsorption onto the plastic.

Previous studies suggested that our MAbs against *M. n. nigrocinctus* PLA_2s recognize different epitopes [20]. We now demonstrate that MAbs 9A and 7H recognize nigroxin-specific epitopes, whereas MAbs 4, 21, 27 and 28 recognize epitopes shared between nigroxins and the most hydrophobic isozymes of the venom.

Structurally, elapid PLA_2s are classified into three subclasses: one includes enzymes from Bungarinae, the second contains PLA_2s from Hydrophiinae and Laticaudinae and the third includes only β-bungarotoxin [3,21]. Nigroxins A and B are structurally most similar to PLA_2s from the first subclass. The amino acid sequence differences between nigroxins A and

Table 2
ELISa titers[1] of MAbs against *Micrurus* PLA_2 with nigroxins

	MAb 4	MAb 7H	MAb 9A	MAb 21	MAb 27	MAb 28
Nigroxin A	5.3	4.2	5.6	4.8	2.7	3.0
Nigroxin B	5.3	4.4	5.6	5.0	2.5	3.0
Nigroxin C1	3.6	2.7	3.4	5.0	–[2]	2.6
Nigroxin C2	3.5	2.2	3.8	5.1	–[2]	2.8
Nigroxin C3	4.7	3.3	4.4	5.0	2.5	2.6

[1] Negative log of the dilution giving 0.3 absorbance units.
[2] Absorbance values lower than 0.3 at a dilution of 5×10^{-3}. At this dilution normal mouse serum gave absorbances lower than 0.1 with all nigroxins.

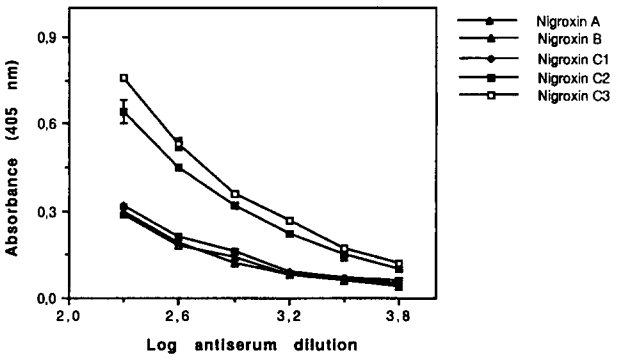

Fig. 3. Immunoreactivities of nigroxins with rabbit antisera to *N. scutatus* notexin in capture ELISA. Normal rabbit serum gave absorbance values lower than 0.13 ± 0.02 at all dilutions tested with all nigroxins. Each point represents the mean ± S.D. of two determinations.

B in the N-terminal segments are not due to single-point mutations, since they cannot arise from single-base changes. The presence of Asp-4 in nigroxin A, in contrast to Gln-4 in nigroxin B, may explain their separation on Mono S, since crystallographic studies of other elapid PLA_2s show that the N-terminal region contributes to the protein surface [15,22].

The α-neurotoxins are competitive antagonists of the AchR and are classified into two types: 'short-chain' and 'long-chain' toxins [4,14]. Short-chain α-neurotoxins are classified into four groups, those of sea kraits (*Laticauda*), true sea snakes/Australian elapids (*Astrotia, Pelamis, Enhydrina, Hydrophis, Lapemis, Aipysurus, Pseudechis* and *Acanthophis*), cobras (*Naja* and *Hemachatus*) and mambas (*Dendroaspis*) [14]. Long-chain α-neurotoxins are classified into six groups, those of sea kraits, true sea snakes, Australian elapids, cobras, mambas and kraits (*Bungarus*) [14]. The four AchR-binding proteins isolated in this study are related to α-neurotoxins from *Naja*. Mnn-9 and Mnn-4 are related to short-chain α-neurotoxins whereas Mnn-3C and Mnn-1A are related to long-chain toxins. In agreement with these results, we previously found that *M. nigrocinctus* antivenoms have higher cross-reactivities with *N. n. oxiana* neurotoxins I and II, than with α-neurotoxins from *Laticauda* and *Bungarus* [8].

This work demonstrates that *M. n. nigrocinctus* venom contains multiple nicotinic AchR-binding proteins and basic PLA_2s with structural and/or antigenic similarities to toxins from *Naja* species. These results contribute to clarify the relationships between *Micrurus* venom proteins and toxins from other elapid taxa and provide information that might be useful to select novel antigenic mixtures better than crude venom for antivenom production.

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