Molecular evolution and structure–function relationships of crotoxin-like and asparagine-6-containing phospholipases A_2 in pit viper venoms

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Some myotoxic or neurotoxic PLA_2s (phospholipases A_2) from pit viper venoms contain characteristic N6 substitutions. Our survey of the venoms of more than ten pit viper genera revealed that N6-PLA_2s exist only in limited Asian pit vipers of two genera, *Protobothrops* and *Gloydius*, and exist as either monomers or the basic subunits of heterodimers in some New World pit vipers. For the newly identified N6-PLA_2s, the neuromuscular blocking activities were assayed with the chick biventer cervicis neuromuscular tissue, whereas the increased serum creatine kinase level assessed their myotoxicities. The purified N6-PLA_2s from *Protobothrops mangshanensis* and *Gloydius intermedius saxatilis* were found to be presynaptic neurotoxins. In contrast, all N6-PLA_2s from the venoms of *Sistrurus miliarius strackeri*, *S. m. barbouri*, *Crotalus viridis viridis*, *C. lepidus lepidus*, *Cerrophidion godmani* and *Bothreichis schlegelii* were myotoxins without neurotoxicity even in the presence of crotoxin A. Crotoxin-like complexes were for the first time purified from the venoms of *Sitrurus catenatus tergeminus*, *C. mitchelli mitchelli*, *C. horridus atricaudatus*, *C. basiliscus* and *C. durissus cumanensis*. The cDNAs encoding six novel N6-PLA_2s and subunits of the crotoxin-like complex from *S. c. tergeminus* were cloned and fully sequenced. Phylogeny analysis showed that two structural subtypes of N6-PLA_2s with either F24 or S24 substitution have been evolved in parallel, possibly descended respectively from species related to present-day *Protobothrops* and *Gloydius*. Calmodulin binds all the N6-PLA_2s but crotoxin A may inhibit its binding to crotoxin B and to other neurotoxic N6-PLA_2s. Structure–activity relationships at various regions of the PLA_2 molecules were extensively discussed.

Key words: cloning, myotoxin, neurotoxin, pit viper venom, phospholipase A_2, phylogenetic analysis.

INTRODUCTION

Owing to the frequent snakebites by the Asian pit vipers and the American rattlesnakes, studies of the related venoms are medically important. On the basis of the snakebite symptom, two types of rattlesnake venoms appear to exist today, one of them being neurotoxic and highly lethal and the other is haemorrhagic and myotoxic but less lethal [1]. As shown previously, potent neurotoxins from rattlesnake venoms (e.g. crotoxin and mojave toxin) are heterodimeric complexes of PLA_2s (phospholipases A_2; EC 3.1.1.4) [2,3]. CA (crotoxin A) and CB (crotoxin B), the acidic and basic subunits of crotoxin, are present in the venom of most South American rattlers. Immunochemical studies have been performed to detect this type of neurotoxin in many of the rattlers, using the antiserum against crotoxin or mojave toxin [4,5]. It has been shown that a CA-like subunit is absent in non-neurotoxic venoms of rattlers [6], whereas myotoxins from the pit viper venoms are usually basic monomeric PLA_2s with either D49 or K49 substitution [7].

Rattlesnakes (*Crotalus* and *Sistrurus*) and other pit vipers of the New World are probably the descendants of some Asian pit vipers [8]. The group II PLA_2s in pit viper venoms have been diversified into distinct pharmacological subtypes, and play different roles such as platelet aggregation inhibitor, anticoagulant, neurotoxin or myotoxin [9,10]. One of the subtypes is the myotoxic/neurotoxic D49-PLA_2 with N6 substitution (hereafter designated as N6-PLA_2). The known members of this venom PLA_2 subtype include the basic subunits of crotoxin and mojave toxin, agkistrodotoxin from *Gloydius halys brevicaudus* (formerly *Agkistrodon halys* Pallas) [11], trimucrotoxin from *Protobothrops mucrosquamatus* [12], Tf-PLA-N from *P. flavoviridis* [13] and the myotoxin from *Crotalus viridis viridis* (designated as Cvv-N6) [14,15]. In the present study, we purified and sequenced six novel N6-PLA_2s from the New World pit viper venoms, and identified several N6-PLA_2 neurotoxins from the Asian pit viper venoms. ESI–MS (electrospray ionization–mass spectrometry), N-terminal sequencing and cloning facilitated the structure determination of these myotoxic or neurotoxic N6-PLA_2s and their functions were characterized using experimental animals. The evolutionary relationships between these N6-PLA_2s were investigated by phylogenetic analysis based on their sequences. Furthermore, the binding specificities of various N6-PLA_2s to calmodulin (one of the receptor candidates) [16] have been tested.

EXPERIMENTAL

Venoms and other materials

Freeze-dried venom samples of *Gloydius intermedius saxatilis* (formerly *Agkistrodon intermedius saxatilis*), *Cerrophidion godmani*, *Bothreichis schlegelii*, *Sistrurus miliarius barbouri*,

Abbreviations used: CA, crotoxin A; CB, crotoxin B; DPPC, L-dipalmitoyl phosphatidylcholine; ESI–MS, electrospray ionization–mass spectrometry; GST, glutathione *S*-transferase; PLA_2, phospholipase A_2; for brevity, the one-letter system for amino acids has been used, N6, e.g. means Asn-6.

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The nucleotide sequence data reported here have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases with accession numbers AF403138 for Cvv-N6, AY355166–AY355170 for Sms-N6, Ceg-N6, Bs-N6, Sct-N6 and sistruxin B respectively and AY508693 for sistruxin A precursor.

Crotalus lepidus lepidus, *C. basiliscus*, *Lachesis muta muta* and *Atropoides nummifer* were purchased from Miami Serpentarium laboratory (FL, U.S.A.). *P. flavoviridis*, *P. tokarensis*, *P. elegans* and *P. mucrosquamatus* were from Latoxan (Valence, France). Venoms of *C. durissus cumanensis*, *C. d. culminatus*, *C. horridus horridus*, *C. mitchelli mitchelli*, *C. vegrandis*, *C. molossus molossus* and *C. v. viridis* were purchased from Kentucky Reptile Zoo (Slade, KY, U.S.A.). The venoms of *P. mangshanensis* and *C. v. oreganus* were gifts from Professor Y.-Y. Shu (Kuangxi Medical University, People's Republic of China) and Professor S. P. Mackessy (Northern Colorado State University, U.S.A.) respectively. The venom of *C. horridus* (neurotoxic or type A, from South Carolina) was a gift from the Kentucky Reptile Zoo. *C. atrox* and *C. adamanatus* venoms were from Sigma (St. Louis, MO, U.S.A.). Live specimens of *B. schlegelii*, *Ce. godmani*, *C. v. viridis*, *Sitrurus catenatus tergeminus* and *S. miliarius strackeri* were purchased from Glades Herp. (Fort Myers, FL, U.S.A.). The venoms were extracted 2 days before the snake was killed and the venom glands were removed immediately for RNA extraction.

The mRNA preparation and the cDNA synthesis kits were purchased from Stratagene. Restriction enzymes and other enzymes were purchased from Promega. Synthetic DPPC (L-dipalmitoyl phosphatidylcholine) was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Other chemicals were of reagent grade.

Purification and characterization of purified PLA_2s

Venom (10–15 mg) was dissolved and fractionated on an FPLC system with a Superdex 75 column (HR10/30; Amersham Biosciences) in 0.1 M ammonium acetate (pH 6.5) at room temperature (25 °C). After freeze drying, the PLA_2-containing fractions were further purified by reverse-phase HPLC using a C8 column (4.5 mm × 250 mm, 10 μ, Vydac, Hesperia, CA, U.S.A.) equilibrated with 0.07 % aqueous trifluoroacetic acid (solvent A), and eluted with a 25–45 % linear gradient of CH_3CN containing 0.07 % trifluoroacetic acid (solvent B). The purified PLA_2s were dried in a vacuum-centrifuge device (Labconco, Kansas city, MO, U.S.A.).

The amino acid sequences of purified PLA_2s were determined by an automated amino acid sequencer (Model 477A; PE Applied Biosystems, Foster City, CA, U.S.A.). The molecular mass of each PLA_2 was determined by ESI–MS (Sciex mass analyser, API100; PerkinElmer) [17].

Cloning and sequencing

The venom gland mRNA and the cDNA were prepared as described previously [15,17]. To amplify cDNA of PLA_2s using the venom gland cDNA as a template, PCR [18] was conducted using SuperTaq DNA Polymerase with a pair of mixed-base oligonucleotide primers (sense primer 1: TCTGGATTSAGGAGGATGAGG and antisense primer 2: GCCTGCAGRACTTAGCA), which were designed according to the highly conserved cDNA regions of the group-II PLA_2s from snake venoms [12,15]. In addition, an antisense primer 3, AAYCTGYTBCARTTYAAYAAAATGATCAAG, was designed according to the N-terminal sequences of the N6-PLA_2s from *Ce. godmani* and *S. m. strackeri*, and was used along with primer 1 in PCR.

After treatment with polynucleotide kinase, the amplified DNA fragment was inserted into the pGEM-T easy vector (Promega Biotech, Madison, WI, U.S.A.). It was transformed into *Escherichia coli* strain JM 109. White transformants were picked up and cDNA clones were selected. The DNA sequencing System model 373A and the Taq-Dye-Dioxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) were used to determine the cDNA sequences by dideoxynucleotide method [19].

Enzymic activity and toxicity assay

PLA_2 concentration was determined by measuring the absorbance at 280 nm, assuming a molar absorption coefficient of 1.5 at 1.0 mg/ml protein. PLA_2 activity was measured using the micellar substrates of DPPC (3 mM) mixed with an equal molar of deoxycholate or 2-fold concentration of Triton X-100 on a pH-stat apparatus (Radiometer RTS 822, Copenhagen, Denmark). The initial reaction rate was followed for 5 min and corrected for non-enzymic spontaneous rate.

For neurotoxicity assay, chick biventer cervicis nerve-muscle preparations [20] were isolated from 4–10-day-old chicks. The tissue was suspended in an organ bath filled with 10 ml of modified Tyrode solution with 2 mM $CaCl_2$ at 37 °C and aerated with 95 % O_2 plus 5 % CO_2. Contractions of the muscle were elicited by stimulation of the nerve with supramaximal rectangular pulses of 0.05–0.1 ms duration on a Grass polygraph [12].

The myotoxic effect of N6-PLA_2s was studied by measuring the serum creatine kinase concentrations after intramuscular injection. Blood (20–40 μl) was drawn by a glass capillary pipette containing EDTA from the retro-orbital sinus of mouse eye 3 h after intramuscular injection of the purified PLA_2 [21]. To assay the serum level of the kinase, a reagent kit from Sigma (Cat. no. 520) was used.

Phylogenetic analysis of crotalid basic D49 PLA_2s

In addition to the full amino acid sequences of six N6-PLA_2s solved in the present study, other N6-PLA_2s were selected by BLASTp search (National Center for Biotechnology Information). The amino acid sequence alignment was made by the PILEUP program. Cladograms were constructed based on these sequences by neighbour-joining algorithm using the PHYLIP program [22], and the degree of confidence for the internal linage was determined by bootstrap methods [23].

Expression of recombinant GST (glutathione *S*-transferase)–calmodulin

The open reading frame of the cDNA of rat calmodulin (accession no. NM-017326) was amplified by PCR, using the primer pair that contains the flanking restriction sites of *Bam*HI and *Xho*I respectively, i.e. *Bam*HI-sense primer GGATCMTGGCTGACCAACTGACTG and *Xho*I-antisense primer CTCGASTACTTCGCTGTCATCATTTG. Marathon-ready cDNAs of rat brain (ClonTech) were used as templates in the PCR to generate full-length cDNA. The PCR products were cleaved with the restriction enzymes, purified and then ligated to pGEX-4T-1 expression vector for the production of GST fusion proteins. The resultant plasmid was transformed into *E. coli* strain BL21 after sequence verification. The recombinant GST–calmodulin was purified on the glutathione–Sepharose according to the manufacturer's instructions (Amersham Biosciences).

Binding to calmodulin

To detect the binding between various N6-PLA_2s and calmodulin [24], two approaches were adopted. One used calmodulin-immobilized Sepharose 4B (Amersham Biosciences) to pull down the toxins directly; the other used glutathione–Sepharose 4B to pull down the reaction mixture of GST–calmodulin and the PLA_2s. The binding buffer used was 10 mM Tris/HCl (pH 8.0) containing 0.15 M NaCl and 2 mM $CaCl_2$. The final concentrations of PLA_2s

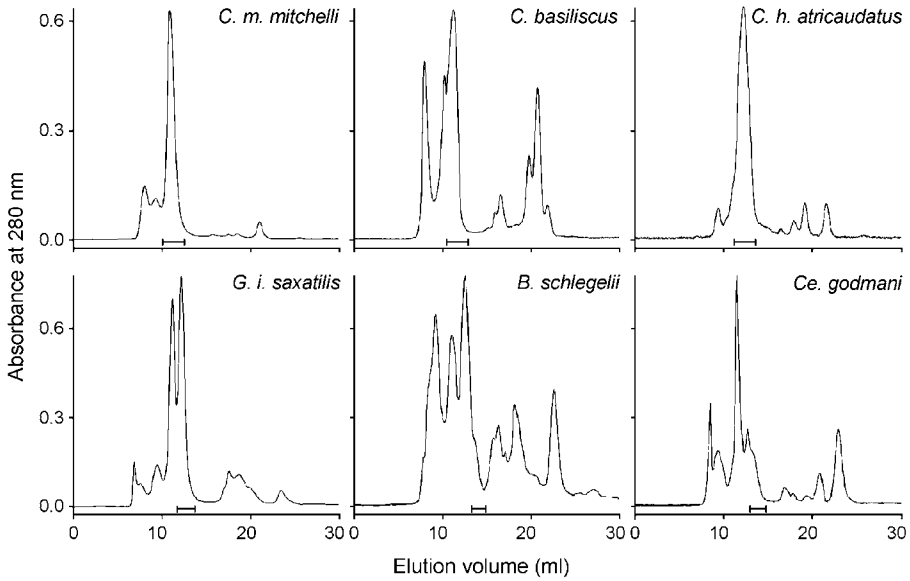

Figure 1 Representative gel filtration profiles of pit viper venoms

Dissolved crude venom was loaded on to a Superdex 75 (HR10/30) column and eluted with 0.1 M ammonium acetate (pH 6.5) at a flow rate of 1.0 ml/min. Fractions containing the dimeric (upper three panels) or the monomeric (lower three panels) PLA_2s were pooled (shown by —).

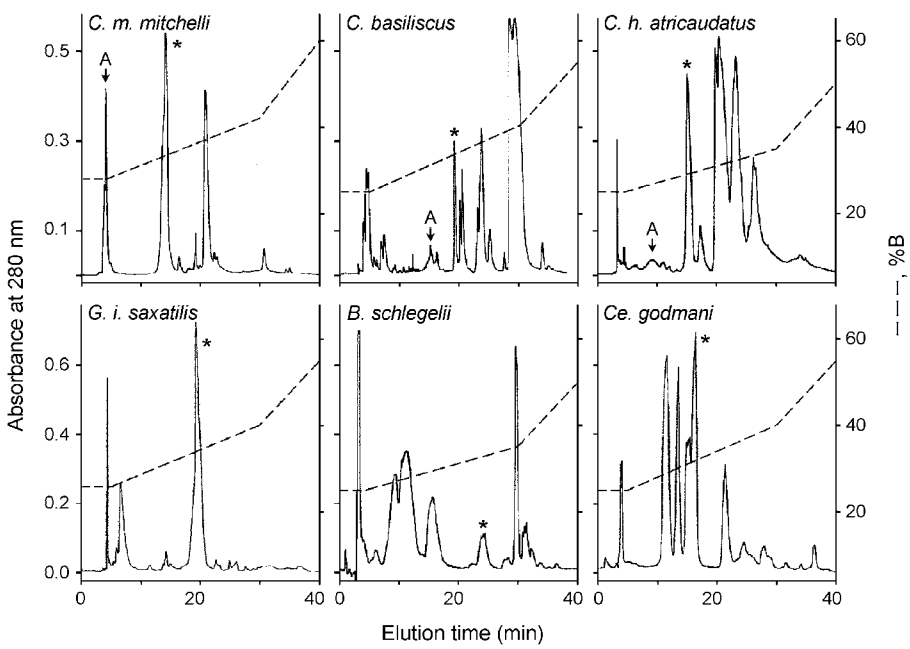

Figure 2 Purification of N6-PLA_2s by reversed-phase HPLC

Each PLA_2-containing fraction from Figure 1 was freeze-dried, re-dissolved and further purified on a Vydac-C8 HPLC column with a gradient of solvent B (acetonitrile with 0.07 % trifluoroacetic acid). *, N6-PLA_2 peak; A, CA-like peak.

and GST–calmodulin applied in the assay were 1.0 and 1.5 μM respectively, whereas the affinity resin (50 % slurry) was 20 μl. The binding reaction was conducted at 4 °C for 2 h with constant mixing, and the matrix was washed three times each with 1 ml of binding buffer, by centrifugation at 1500 ***g*** for 2 min. The pellet was then boiled in SDS sample buffer; the bound material was resolved by SDS/PAGE (12 % gel) followed by Coomassie Blue staining.

RESULTS

Purification and identification of the N6-PLA_2s

Gel filtration was used to isolate the PLA_2-containing fractions from crude venom of more than 20 species. The 11–14 and 22–27 kDa fractions of the venoms (Figure 1) presumably contained monomeric and dimeric PLA_2s respectively. The proteins were further purified by HPLC (Figure 2). The molecular mass of each

Table 1 List of N6-PLA$_2$s purified from the Asian pit viper venoms

Molecular masses of PLA$_2$s were determined by ESI–MS and the N-terminal sequences were determined by the amino acid sequencer. Residues identical with the first line were shown by dots, special substitutions are shown in bold.

Toxin or PLA$_2$	Venom species	Mass (kDa)	N-terminal sequences 1–23
Trimucrotoxin	*P. mucrosquamatus*	13.902	NLLQF**N**KMIK**IM**T**K**KNAIPFY**SS**
Mangshantoxin	*P. mangshanensis*	13.902	·····
Tf-PLA-N	*P. flavoviridis*	14.033	·····GF···**T**·
Pto-N6	*P. tokarensis*	14.033	·····GF···**T**·
DAV-N6	*D. acutus*	14.000	H·····R···F···**T**·
Agkistrodotoxin	*G. b. brevicaudus*	13.938	·····EE·G·····AF
Intermexin	*G. i. saxatilis*	14.145	·····VE·G·····AF

Table 2 List of N6-PLA$_2$s purified from the New World pit viper venoms

Other details are the same as given in the legend of Table 1.

PLA$_2$ or subunit	Venom species	Mass (kDa)	N-terminal sequences 1–23
CB1	*C. d. terrificus*	14.183 ± 0.003	**H**LLQF**N**KMIK**FE**TRKNAIPFYA**F**
	C. basiliscus		
	C. vegrandis		
	C. s. scutulatus		
	C. d. cumanensis		
CB2	*C. d. terrificus*	14.244 ± 0.002	**S**·····
	C. d. cumanensis		
	C. m. mitchelli		
CB3	*C. d. terrificus* (Paraguay)	14.038	·····G·····
Canebraxin B	*C. h. atricaudatus*	14.154	·····
Sistruxin B	*S. c. tergeminus*	14.122	N·····NA·····
Monomeric myotoxin	*C. v. viridis*	14.200	N·····M··K···F····S
	C. l. lepidus	13.861	N·····IM·K·····TS
	S. m. barbouri	13.948	N·····IM·K·····TS
	S. m. strackeri	13.893	N·····IM·K····S·TS
	Ce. godmani	14.012	N·····IM·K···V···TS
	B. schlegelii	13.785	N·····IM····G··Y·SS

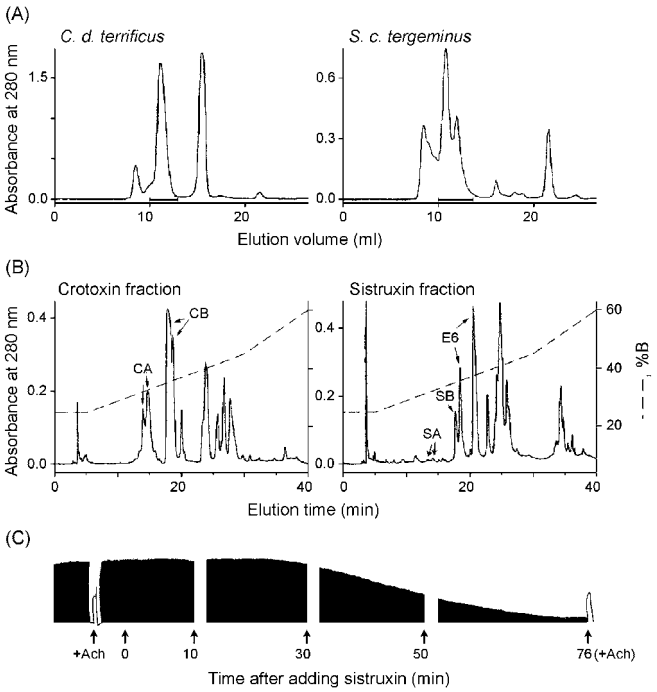

Figure 3 Purification of crotoxin and sistruxin subunits and neurotoxicity assay of the sistruxin complex

(**A**) Gel filtration of the crude venom by a Superdex 75 column. The neurotoxin fractions (shown by —) were pooled. (**B**) CA and CB subunits of crotoxin and SA and SB subunits of sistruxin were purified by reversed-phase HPLC on a Vydac-C8 column. The elution conditions were the same as in Figures 1 and 2. (**C**) Neurotoxicity assay of the complex of sistruxins A and B (0.1 μg/ml) using chick biventer cervicis neuromuscular tissue. Acetylcholine (Ach) was added to a final concentration of 10 μg/ml.

Table 3 Enzymic activities of F24-N6PLA$_2$s in the presence and absence of CA and their protein contents in the venom

Enzymes were assayed in the presence of 10 mM $CaCl_2$ at 37 °C, pH 7.4. The concentration of added CA was eight times higher than that of N6PLA$_2$.

		Specific activity (μmol · mg^{-1} · min^{-1})	
PLA$_2$ or subunit	Content (%, w/w)	DPPC + deoxycholate	DPPC + Triton X-100
Intermexin	13	392 ± 15	222 ± 6
Intermexin + CA	–	359 ± 15	106 ± 2
CB1 of various rattlesnakes	4–14	652 ± 32	226 ± 24
CB2 of *C. m. mitchelli*	30	430 ± 4	123 ± 6
CB3 of *C. d. terrificus*	20	672 ± 44	231 ± 20
Crotoxin complex	35–50	388 ± 25	58 ± 2
Canebraxin B	8	480 ± 13	195 ± 16
Canebraxin complex	11	355 ± 15	63 ± 2
Sistruxin B	3	385 ± 38	192 ± 6

purified protein peak was determined by ESI–MS and each protein of 13.9 ± 0.4 kDa was subjected to N-terminal sequencing and enzyme assay. Our survey revealed that N6-PLA$_2$s are absent from the venoms of the Old World pit viper genera *Calloselasma*, *Hypnale*, *Tropidolaemus*, *Ovophis* and *Trimeresurus* (*sensu stricto*) and those of the New World *Agkistrodon*, *Lachesis*, *Atropoides* and *Bothrops*.

We found that N6-PLA$_2$s are present only in the venom of the Asian pit vipers under two genera, *Protobothrops* and *Gloydius*. N6-PLA$_2$s with identical masses and sequences could be isolated from the venoms of *P. tokarensis* (i.e. Pto-N6) and *P. flavoviridis* (i.e. Tf-PLA-N) and from those of *P. mangshanensis* and *P. mucrosquamatus* (Table 1). However, we did not find N6-PLA$_2$ in *P. elegans* venom. Table 2 lists the molecular masses and N-terminal sequences of the purified N6-PLA$_2$s from the venoms of the New World pit vipers. Crotoxin-like heterodimers were found in the venoms of *C. d. terrificus*, *C. scutulatus scutulatus*, *C. d. cumanensis*, *C. vegrandis*, *C. m. mitchelli*, *C. basiliscus basiliscus*, *C. horridus* (type A) and *S. c. tergeminus*. On the other hand, monomeric N6-PLA$_2$s were identified in the venoms of *B. schlegelii*, *Ce. godmani*, *C. l. lepidus* and *C. v. viridis*, and were abbreviated as Bs-N6, Ceg-N6 etc. respectively (Tables 2 and 4).

CA and CB were purified from *C. d. terrificus* and related rattlesnakes (Figure 3A). Sistruxins A and B as well as a major acidic PLA$_2$ (i.e. E6) were purified from the venom of an individual *S. c. tergeminus* (Figure 3B). Moreover, the reconstituted sistruxin complex was found to be as potent as the native heterodimer purified by monoQ column (results not shown).

Assay and functional characterization

The enzymic activities of the purified N6-PLA$_2$s towards cationic and zwitter-ionic micellar substrates were studied (Tables 3

Table 4 Enzymic activities of S24-N6PLA$_2$s in the presence and absence of CA and their protein contents in the venom

Legend is same as in Table 3.

		Specific activity (μmol · mg^{-1} · min^{-1})	
PLA$_2$ toxin or subunit	Content (%, w/w)	DPPC + deoxycholate	DPPC + Triton X-100
Trimucrotoxin	10	300 ± 15	97 ± 1
Trimucrotoxin + CA	–	280 ± 25	45 ± 4
Mangshantoxin	3	521 ± 15	155 ± 4
Mangshantoxin + CA	–	468 ± 11	15 ± 2
Cvv-N6	1–4	280 ± 8	88 ± 4
Cvv-N6 + CA	–	246 ± 1	86 ± 5
CII-N6	8	294 ± 8	7 ± 1
CII-N6 + CA	–	279 ± 18	6 ± 1
Ceg-N6	2	251 ± 15	< 1
Ceg-N6 + CA	–	248 ± 17	< 1
Bs-N6	1–2	208 ± 7	116 ± 2
Smb-N6	2	324 ± 13	< 1

Table 5 Neurotoxicity of N6-PLA$_2$ towards the chick biventer cervicis tissue

The numbers in parentheses represent the number of experiments.

N6-PLA$_2$ toxin	Dose (μg/ml)	Time of 90 % inhibition of the twitch (min)
Trimucrotoxin	2.0	38 ± 2 (n = 4)
	1.0	76 ± 13 (n = 5)
	0.3	106 ± 2 (n = 3)
Mangshantoxin	1.0	77 ± 3 (n = 2)
Agkistrodotoxin	0.3	75 ± 3 (n = 2)
Intermexin	2.0	80 ± 5 (n = 2)
	1.0	150 ± 5 (n = 3)
CB subunit	2.0	80 ± 20 (n = 2 for each venom species)
	1.0	170 ± 30 (n = 2 for each venom species)
Canebraxin B	2.0	63 ± 3 (n = 2)
	1.0	163 ± 1 (n = 2)
Sistruxin B	0.3	230 (n = 1)
Crotoxin complex	0.10	65 ± 2 (n = 2)
	0.05	95 ± 7 (n = 4)
	0.03	150 ± 10 (n = 2)
Canebraxin complex	0.1	83 ± 7 (n = 3)
Sistruxin complex	0.3	50 ± 3 (n = 2)
	0.1	76 ± 4 (n = 2)
Cvv-N6 ± CA	4.0	> 240 (n = 2)

and 4). All the S24-N6-PLA$_2$s have similar catalytic activities, which are lower than those of various F24-N6-PLA$_2$s. The activities of S24-N6-PLA$_2$s were especially low when Triton X-100 micelles were the substrates. Effects of CA on the catalytic activities were also investigated.

Chick biventer cervicis neuromuscular tissue has been shown to be a very sensitive target for crotoxin in electrophysiological test [25,26] and therefore was used to study the neurotoxicity of the newly found sistruxin (Figure 3C) and other N6-PLA$_2$s. Neurotoxicity of the CB subunit was more than 30 times weaker than the intact crotoxin-like complex towards the chick tissue (Table 5).

Myotoxicity of the N6-PLA$_2$ towards mice was studied. Table 6 listed the serum level of creatine kinase after intramuscular injection of N6-PLA$_2$s into the extensor digitorum longus muscle of mouse legs.

Table 6 Comparison of myotoxic effects of N6-PLA$_2$s

The creatine kinase levels in mouse serum were measured 2 or 3 h after intramuscular injection of 16 μg of venom PLA$_2$ in sterile PBS. Definition of unit followed that of the Sigma kit and values are the means ± S.D. for duplicate experiments.

	Creatine kinase (units/ml)	
PLA$_2$	2 h	3 h
Trimucrotoxin	4.73 ± 0.01	5.35 ± 0.20
Intermexin	1.91 ± 1.11	2.70 ± 0.95
Cvv-N6	5.83 ± 1.66	7.45 ± 0.98
PBS (control)	0.26 ± 0.21	0.38 ± 0.01

Cloning and complete sequencing of PLA$_2$s

Using specifically designed PCR primer, cDNAs of many PLA$_2$s could be amplified and cloned. The success of this approach further confirms that the venom PLA$_2$ genes of various pit vipers share highly conserved 5′ and 3′ non-coding regions [13]. The complete amino acid sequences of these N6-PLA$_2$s were predicted from the cDNA sequences and matched against the mass and N-terminal sequences of the purified PLA$_2$s (Tables 1 and 2). Alignment of all the N6-PLA$_2$ sequences obtained from the present study and previous works [11–13,27–30] shows that their sequence identities are approx. 73–94 % (Figure 4A).

In addition, the precursor for sistruxin A (the acidic subunit of sistruxin) was also cloned from the venom gland of *S. c. tergeminus*. Its deduced sequence was aligned with the precursors of other CA-like proteins, as well as other homologous acidic E6-PLA$_2$s obtained by BLASTp (Figure 4B). The sequence of mature sistruxin A differs from that of CA in only five semi-conserved amino acids, and appears to have similar functions and specificities. On the other hand, residues 1–32 of the major PLA$_2$ of *S. c. tergeminus* are NLIQFETLILKVAKKSGMFSYSAYG-CYCGWGG/, which differs from that of the acidic PLA$_2$s of *Gloydius blomhoffi brevicaudus* [28] by only three substitutions.

Phylogenetic tree of the N6-PLA$_2$s and G6-PLA$_2$s

A phylogenetic tree based on the protein sequences of all the N6-PLA$_2$s and the G6 or A6-myotoxic PLA$_2$s [31–34] from pit viper venoms is shown in Figure 5. The W6D49-PLA$_2$ of *C. rhodostoma* venom [17] was used as out-group. The tree shows the clustering of all the N6-PLA$_2$s, separated from the G6D49-PLA$_2$s and the existence of two lineages of N6-PLA$_2$s, denoted as F24 and S24 respectively. The bootstrap values higher than 50 at each node support the robustness of the cladogram.

Binding of N6-PLA$_2$s to calmodulin and the effect of CA

Although it was shown that highly neurotoxic PLA$_2$s may bind to calmodulin with high affinity ($K_d = 10^{-8}$–10^{-9} M) [24], no direct correlation between the toxic potency of PLA$_2$s and their binding affinity for calmodulin was observed in previous experiments. Results of our pull-down assay showed that *in vitro* calmodulin binds strongly to all the N6-PLA$_2$s in the absence of CA (Figure 6). However, the binding of calmodulin to the intact complex (e.g. crotoxin and canebraxin) was much weaker. Thus the binding is not specific for the neurotoxic PLA$_2$s. We found that CA may specifically protect neurotoxic N6-PLA$_2$s, including CB, sistruxin B and trimucrotoxin, from binding calmodulin; however, no CA protection was found with the myotoxic Cvv-N6 (Figure 6C) and other non-neurotoxic N6-PLA$_2$s (results not shown).

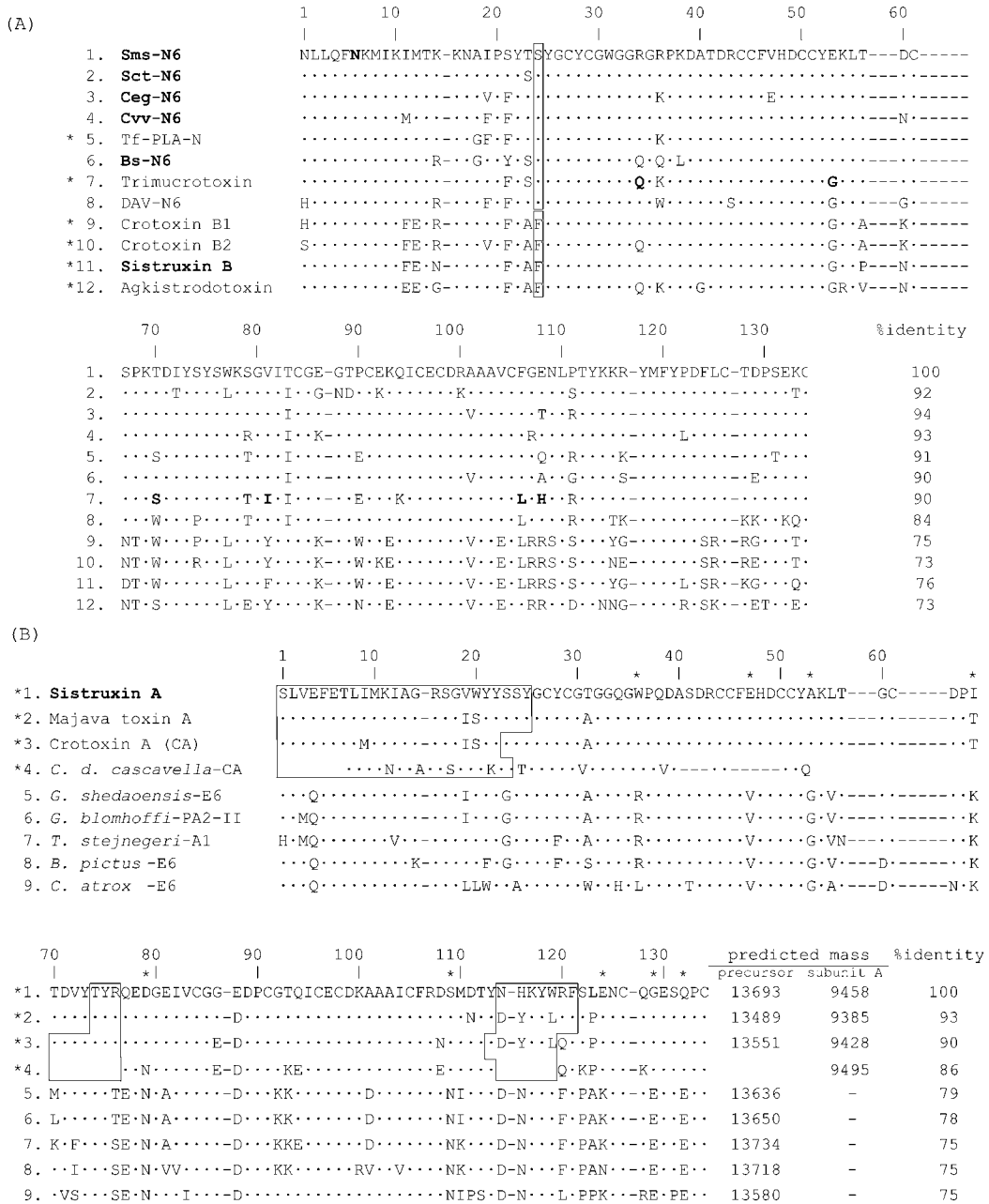

Figure 4 Alignment of the amino acid sequences of (A) N6-PLA$_2$s or CB-like subunits and (B) the precursor of sistruxin A and related acidic PLA$_2$s

Novel sequences deduced in the present study are shown in boldface. Single-letter codes are used, and the numbering system followed that commonly used [30]. Residues identical with those in the top line are denoted with dots and gaps are marked with hyphens. Neurotoxins or their subunits are denoted with asterisks, and special substitutions possibly related to the neurotoxicity of trimucrotoxin are shown in boldface. (**B**) GenBank® accession numbers for sequences 5–9 are AAR11860, P20249, P20476, Q9I8F8 and P00624 respectively; regions processed away during the maturation of CA-like subunits were blocked. Substitutions possibly important for the chaperone role of CA are marked with asterisks.

DISCUSSION

Phylogeography and evolution of venom N6-PLA$_2$s

Originally, the name of crotoxins has been designated to the neurotoxic PLA$_2$ complex from the venoms of the South American rattlesnakes including *C. d. terrificus*, *C. d. durissus*, *C. d. cumanensis* [3,4], *C. d. cascavella* and *C. d. collilineatus* [35], whereas mojave toxin was named for the neurotoxin in the North American Mojave rattlesnake (*C. s. scutulatus*) [6,7] (Table 2). As shown in various reports, crotoxin homologues are also present in the venom of several other rattlesnakes, e.g. *C. v. concolor*, *C. vegrandis* [36] and canebrake or timber rattlesnake (*C. horridus*) [37]. Depending on the content of these neurotoxins, the venoms show high or moderate lethal potency with LD_{50} values of 0.2–0.6 μg/g, which are approx. ten times lower than non-neurotoxic rattlers [1,2]. We here surveyed the presence of N6-PLA$_2$s in all pit viper venoms available, and purified for the first time the crotoxin-like toxins in the venoms of *S. c. tergeminus*, *C. basiliscus* and *C. m. mitchelli* (Table 2).

Two isoforms of CB with molecular masses of 14.180 kDa (CB1) and 14.244 kDa (CB2) were previously identified in the pooled venom of *C. d. terrificus* [3,38]. We found that CB

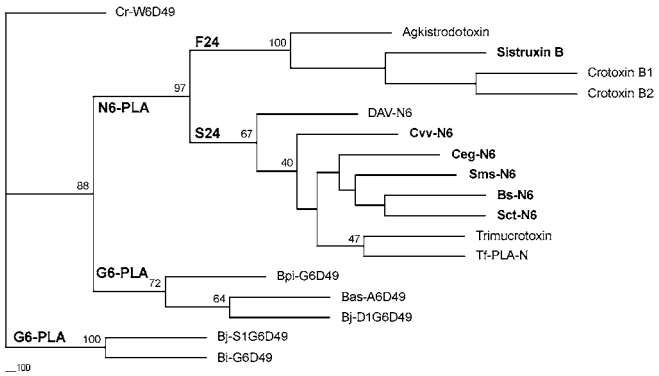

Figure 5 Molecular phylogeny of basic crotalid D49 PLA_2s

Data used include all the sequences listed in Figure 4(A) and the G6D49-PLA_2 sequences from four species. The PLA_2, GenBank® accession numbers and the venom species are agkistrodotoxin (P14421), CB (P07517, P24027), trimucrotoxin (× 77 645), DAV-N6 (× 77 649) from *Deinagkistrodon acutus*, Tf-PLA-N (AB102728) from *P. flavoviridis*, Bpi-G6D49 (P58464) from *B. pirajai*, Bas-A6D49 (P20474) from *B. asper*, Bj-D1G6D49 (AY185201), Bj-S1G6D49 (AY145836) from *B. jararacussu*, and Bi-G6D49 (AF490535) from *B. insularis*. Sequence data of Sct-N6, sistruxin B, Sms-N6, Cvv-N6, Ceg-N6 and Bs-N6 from Figure 4(A) are shown in boldface.

isoforms purified from various *Crotalus* venoms are highly conserved. Apparently, CB1 and CB2 were expressed in the venom differentially, and *C. m. mitchelli* venom expressed only CB2 (Table 2), which possibly explains the lower lethality of this species towards mice when compared with *C. durissus* containing more CB1 [2,3]. Some other CB variants (e.g. CB3; Table 2) were, however, identified in special geographic samples of *C. d. terrificus* [39,40]. The basic subunit of canebraxin (from neurotoxic population of *C. horridus*) has its N-terminal sequence identical with that of CB1 but has a distinct mass of 14.154 kDa. Although the toxicities of sistruxin and canebraxin are as potent as crotoxin (Table 5), their contents in the venom are relatively low (5–12 %). Thus envenomings by Pigmy rattlers or Timber rattlers usually do not show neurotoxic symptoms like those by Mojave or neotropical rattlers, which contain crotoxin up to 50 % of the total venom content [35,39].

Apparently, the sequences of N6-PLA_2 subunits of crotoxins are similar to the sequence of agkistrodotoxin from the Asian *Gloydius*, and the sequences of CA are similar to those of the E6-PLA_2s from *Gloydius* venoms (Figure 4B). In contrast, the sequences of the monomeric N6-PLA_2s from the New World pit vipers are closely related to that of the trimucrotoxin. Thus ancient pit vipers related to the present-day *Gloydius* and *Protobothrops* possibly migrated to the New World and evolved into various rattlesnakes. The evolutionary relationships of two lineages of N6-PLA_2s (i.e. F24 versus S24) are also supported by the cladogram (Figure 5). Most of the crotalid species express only one of the subtypes, but both subtypes of N6-PLA_2s have been cloned from *S. c. tergeminus*, although only the F24 subtype is expressed in detectable quantity. The origin of *S. c. tergeminus* remains puzzling as long as the Asiatic pit viper containing both N6-PLA_2 subtypes has not been discovered.

CA does not recognize non-neurotoxic N6-PLA_2s

CA has been known to form a complex with CB with high affinity ($K_d = 10^{-8}$–10^{-9} M) [38], and prevent non-specific binding of CB [25–27]. CA may increase the toxicity of CB more than 30-fold and those of trimucrotoxin and agkistrodotoxin severalfold towards mouse or chick tissue [12,27]. CA also inhibited the *in vitro*

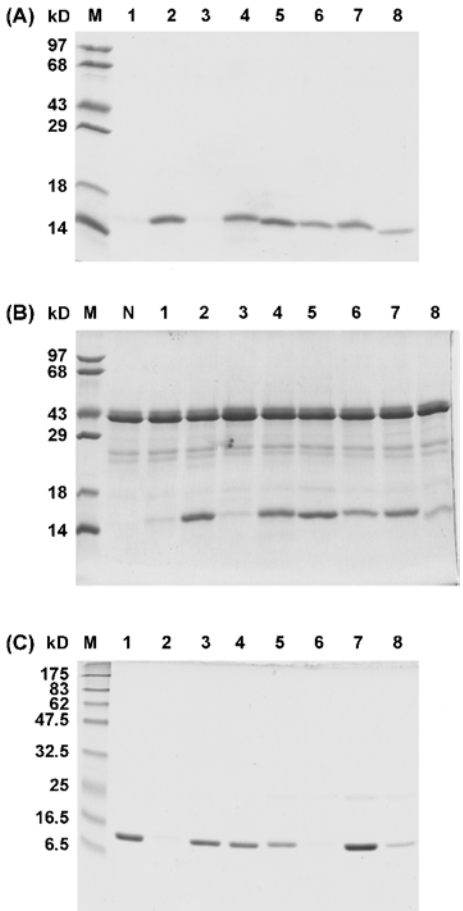

Figure 6 Calmodulin binding of N6-PLA_2s and the effect of CA

(**A**) Pull-down assay of purified N6-PLA_2s (1.5 μM) with calmodulin–Sepharose gel. (**B**) Pull-down assay by incubating glutathione resin with GST–calmodulin (1.0 μM) and the PLA_2 (1.5 μM). Lane M, protein standards; lane N, control without PLA_2; lane 1, intact crotoxin; lane 2, CB; lane 3, intact canebraxin; lane 4, canebraxin B; lanes 5–8, Cvv-N6, Smb-N6, Ceg-N6 and Bs-N6 respectively. (**C**) Pull-down assay with calmodulin–Sepharose, in the presence (lanes 2, 4, 6 and 8) and absence (lanes 1, 3, 5 and 7) of CA. Lanes 1 and 2, CB; lanes 3 and 4, Cvv-N6; lanes 5 and 6, canebraxin B; lanes 7 and 8, trimucrotoxin.

enzymic activities of CB isoforms, and the inhibition was weak in the presence of deoxycholate because the anionic detergent interfered with the binding between CA and CB [41]. However, the inhibition was prominent with the Triton X-100 micellar substrate (Table 3). Notably, the esterase activities of the basic subunits of canebraxin and sistruxin were inhibited by the CA-like subunit significantly, as also reported for the case of crotoxin complex [40]. However, CA apparently binds poorly to the monomeric Ceg-N6, Cll-N6 and Cvv-N6, since CA at 8-fold molar excess neither inhibited on the enzymic activities of these PLA_2s (Table 4), nor did it increase their neurotoxicities towards chick biventer cervicis tissue. Since these N6 myotoxins do not form a complex with CA, it may be concluded that they lack a binding site for CA. The critical evolution of the CA precursor from an E6-PLA_2 (Figure 4B) may be a turning point leading to great potentiality of the neurotoxicity of N6-PLA_2 in some rattlesnake venoms [6].

Structure–function relationships

All of the N6-PLA_2 toxins are basic enzymes and contain 122 amino acid residues and seven conserved disulphide bonds.

Their sequences have > 80 % identity (Figure 4A). The three-dimensional structure model of Cvv-N6 has a carbon backbone almost identical with that of trimucrotoxin (results not shown). Notably, high doses of the neurotoxic trimucrotoxin showed myotoxicity [42]; CB alone also showed myotoxic, oedema-inducing and anticoagulant activities [43]. Thus some neurotoxic sites on the N6-PLA_2 molecules possibly overlap with that of the myotoxic sites.

N6-PLA_2s may be classified into two structural subtypes (i.e. S24- and F24-N6-PLA_2s) by careful sequence alignment and comparison (Figure 4A) and the molecular phylogeny (Figure 5). Easily distinguishable at the N-terminal regions, N1, I11, K14 and T/S23 are conserved in the S24 subtype, whereas H/S1, F11, R14 and A23 are conserved in the F24 subtype (Figure 4A). Moreover, the two subtypes probably show different surface epitopes and are immunologically distinct. Screening of many rattlesnake venoms with anti-CB antibodies showed positive results for only approx. one-third of them [4,6]. It was also shown that all the *C. v. viridis* venoms collected from the seven States contained N6-PLA_2s [15], but the venoms failed to react to the antibodies against crotoxin or mojave toxin [4]. Thus Cvv-N6 (a S24-N6-PLA_2) and CB (a F24-N6-PLA_2) apparently have different epitopes.

Although all S24-N6-PLA_2s from the New World pit vipers are devoid of any neurotoxicity, S24 should not be directly responsible for the lack of neurotoxicity since trimucrotoxin containing S24 is 2–3-fold more neurotoxic when compared with CB or sistruxin B-containing F24 (Table 5). However, the side-chain of F24 has been proven to be important for the toxicity of ammodytoxins A and C [44], and modification of Y22 by tetranitromethane greatly reduces the specific binding of crotoxin to synaptosomal vesicles [45]. In addition, the residues 6–8 have been shown to be essential for the neurotoxicity of trimucrotoxin by site-directed mutagenesis [46] and the S24F mutant of trimucrotoxin exhibited lower toxicity compared with the wild-type (results not shown). Thus the N-terminal region is important for the neurotoxicities of N6-PLA_2s.

A natural variant of agkistrodotoxin with two substitutions (V56P and N90W) causes a decrease in lethality (on mice) by approx. 7-fold [29]. These two mutations in agkistrodotoxin make it more similar to sistruxin B (85 % identities; Figure 4A), which in the absence of sistruxin A is a very weak neurotoxin. Crystallographic structure of agkistrodotoxin also indicated that regions 53–70 and 85–91 are special in this neurotoxic PLA_2 [47]. Both regions and the residues 90–108 of CB have been suggested to be involved in toxicity and the interaction between CB and CA, based on the monoclonal antibody mapping methods [48]. However, the modification of residues W70 and W90 only partially reduced its lethality and myotoxicity [43].

Notably, neurotoxic S24-N6-PLA_2s from the Old World *Protobothrops* show up to 90 % sequence identity with many of the non-neurotoxic S24-N6-PLA_2s from the New World pit vipers (Figure 4A). Distinct substitutions Q34, G53, I81, L106 and H108 in trimucrotoxin are possibly related to its binding to presynaptic sites [12] and with CA (Figure 6C). It has been suggested that the basic and hydrophobic residues of CB may be involved in the myotoxicity [7]. Thus different sets of basic and hydrophobic residues appear to be involved in their distinct functions, e.g. the myotoxins contain R34, K93, K115 and R116, whereas the neurotoxins contain K86, R107, R or H108 as well as hydrophobic F11, F24, Q34, G53, W70, W90 and Y or N115 (Figure 4A). The contribution of these residues to the toxicity remains to be confirmed by site-directed mutagenesis and binding assay. Recently, an *in vitro* R34Q mutation in a basic PLA_2 from a Chinese pit viper venom was found to reduce its haemolytic activity [49], suggesting that R34 in the group II PLA_2 is favourable for membrane binding, whereas Q34 may be more favourable for binding to specific neuronal proteins or receptors.

On the basis of the sequence comparison of CA-like acidic PLA_2s (Figure 4B), we may predict that the unique acidic residues E47, D79 and E124 as well as neutral residues W36, A53 and G129 of CA may be involved in its recognition and binding with basic CB-like PLA_2s. It is also interesting to note that unique basic residues at positions 23, 76 and 119–122 in preCA or presistruxin A (Figure 4B) may be crucial for protease hydrolysis during the post-translational processing of CA.

Binding with calmodulin

Several binding proteins of crotoxin on the neuronal or presynaptic membrane of mammalian brain have been characterized [16,50], but the mechanism by which crotoxin blocks neurotransmitter release remains obscure [51]. Calmodulin, a ubiquitous and cytosolic protein, binds a large range of proteins [16], probably via its hydrophobic and acidic domains [52,53]. The role of calmodulin in the process of neurotoxicity remains questionable. It has been shown that a chimaeric ammodytin L (non-neurotoxic) containing the C-terminal 26 residues of ammodytoxin (a neurotoxin) could bind calmodulin better but did not become neurotoxic. Moreover, the substantial increase in the binding affinity for calmodulin observed by introducing YIRN (positions 115–119 of ammodytoxin) into RVV-PL-VIII also failed to increase the toxicity of the latter [24,54]. In fact, RVV-PL-VIII and most of the non-neurotoxic N6-PLA_2s contain KK or KR at positions 115–116, whereas CB has YG. Thus the positively charged 115–116 residues probably contribute to the myotoxicity rather than neurotoxicity of the PLA_2s. Other specific binding proteins of crotoxin on the presynaptic membrane [50] related to exocytosis should be investigated.

The finding that CA partially protects monomeric neurotoxins such as trimucrotoxin from binding to calmodulin (Figure 6C) is in agreement with previous reports that addition of CA increased the neuromuscular blocking effects of trimucrotoxin [12] and agkistrodotoxin [27], but not Cvv-N6 (Table 5). This is also consistent with the result of calmodulin pull-down assay that CA could not bind or protect Cvv-N6 from calmodulin binding. Thus CA has a neurotoxin-recognizing specificity, that calmodulin does not have. In practice, this assay of protection from binding with calmodulin may be a convenient test for the binding of various N6-PLA_2s with CA. Since CA is released after the binding of crotoxin to presynaptic acceptors [16,26,50], CA possibly binds to similar surface residues of N6-PLA_2 as the presynaptic acceptors.

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