Two phospholipase A₂ inhibitors from the plasma of *Cerrophidion (Bothrops) godmani* which selectively inhibit two different group-II phospholipase A₂ myotoxins from its own venom: isolation, molecular cloning and biological properties

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Myotoxic phospholipases A₂ (PLA₂s; group II) account for most of the muscle-tissue damage that results from envenomation by viperid snakes. In the venom of the Godman's viper (*Cerrophidion godmani*, formerly *Bothrops godmani*), an enzymically active PLA₂ (myotoxin I) and an inactive, Lys-49 variant (myotoxin II) induce extensive muscle damage and oedema. In this study, two distinct myotoxin inhibitor proteins of *C. godmani*, CgMIP-I and CgMIP-II, were purified directly from blood plasma by selective binding to affinity columns containing either myotoxin I or myotoxin II, respectively. Both proteins are glycosylated, acidic (pI = 4) and composed of 20–25-kDa subunits that form oligomers of 110 kDa (CgMIP-I) or 180 kDa (CgMIP-II). In inhibition studies, CgMIP-I specifically neutralized the PLA₂ and the myotoxic, oedema-forming and cytolytic activities of myotoxins I, whereas CgMIP-II selectively inhibited the toxic proper-

ties of myotoxin II. N-terminal amino acid sequence analysis and sequencing of cDNAs encoding the two inhibitors revealed that CgMIP-I is similar to γ -type inhibitors, which share a pattern of cysteine residues present in the Ly-6 superfamily of proteins, whereas CgMIP-II shares sequence identity with α -type inhibitors that contain carbohydrate-recognition-like domains, also found in C-type lectins and mammalian PLA₂ receptors. N-terminal sequencing of myotoxin I revealed a different primary structure from myotoxin II [De Sousa, Morhy, Arni, Ward, Díaz and Gutiérrez (1998) *Biochim. Biophys. Acta* **1384**, 204–208], which provides insight into the nature of such pharmacological specificity.

Key words: carbohydrate-recognition domain, Crotalinae, myotoxicity, three-finger domain, toxin inhibitor.

INTRODUCTION

Phospholipases A₂ (PLA₂; EC 3.1.1.4) catalyse the Ca²⁺-dependent hydrolysis of *sn*-2 linkages in phosphoglycerides. Although all PLA₂s catalyse essentially the same reaction, they may vary according to their localization (e.g. secreted or cytoplasmic) and structural criteria, the latter of which has led to the classification of at least 10 major groups [1,2]. Animal venoms contain secreted PLA₂s comprising groups I (elapid snakes), II (viperid and crotalid snakes) and III (bees and lizards) [3].

Although not all PLA₂s found in snake venoms are toxic, some are associated with a variety of pharmacological effects, such as muscle-tissue damage (myotoxicity), neurotoxicity, anti-coagulant activity and inflammation [4]. Myotoxic PLA₂s have been described in the venoms of crotalid snake genera (family Viperidae, subfamily Crotalinae) such as *Bothrops*, *Porthidium*, *Agkistrodon*, *Trimeresurus*, *Crotalus*, *Bothriechis*, *Bothriopsis* and *Cerrophidion* [5,6]. Some of these toxins are Asp-49 enzymically-active PLA₂s, whereas others contain Lys-49, a critical substitution at the calcium-binding loop of the protein that renders it inactive or leaves it with extremely low PLA₂ activity [7].

In recent years, a growing number of inhibitors of venom PLA₂ activity have been isolated from the blood plasma of

venomous snakes. Together with other anti-toxic factors such as anti-haemorrhagins, these proteins are believed to contribute to the natural resistance of snakes to their own venom [8]. They are invariably acidic (pI = 4–5) α -globulin glycoproteins that inactivate PLA₂s by forming soluble complexes. In addition to their anti-PLA₂ properties, some of these factors also neutralize the toxic effects induced by particular PLA₂s. Such is the case of the *Bothrops asper* myotoxin inhibitory protein (BaMIP) which neutralizes the myotoxic and pro-inflammatory effects of basic PLA₂ myotoxins from the venom of this snake [9]. In addition, *Crotalus durissus terrificus* crotoxin neutralizing factor (CNF) inhibits the lethal effects caused by crotoxin, a potent presynaptic β -type neurotoxin [10].

Based on amino acid sequence comparisons with other proteins, snake-plasma PLA₂ inhibitors (PLIs) have been classified into three major groups [11]: α , β and γ . The α inhibitors contain carbohydrate-recognition domains (CRDs) similar to those of Ca²⁺-dependent (C-type) lectins (for example, mannose-binding protein and pulmonary surfactant apoprotein) [12] as well as to mammalian muscle (M)-type PLA₂ receptors that bind pancreatic group-I and group-II PLA₂s [2]. This group includes the heterotrimeric PLI of the Habu snake (*Trimeresurus flavoviridis*) [13], the PLI- α of the Chinese mamushi (*Agkistrodon blomhoffii*

Abbreviations used: PLA₂, phospholipase A₂; PLI, PLA₂ inhibitor; BaMIP, *Bothrops asper* myotoxin inhibitory protein; IEF, isoelectric focusing; CK, creatine kinase; CNF, crotoxin neutralizing factor; CRD, carbohydrate-recognition domain; M-type receptor, muscle-type receptor; u-PAR, urokinase plasminogen activator receptor; CgMIP, *C. godmani* myotoxin inhibitory protein.

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siniticus) [14] and BaMIP [9]. Group β is represented by the PLI- β of *A. b. siniticus*, which contains leucine-rich repeats and shares 33 % sequence identity with the leucine-rich α -glycoprotein from human serum [15]. Finally, the γ inhibitors share a pattern of cysteine residues constituting two internal repeats of the characteristic three-finger-shaped motifs of the urokinase plasminogen activator receptor (u-PAR) and the Ly-6 superfamily of proteins [16]. This group includes inhibitors from viperids such as CNF [17,18], the PLI- γ inhibitor of *A. b. siniticus* [11], and the PLI-I isoform of *T. flavoviridis* [19] as well as inhibitors from elapids such as the heteromeric 90-kDa PLI of the Thailand cobra (*Naja naja kaouthia*) [20,21], and the recently described inhibitors from the sea krait, *Laticauda semifasciata* [22], and the colubrid *Elaphe quadrigata* [23]. It can thus be concluded that the distribution of inhibitors such as the α and β types is not species-specific: in fact, snakes such as *T. flavoviridis* and *A. b. siniticus* have been shown to contain more than one type of inhibitor [11,19]. Moreover, at least the γ -type inhibitors are present in representatives of four families of snakes: Elapidae (*N. n. kaouthia*), Hydrophiidae (*L. semifasciata*), Viperidae (*C. d. terrificus*, *A. b. siniticus* and *T. flavoviridis*) and Colubridae (*E. quadrigata*).

Such distribution may reflect the evolution of specialized inhibitory proteins adapted to neutralize different types of toxic PLA₂s present in the venom of the same snake or other venomous snakes. The α (CRD-like) PLIs of vipers *A. b. siniticus* and *T. flavoviridis*, which have group-II PLA₂s in their venom, have been reported to specifically bind and inhibit only group-II PLA₂s, particularly the acidic group-II PLA₂s of their own venoms [19,24]. On the other hand, the γ inhibitors are apparently less specific, as the factors of *N. n. kaouthia*, *L. semifasciata* and *E. quadrigata* inhibit group-I, -II and -III PLA₂s [22,23,25], with the former exhibiting stronger inhibitory and affinity constants towards elapid group-I PLA₂s, particularly the CM-II PLA₂ from the venom of the same snake [25]. However, the γ -factor CNF of *C. d. terrificus* inhibits the activity of crotoxin, a group-II PLA₂ from its venom, and to a lesser extent that of group-III bee venom PLA₂, but not that of mammalian pancreatic PLA₂ (group I) [17]. Although these observations have been limited to a relatively small number of mostly venom PLA₂s and have only focused on α and γ inhibitors, they suggest that inhibitors are species-dependent rather than PLA₂-group-dependent. Moreover, most studies have not addressed the specificity of inhibition of toxic properties related to venom PLA₂s, which in some cases, such as with basic PLA₂ myotoxins, are not absolutely nor necessarily dependent on PLA₂ activity [26].

In this study, we report the isolation, cloning, and biochemical and biological properties of two distinct (α and γ) PLIs from the plasma of the Godman's viper (*Cerrophidion godmani*). These exhibit differential affinities as well as pharmacological specificity towards two basic PLA₂ myotoxin isoforms from its own venom and with the same structural classification (group II): an enzymically-active PLA₂ (myotoxin I) and a catalytically-inactive Lys-49 isoform (myotoxin II) [27,28]. The differential neutralization patterns of these inhibitors against toxins of the same structural group provide a new model to analyse the molecular basis of myotoxicity and the molecular mechanisms of inhibitor action.

EXPERIMENTAL PROCEDURES

Plasma and toxins

C. godmani blood was obtained from specimens captured in Costa Rica and kept at the serpentarium of the Instituto Clodomiro Picado, University of Costa Rica, San José, Costa

Rica, using 0.38 % sodium citrate as an anti-coagulant. Plasma was separated by centrifugation and stored at -20°C . Myotoxins I and II were isolated from *C. godmani* venom by ion-exchange chromatography on carboxymethyl-Sephadex (Pharmacia) as described previously [27]. Crotoxin and crotoxin PLA₂ subunit B were provided kindly by Dr José R. Giglio (University of São Paulo, Brazil).

Affinity chromatography procedures

Myotoxins I or II (50 mg) were coupled to 10 ml of CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer. The resulting affinity matrices were packed in Econopak columns (Bio-Rad, Hercules, CA, U.S.A.), washed and equilibrated with 0.1 M sodium phosphate buffer, pH 7.2. Plasma from *C. godmani* (10 ml) was diluted with 5 vol. of 0.1 M sodium phosphate buffer and first recirculated through the Sepharose-myotoxin II column at a rate of 2 ml/min, for 2 h. Flow-through absorbance was monitored at 280 nm. After extensive washing with 0.1 M sodium phosphate buffer, the bound fraction was eluted with 0.1 M glycine/HCl, pH 2.8, and collected in 2 ml of 0.5 M Tris/HCl, pH 8.0, to neutralize the pH. This fraction was dialysed against PBS (0.04 M sodium phosphate/0.12 M NaCl, pH 7.2) at 4°C , and concentrated by filtration on 50-ml-capacity ultrafiltration cells (Amicon, Beverly, MA, U.S.A.) fitted with size-exclusion membranes with a molecular-mass cutoff of 10 kDa. This concentrated material will be referred to subsequently as *C. godmani* myotoxin inhibitory protein II (CgMIP-II). The unbound plasma fraction from the Sepharose-myotoxin II chromatography was subsequently applied to the Sepharose-myotoxin I column, and processed as described above, in order to purify CgMIP-I. Protein concentrations of these fractions were determined by the micro-Bradford method [29], and subsequently aliquoted and stored at -70°C .

Electrophoretic analyses

The affinity-purified factors CgMIP-I and CgMIP-II were analysed by SDS/PAGE on 12 % gels [30] under reducing (2-mercaptoethanol) or non-reducing conditions. Gels were stained with Coomassie Brilliant Blue R-250. Isoelectric focusing (IEF) was performed using polyacrylamide gels containing ampholytes in the pH 3–10 range, on a mini-IEF cell (Bio-Rad) as described by the manufacturer.

FPLC and molecular-mass determinations

The molecular masses of CgMIP-I and CgMIP-II under native conditions were estimated by analytical gel filtration on an FPLC system, using a Superdex 200 HR10/30 column (Pharmacia). PBS was utilized as eluent, at a flow rate of 0.4 ml/min. Molecular mass was estimated relative to the calibration curve derived from protein standards (Pharmacia) by linear regression. K_{av} was determined by the following equation: $K_{av} = V_e - V_0/V_t - V_0$, where V_e is elution volume, V_0 is void volume and V_t is total column volume. Experiments were performed in triplicate.

Deglycosylation analysis

To test glycosylation, CgMIP-I and CgMIP-II were subjected to enzymic deglycosylation with neuraminidase (sialidase) from *Arthrobacter ureafaciens* and recombinant N-glycosidase F from *Flavobacterium meningosepticum* (expressed in *Escherichia coli*; Roche Molecular Biochemicals). A sample of each inhibitor (2 μg) was incubated with neuraminidase or N-glycosidase F

(10 m-units of either enzyme) in 100 mM NaH₂PO₄/50 mM EDTA (pH 7.0) to a final reaction volume of 20 μ l for 18 h at 37 °C. After incubation, 5 μ l of 5 $\times$ sample gel buffer [30] was added and separated by SDS/PAGE on 15% polyacrylamide gels. Changes in molecular mass were determined by comparing the migration of bands relative to the undigested inhibitor and standard molecular-mass markers (Promega, Madison, WI, U.S.A.).

N-terminal sequencing

N-terminal sequencing of CgMIP-I and CgMIP-II was performed on a Perkin-Elmer Procise protein sequencer according to the manufacturer's instructions. The N-terminal sequencing of myotoxin I was performed on an Applied Biosystems Model 477A protein sequencer.

cDNA cloning and sequencing

Poly(A)⁺-enriched RNA was purified from fresh liver extracts by using the oligo(dT) RNA extraction kit from Perkin Elmer-Cetus according to the manufacturer's instructions. Oligo(dT)-primed synthesis of cDNA was carried out using a ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA, U.S.A.). The cDNA synthesis reaction was used as a template for PCR amplification [31] using oligonucleotide primers complementary to the non-coding sequences flanking the reading frames of reported α and γ PLI sequences [17,32]. For CgMIP-I, a γ -type inhibitor, the following primers were used: γ -F (5'-ATCCTCACTAAAGAGCCA-3') and γ -R (5'-GGATGAAATTGCTGACCT-3'). For CgMIP-II, an α -type inhibitor, α -F (5'-TGTACAGGTTTAAAGATG-3') and α -R (5'-CATGCAGGTCTTCCTTC-3') primers were used. PCR amplification was performed using the low-error-rate *Pwo* (*Pyrococcus woese*) DNA polymerase (Boehringer Mannheim) with 3' $\rightarrow$ 5' proofreading capacity on 50- μ l reactions with approx. 0.1 μ g of cDNA template, dNTPs (0.2 mM), and primer combinations (α -F/ α -R or γ -F/ γ -R) at a concentration of 0.5 μ g/reaction for each primer. PCR thermal cycling conditions were as follows: denaturing at 95 °C for 5 min, followed by 25 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. *Taq* polymerase (5 units; Gibco-BRL) was added at the end of the cycling procedure and the reactions incubated for an additional 5 min at 72 °C in order to incorporate 3' adenyl overhangs on to the blunt-ended PCR products for the cloning step (see below). Part of the PCR reaction (5 μ l) was analysed by electrophoresis on 1% agarose gels with 40 mM Tris/acetate/1 mM EDTA (pH 8.0), and the rest (45 μ l) was purified on the Wizard PCR purification kit (Promega). The purified PCR product was ligated to pGEMT (Promega) and used to transform DH10B *E. coli* cells (ATCC). DNA minipreps of these clones were made using a Wizard miniprep system (Promega) and sequenced on an ABI Prism automatic DNA sequencer model 377 (Applied Biosystems) according to manufacturer's instructions. Two separate PCR reactions and cloning procedures were performed on the same cDNA construct, and at least two clones from each reaction were sequenced in opposite directions using primers complementary to the SP6 and T7 promoter sites, which flank the cloning site of pGEMT. DNA sequence data were analysed with SeqEd software (version 1.0.3; Applied Biosystems) and protein sequence alignments were performed on BLAST software [33] on the National Center for Biotechnology Information (NCBI) protein database.

Inhibition of myotoxicity *in vivo*

Myotoxic activity was estimated by determining the plasma levels of creatine kinase (CK; EC 2.7.3.2) in groups of four Swiss-Webster mice (18–20 g), which received an intramuscular injection (in the gastrocnemius) of myotoxins I or II alone (75 μ g), or mixtures of either myotoxin with either CgMIP-I or CgMIP-II, at different molar ratios, incubated previously for 30 min at 37 °C. A control group received an identical injection of PBS alone. Blood samples were collected from the tail 3 h after injection. Plasma CK activity was determined using the Sigma kit 520, and expressed in units/ml (1 unit defined as the amount of enzyme required to phosphorylate 1 nmol of creatine/min at 25 °C).

Inhibition of cytotoxicity *in vitro*

Cytotoxic activity was assayed on murine skeletal-muscle C2C12 myoblasts (ATCC CRL-1772) grown in 96-well plates, as described previously [34]. Myotoxins I or II alone, or mixtures of myotoxin I/CgMIP-I and myotoxin I/CgMIP-II, at different molar ratios, were incubated for 30 min at 37 °C. Then, 150- μ l aliquots (containing 20 μ g of toxins) were applied to the cultures, after aspirating their growth medium. After 3 h of incubation at 37 °C, lactate dehydrogenase released to supernatants was determined with the Sigma kit 500C. Controls for 0 and 100% cytotoxicity consisted of medium and 0.1% Triton X-100, respectively. Additional controls consisted of cells incubated with CgMIP-I or CgMIP-II in the absence of myotoxin. Assays were carried out in triplicate.

Inhibition of oedema-forming activity

Oedema was determined by the footpad assay [35] in groups of four mice (18–20 g), which received a subcutaneous injection of myotoxins I or II alone (15 μ g), or mixtures of myotoxin I/CgMIP-I and myotoxin I/CgMIP-II, at different molar ratios, incubated previously for 30 min at 37 °C. An identical injection of PBS alone was made into the contralateral footpad as a control. Oedema was expressed as the percentage increase in the mass of the toxin-injected footpad after 1 h of incubation relative to the PBS-injected footpad.

Inhibition of PLA₂, anti-coagulant and intracerebral lethality activities

PLA₂ activity was assayed by titration of the fatty acids released from an egg yolk suspension, as described previously [36]. Myotoxin I alone or mixtures of myotoxin I/CgMIP-I and myotoxin I/CgMIP-II, at different molar ratios, were incubated for 30 min at 37 °C. Then, 100- μ l aliquots (containing 10 μ g of toxin) were assayed for PLA₂ activity. The same procedure was followed for the measurement of PLA₂ activity of crotoxin.

The anti-coagulant effect of myotoxin I was determined using platelet-depleted sheep plasma [37]. Myotoxin I alone or mixtures of myotoxin I/CgMIP-I and myotoxin I/CgMIP-II, at different molar ratios, were incubated for 30 min at 37 °C. Then, 100- μ l aliquots (containing 5 μ g of toxin) were added to 0.5 ml of plasma, incubated for 5 min at 37 °C, and coagulation times were recorded after the addition of CaCl₂.

Groups of four mice (16–18 g) received an intracerebroventricular injection (5 μ l) of myotoxin I alone (2 μ g) or mixtures of either myotoxin with CgMIP-I or CgMIP-II, at different molar ratios, incubated previously for 30 min at 37 °C. A control group of mice received an identical injection of PBS alone. Deaths were scored at 24 and 48 h [37].

RESULTS

Isolation and biochemical characterization of CgMIP-I and CgMIP-II

Two myotoxin-binding protein factors, named CgMIP-I and CgMIP-II, were isolated from the plasma of *C. godmani* by means of affinity chromatography with immobilized myotoxins I and II from venom of the same species. SDS/PAGE analysis under reducing conditions showed a band migrating within the range of 20–25 kDa for both CgMIP-I and CgMIP-II (Figure 1, lanes B and D). FPLC gel-filtration analysis on Superdex 200 HR indicated approximate molecular masses of 110 and 180 kDa for CgMIP-I and CgMIP-II, respectively (results not shown). Since SDS/PAGE under non-reducing conditions did not result in a higher migration of the bands, it is likely that this oligomeric conformation is stabilized by non-covalent interactions, and that disulphide bridges occur internally within each subunit, as suggested by the migration of the bands to a lower position (Figure 1, lanes C and E). Both proteins displayed a pI value of approx. 4.0, as estimated by IEF (results not shown).

Treatment of CgMIP-I with N-glycosidase F reduced its molecular mass from 25 kDa to approx. 22 kDa (Figure 2, lanes 1 and 3), whereas partial digestion of CgMIP-II was detected with the same enzyme, resulting in a reduction in size to about 16.5 kDa (Figure 2, lanes 5 and 7). Neuraminidase treatment of

Figure 1 SDS/PAGE analysis of CgMIP-I and CgMIP-II

SDS/PAGE was performed on discontinuous 12% (w/v) polyacrylamide gels of samples under reducing (2-mercaptoethanol) or non-reducing conditions and stained with Coomassie Brilliant Blue R-250. Lane A, molecular-mass markers; lane B, reduced CgMIP-I; lane C, non-reduced CgMIP-I; lane D, reduced CgMIP-II; and lane E, non-reduced CgMIP-II.

Figure 2 Enzymic deglycosylation of CgMIP-I and CgMIP-II

SDS/PAGE was performed on discontinuous 12% (w/v) polyacrylamide gels of samples under reducing conditions. Each inhibitor (2 µg) was incubated for 18 h at 37 °C with neuraminidase or N-glycosidase F (see the Experimental procedures section). Lane 1, CgMIP-I with N-glycosidase F; lane 2, CgMIP-I with neuraminidase; lane 3, undigested CgMIP-I; lane 4, molecular-mass markers; lane 5, CgMIP-II with N-glycosidase F; lane 6, CgMIP-II with neuraminidase; lane 7, undigested CgMIP-II.

A

ATCCTCACTAAAGAGGCC										ATG	AAA	TAT	CTA	CAC	ACC	ATC	TGC	CTT	CTT	
										M	K	Y	L	H	T	I	C	L	L	
TTC	ATT	TTC	GTA	GCC	AGA	GGA	AAC	TCT	CGC	TCA	TGT	GAC	TTT	TGT						6
F	I	F	V	A	R	G	N	S	R	S	C	D	F	C						
										→										
CAC	AAC	ATA	GGA	AAA	GAG	TGT	GAT	GGT	TAC	GAA	AAG	GAA	TGT	TCC						
H	N	I	G	K	E	C	D	G	Y	E	K	E	C	S						21
H	N	I	G	K	E	C	D	G	V*	E	K	E	C	S						
TCT	CCA	GAA	GAT	GTA	TGT	GGC	AAG	GTC	TTG	CTG	GAG	ATT	TCA	TCA						
S	P	E	D	V	C	G	K	V	L	L	E	I	S	S						36
D*	P	E	D	V	C	G	K	V												
GCA	TCG	CTG	TCA	GTC	CGA	ACT	GTG	CAT	AAG	AAC	TGT	TTC	TCA	TCC						
A	S	L	S	V	R	T	V	H	K	N	C	F	S	S						51
AGC	GTG	TGC	AAA	CTT	GAG	CAA	TTT	GAT	GTA	AAT	ATT	GGG	CAT	CAC						
S	V	C	K	L	E	Q	F	D	V	N	I	G	H	H						66
TCA	TAT	ATA	AGA	GGA	AGA	ATC	AAT	TGC	TGT	GAG	AAA	GAA	CAG	TGT						
S	Y	I	R	G	R	I	N	C	C	E	K	E	Q	C						81
GAA	GAC	CGA	CCG	TTT	CCG	GGA	CTG	CCC	CTC	TCC	CAA	CCA	AAT	GGA						
E	D	R	P	F	P	G	L	P	L	S	Q	P	N	G						96
TAC	TAT	TGC	CCT	GGC	GCA	TTG	GGC	CTT	TTT	ACA	GAG	GAC	AGC	ACT						
Y	Y	C	P	G	A	L	G	L	F	T	E	D	S	T						111
GAA	TTT	GAA	GCT	ATT	TGC	AAA	GGA	ACT	GAG	ACT	AAG	TGC	ATT	AAC						
E	F	E	A	I	C	K	G	T	E	T	K	C	I	N						126
ATC	GTG	GGA	CAC	AGA	CAT	GAA	GAT	TTT	CCT	GGA	GAC	ATC	TCT	TAC						
I	V	G	H	R	H	E	D	F	P	G	D	I	S	Y						141
AAT	CTC	AAA	GGC	TGC	ATT	TCT	TCC	TGT	CCT	CTG	CTG	AGT	TTG	AGC						
N	L	K	G	C	I	S	S	C	P	L	E	D	S	L						156
AAT	GCA	ACC	CAT	GAA	GAA	AAC	AGA	AAT	TAT	CTG	GAG	AAA	GTT	GAA						
N	A	T	H	E	E	N	R	N	Y	L	E	K	V	E						171
TGT	AAG	GAC	GCC	TTC	CAA	TTA	GCA	AGA	CTC	TGA	AGGTCAGCAATTTTCATCC									
C	K	D	A	F	Q	L	A	R	L	END										

B

TGTACAGGTTTAAAG																ATG	CGT	CTG	ATT	CTG	CTT	TCC	GGT	CTT	CTT	CTT
																M	R	L	I	L	L	S	G	L	L	L
TTG	GGA	ACC	TTT	CTG	GCC	AAC	GGA	GAT	GAG	AAA	GAT	TCT	GAA	ATA												7
L	G	T	F	L	A	N	G	D	E	K	D	S	E	I												
CAA	TTG	CTG	AAT	TCG	ATG	ATT	GAA	GCC	GTA	ATG	ATA	CTT	CAA	AGA												22
Q	L	L	N	S	M	I	E	A	V	M	I	L	Q	R												
Q	L	L	N	S	M	I	E	A	V	M	I	L	Q	R												
GAC	TTC	TCC	AAC	CTG	AGA	TAT	GCC	TTG	ATG	ACA	GTC	CAC	AAC	GCC												37
D	F	S	N	L	R	Y	A	L	M	T	V	H	N	A												
H*	V*	S	N	L																						
CGA	TCC	TTT	GGG	AGT	GGC	AGT	GAA	AGA	GTG	TAC	GTG	AGC	AAC	AAG												52
R	S	F	G	S	G	S	E	R	V	S	V	S	N	K												
GAA	GTC	AGT	AAG	TTT	GAA	GGT	CTC	GAA	GAA	ATT	TGT	AGC	CAA	GCC												67
E	V	S	K	F	E	G	L	E	E	I	C	S	Q	A												
GGG	GGC	CAT	ATC	CCT	TCC	CCT	CAA	CTC	GAA	AAT	CAG	AAC	AAG	GCC												82
G	G	H	I	P	S	P	Q	L	E	N	Q	N	K	A												
TTT	GCA	GAT	GTT	CTG	GAG	AGG	CAC	AAC	AAA	GCA	GCC	TAC	CTT	GTC												97
F	A	D	V	L	E	R	H	N	K	A	A	G	H	P												
GTG	GGT	GAC	TCA	GCA	AAC	TTC	ACC	AAC	TGG	GCT	GCG	GGA	CAC	CCG												112
V	G	D	S	A	N	F	T	N	W	A	A	G	H	P												
AAT	GAG	GCT	GAT	GGG	ACC	TGT	GTG	AAA	GCA	GAT	ACA	CAC	GGA	TCC												127
N	E	A	D	G	T	C	V	K	A	D	T	H	G	S												
TGG	CAC	TCT	GCA	TCC	TGT	GAT	GAC	AAC	CTC	TTA	GTC	GTG	TGT	GAG												142
W	H	S	A	S	C	D	D	N	L	V	V	V	C	E												
TTT	TAT	TTC	ATT	TTA	TGA	GAAGGAAGACCTGCATG																				
F	Y	F	I	L	END																					

Figure 3 cDNA and N-terminal-derived sequences of (A) CgMIP-I and (B) CgMIP-II

The DNA sequences were obtained by PCR amplification from double-stranded liver cDNA using primers to the non-coding sequences (italics) flanking the open reading frame, which is delineated by start and stop codons (underlined). The underlined amino acid sequence corresponds to the signal peptide. The underlined sequence (bold) corresponds to the N-terminal amino acid sequence determined from the affinity-purified fractions of CgMIP-I and II. Asterisks (*) denote residues of the N-terminal-derived sequence that are different from the cDNA-translated sequence.

A

B

Figure 4 Sequence alignments of CgMIP-I and CgMIP-II with related PLIs

Amino acid residues identical to those of CgMIP-I or CgMIP-II are shown white on a black background. -, Absent sequences; *, N-glycosylation sites; arrows, CRD-like domain. (A) Alignment of CgMIP-I with γ -type inhibitors; T.f. PLI-I, *T. flavoviridis* PLI-I [19]; L.s. PLI-A and -B, *L. semifasciata* PLI subunits A and B [22]; N.n.k. 25 and 31 kDa, *N. n. kaouthia* PLI 25- and 31-kDa subunits [20]. (B) Alignment of CgMIP-II with α -type inhibitors; A.b.s. PLI α , *A. b. siniticus* PLI- α [14], and BaMIP [9].

both inhibitors (Figure 2, lanes 2 and 6) resulted in a slight decrease in size, suggesting that the glycosidic chain contained superficial sialic acid residues.

cDNA and N-terminal sequencing

Figures 3 and 4 show the cDNA and deduced amino acid sequences of CgMIP-I and CgMIP-II, aligned with the amino acid sequences derived from the N-terminal sequencing of the affinity fractions containing either inhibitor. In the case of CgMIP-I, the cDNA-derived sequence of the first 30 amino acids coincides with the N-terminal-derived sequence, with the exception of amino acids 16 and 22 of the mature protein (Figure 3A). Similarly, residues 23 and 24 of the N-terminus of CgMIP-II are different from the amino acids at the same position from the cDNA deduced sequence (Figure 3B). These differences may reflect possible micro-variations among *C. godmani* specimens within a single species or the existence of isoforms of these inhibitors in the serum rather than errors during the PCR cloning, as the possibility of mis-incorporations was minimized by using a low-error-rate polymerase (*Pwo*) and the sequence was identical when derived from at least two clones from two separate PCR reactions (results not shown).

Figure 4 shows the alignments of the deduced cDNA sequences of CgMIP-I and CgMIP-II with γ - and α -type inhibitors of known sequence, respectively. CgMIP-I showed a high (> 85%) sequence identity with factors from viperid snakes, such as the CNF of *C. d. terrificus* and the PLI-I from *T. flavoviridis* (Figure

4A). In contrast, there was considerably lower sequence identity between CgMIP-I and the sequences from the PLIs of hydrophid and elapid origins, particularly subunit B of the *L. semifasciata* inhibitor and the 25-kDa subunit of *N. n. kaouthia* PLI. The degree of sequence identity between CgMIP-II and the other α inhibitors, on the other hand, ranged consistently between 76 and 80% (Figure 4B), with most of the sequence heterogeneity lying within the first 30 N-terminal amino acids. Both types of inhibitor conserve the single N-linked glycosylation site that is present at alignment position 157 (Asn-157) in γ -type inhibitors [20] and position 103 (Asn-103) in α -type inhibitors [14], thus correlating with the enzymic deglycosylation (Figure 2).

Inhibition of PLA₂ activity

CgMIP-I inhibited the PLA₂ activity of myotoxin I, as demonstrated by a linear decrease ($r^2 = 0.99$) in the release of fatty acids by the Dole [36] titrimetric assay (Figure 5A) using 10 μ g of myotoxin (approx. 0.7 nmol) and increasing molar concentrations of CgMIP-I based on the 180-kDa molecular mass of the CgMIP-I oligomer up to a CgMIP-I/myotoxin I molar ratio of 0.4:1, at which a more than 90% reduction of PLA₂ activity was achieved. In addition, this inhibitor completely abrogated the anti-coagulant effect of myotoxin I as well as its intracerebral lethal effect (results not shown). Similar results were obtained upon incubation of CgMIP-I with the crotoxin heterodimer form or with subunit B alone (Figure 5B). In contrast, CgMIP-II did not inhibit the PLA₂ activities of myotoxin I nor the crotoxin

Figure 5 Selective inhibition of the PLA₂ activity of *C. godmani* myotoxin I and crotoxin by CgMIP-I

PLA₂ activity was determined according to Dole [36]. The full activity of myotoxin I was defined as 100%, which corresponded to 24 μ Eq of NaOH/min per mg of myotoxin. The inhibitor/myotoxin ratios were based on the molecular mass of either inhibitor as determined by FPLC gel filtration (Figure 2) and the reported molecular masses of myotoxin I [27] and crotoxin [50]. (A) PLA₂ activity curves of myotoxin I incubated with CgMIP-I (●) and CgMIP-II (□). (B) PLA₂ activity curves of the crotoxin complex (composed of subunits A and B) incubated with CgMIP-I (◆) and CgMIP-II (○), and PLA₂ activity curves of the crotoxin PLA₂ subunit B incubated with CgMIP-I (■) and CgMIP-II (▲).

forms (Figure 5) under the same conditions, and did not neutralize the anti-coagulant or lethal activities of myotoxin I (results not shown).

Neither of the *C. godmani* inhibitors was effective in blocking the enzymic activity of a crude acidic PLA₂ fraction from this venom (results not shown). Myotoxin II, which lacks PLA₂, anti-coagulant or lethal activities, was not included in these inhibition experiments.

Inhibition of toxic, lethal and pro-inflammatory effects of myotoxins I and II

Cytotoxicity experiments using cultured C2C12 myoblasts showed a pattern of selective inhibition by CgMIP-I and CgMIP-II of more than 90% of the cytotoxicity induced by the corresponding myotoxin utilized in their affinity purification (Figures 6A and 6B). With this technique, however, partial reduction of cytotoxic activity is also observed when myotoxin I is incubated with CgMIP-II (Figure 6A), and when myotoxin II is incubated with CgMIP-I (Figure 6B). Such reduction is stronger for the inhibition of myotoxin I with CgMIP-II (approx. 50%) than for the neutralization of myotoxin II by CgMIP-I (36%), yet they are significantly less than for the inhibition of each myotoxin with their respective affinity ligands. The data shown in Figures 6(A) and 6(B) show a representative inhibition

profile of the two inhibitors against the myotoxins at an inhibitor/myotoxin ratio of 0.3:1, which correlates with the PLA₂ inhibition of myotoxin I by CgMIP-I (Figure 5) and is consistent with previous observations of inhibition of basic myotoxic PLA₂s of BaMIP at similar molar ratios [9].

Intramuscular injection of *C. godmani* myotoxins I or II into mice resulted in a drastic increase in plasma CK levels at 3 h, in comparison with controls receiving PBS only. When these toxins were incubated with the inhibitors prior to injection, CgMIP-I neutralized the effect of myotoxin I (Figure 6C), whereas no significant effect was observed with myotoxin II (Figure 6D). Conversely, CgMIP-II completely inhibited the myotoxicity of myotoxin II (Figure 6D), yet had only a slight effect on the CK release induced by myotoxin I at the same molar ratio (Figure 6C). Similar findings were obtained when testing the inhibition of oedema-forming activity of myotoxins (results not shown).

N-terminal analysis of *C. godmani* myotoxin I

Figure 7 shows an alignment of the first 40 N-terminal amino acids of *C. godmani* myotoxin I obtained by BLAST analysis on the NCBI protein database. Sequences are grouped into two sections based on relative sequence identities. Highest identities were observed with *T. flavoviridis* PLA₂-X as well as other PLA₂s from the venoms of crotalid snakes of different genera. The crotoxin B subunit, which is neutralized by CgMIP-I but not by CgMIP-II, shows a higher identity with *C. godmani* myotoxin I (67.5% of the first 40 N-terminal amino acids) than with the full-length myotoxin II [28], which showed 55% sequence identity (alignment not shown). Lower identity was detected between myotoxin I and a number of myotoxic PLA₂s of the same species or from the taxonomically related *Bothrops* species.

DISCUSSION

Within the last decade, a growing number of inhibitors of snake venom PLA₂ activity have been isolated from the blood of snakes and classified into three major groups: α , β and γ (Table 1). These natural components of snake blood are assumed to provide the snake with a natural resistance against the potential toxic effects of its own venom, and perhaps that of other venomous snakes. Whether these factors play a particular physiological function or have emerged solely as a result of evolutionary pressure to counteract toxic effects within venomous organisms is not known. However, the observations that several types of PLA₂ inhibitory factor are present in the blood of a single snake species [11,19] and neutralize specific toxic PLA₂s within the same snake (this study) suggest that several endogenous snake proteins have evolved to neutralize a wide array of PLA₂ components present in snake venoms.

This study shows that two plasma PLIs of the α and γ groups in *C. godmani* (CgMIP-I and CgMIP-II, respectively), bind to and inhibit two different PLA₂s from its venom that belong to the same structural group and share similar myotoxic properties. Such pharmacological selectivity may be explained by critical differences within the structure of both inhibitors and group-II PLA₂s. In the case of α inhibitor CgMIP-II, the CRD-like structure is likely to account for the interaction with the PLA₂ myotoxins. The CRD-like region of α -type inhibitors comprises approx. two-thirds of the length of each subunit, residues 49–143, including nine out of fourteen amino acid residues that are conserved among all CRD domains [14]. Studies with the rabbit M-type PLA₂ receptor have shown that particular CRD domains are responsible for binding to PLA₂ ligands [38]. Furthermore, the mammalian pulmonary surfactant apoprotein (SP-A), which also contains CRD motifs, binds and inhibits a PLA₂ of *T.*

Figure 6 Neutralization of the cytotoxicity *in vitro* and myotoxicity *in vivo* of *C. godmani* myotoxins I and II by CgMIP-I and CgMIP-II

Cytotoxicity *in vitro* was determined by measuring the release of lactate dehydrogenase from a monolayer of C2C12 cells incubated with myotoxin I (Mt-I) alone or with inhibitors (**A**), or myotoxin II (Mt-II) alone or with inhibitors (**B**; see the Experimental procedures section). In each case, a dose of 20 μ g of myotoxin was used, and the molar ratio of inhibitor to myotoxin was 0.4:1. Full activity (100%) was defined as the amount of lactate dehydrogenase released upon lysis of monolayer controls by addition of 0.1% Triton X-100. Incubations were performed in triplicate. Myotoxicity was determined by measuring the CK activity in mice bled 3 h after intramuscular injection of a fixed dose of either myotoxin I or II (75 μ g in an injection volume of 150 μ l), or mixtures of myotoxin with either inhibitor at a defined inhibitor/myotoxin molar ratio of 0.4:1, according to the molecular mass of each inhibitor derived from gel-filtration analysis (Figure 2). (**C**) Myotoxin I; (**D**) myotoxin II. Samples were preincubated for 30 min at 37 °C in PBS prior to injection. Groups of four mice were used for each condition. CK activity is expressed as units/ml of plasma.

PLA ₂	10	20	30	40	% identity
<i>C. godmani</i> myotoxin I	HLFQFREMIK	EMTGKEPVVS	YAFYGCYCGK	GGRGKPK-DAT	
[45] <i>T. flavoviridis</i> PL-X	HLLQFRKMIK	KMTGKEPIVS	YAFYGCYCGK	GGRGKPK-DAT	87.5
[46] <i>A.h. pallas</i> chain A	HLLQFRKMIK	KMTGKEPVVS	YAFYGCYCGS	GGRGKPK-DAT	87.5
[47] <i>A.b. brevicaudus</i> β AgTx2	NLLQFNKMIK	ETGKNAYPF	YAFYGCYCGW	GGQGKPK	69.4
[44] <i>C.d. terrificus</i> crot. B	HLLQFNKMIK	FETRKNAIPF	YAFYGCYCGW	GGRGKPK-DAT	67.5
[42] <i>B. asper</i> myotoxin II	SLFELGKMIL	QETGKNPAKS	YGAYGCNCGV	LGRGKPK-DAT	57.5
[43] <i>B. jararacussu</i> BthTX-I	SLFELGKMIL	QETGKNPAKS	YGAYGCNCGV	LGRGKPK-DAT	57.5
[41] <i>B. asper</i> myotoxin III	SLIEFEKMIL	ETKRLPFPY	YTYGCYCGW	GGQGQPK-DAT	52.5
[28] <i>C. godmani</i> myotoxin II	SMYQLWKMIK	QETGKNVAPS	YGLYGCNCGV	GSRGKPK-DAT	52.5

Figure 7 N-terminal sequence of *C. godmani* myotoxin I

The first 40 N-terminal amino acids of myotoxin I are compared with the N-termini of selected PLA₂s. Sequence alignments were performed using BLAST software on the NCBI protein database. Identical amino acids are shown as white on black.

flavoviridis [39]. A recent study using recombinant deletion mutants of the α inhibitor of *T. flavoviridis* indicated that the CRD region is involved in binding to a PLA₂ from the same snake [40].

The α inhibitors share a consistent sequence identity with each other (Figure 4B), with the greatest heterogeneity (40–52% sequence identity) concentrated within the first 25 N-terminal amino acids. In contrast, the CRD-like regions between residues 49 and 143, which include the Asn-103 glycosylation site, are highly conserved (80–90% sequence identity). This general composition of the α inhibitors is reminiscent of the structural organization of the CRD domains of M-type and mannose receptors, which are highly conserved globular domains of about 110 amino acids and spaced by less-conserved linker regions [2]. It is thus possible that the N-terminus of α -type inhibitors corresponds to this heterogeneous linker-type region, and may perhaps be unrelated to PLA₂ binding. This, however, is not consistent with recent evidence that peptide regions comprising

residues 1–37 of the *T. flavoviridis* PLI subunits (A and B) reportedly bind to PLA₂s in venoms from the same species [40].

As far as PLA₂ specificity is concerned, studies with α -type PLIs of *T. flavoviridis* and *A. b. siniticus* have reported an apparent specificity towards group-II PLA₂s from their respective venoms but not the group-I and -III PLA₂s from elapid or bee venoms, respectively [19]. This apparent restriction to group-II PLA₂s correlates with the fact that viperid snakes have group-II PLA₂s in their venoms, and may indicate that their CRD domains recognize particular structural motifs within this group. However, this is not in accordance with observations that the M-type receptor with CRD-like domains binds secreted PLA₂s from both groups I and II [2]. Furthermore, this study demonstrates that selectivity of an α inhibitor is subject to more subtle differences among PLA₂s of the same structural group, as indicated by the specificity of CgMIP-II towards *C. godmani* myotoxin II but not its myotoxin I. *C. godmani* myotoxin II, a Lys-49 PLA₂, shares close to 80% sequence identity with the

Table 1 Classification of snake-blood PLIs

Nomenclature is based on the proposed classification made by Ohkura and co-workers upon description of the three inhibitor types in the blood plasma of *A. b. siniticus* [11]. The α -type inhibitors include those containing CRD-like sequences characteristic of C-type lectins and the mammalian M-type PLA₂ receptor. The β inhibitor type contains a leucine-rich domain homologous to that of the human leucine-rich α_2 -glycoprotein. The γ -type inhibitors share a pattern of cysteine residues typical of the Ly-6 superfamily of proteins as well as the u-PAR.

Inhibitor type	PLI	Reference
α	BaMIP	[9]
	<i>T. flavoviridis</i> PLI subunits A and B	[3]
	<i>A. b. siniticus</i> PLI- α	[14]
	CgMIP-II	This paper
β	<i>A. b. siniticus</i> PLI- β	[15]
γ	<i>A. b. siniticus</i> PLI- γ	[11]
	<i>T. flavoviridis</i> PLI-I	[19]
	<i>C. d. terrificus</i> CNF	[17]
	<i>N. n. kaouthia</i> PLI	[20]
	<i>L. semifasciata</i> PLI	[22]
	<i>E. quadrigata</i> PLI	[23]
	CgMIP-I	This paper

Lys-49 myotoxin II of *B. asper* [28], which is inhibited by another α factor, BaMIP [9]. However, the Lys-49 or Asp-49 variation is not the determining factor for the binding of these α -type inhibitors, as BaMIP inhibits both Lys-49 and Asp-49 isoforms of its own venom, and CgMIP-II inhibits the Asp-49 myotoxin III of *B. asper*, even though this PLA₂ shares only 58 % homology with *C. godmani* myotoxin II (S. Lizano, unpublished work). Therefore, independent of catalytic activity, common structural motifs within these PLA₂s, which are probably different or absent in *C. godmani* myotoxin I, may be recognized by the CRD-like domains of these α inhibitors. Future cross-species inhibition studies of α -type inhibitors against PLA₂ toxins of known sequence should enable a more direct assessment of the structural elements of the inhibitor-PLA₂ interaction.

In contrast, *C. godmani* myotoxin I, which is inhibited selectively by the γ factor CgMIP-I but not CgMIP-II, shows high enzymic activity, yet has similar myotoxic properties to myotoxin II [27]. Preliminary sequence analysis of this PLA₂ (Figure 7) revealed significant differences with myotoxin II of *C. godmani*, as well as with other myotoxic PLA₂s from the venoms of *Bothrops* species, such as the Asp-49 and Lys-49 isoforms of *B. asper* [41,42] and the bothropstoxin I of *B. jararacussu* [43]. Instead, *C. godmani* myotoxin I shares considerable sequence identity with the B subunit of crotoxin [44], a neurotoxic PLA₂ which is also neutralized by CgMIP-I, but not by the CRD-containing CgMIP-II (Figure 5). Crotoxin is inhibited by another γ -type inhibitor, CNF [17], which shares 90 % sequence identity with CgMIP-I. In addition to crotoxin, *C. godmani* myotoxin I is closely related to other toxic group-II PLA₂s [45–47]. Some of these PLA₂s are presynaptic neurotoxins and share less identity with the classic *Bothrops* myotoxins (Figure 7), including *C. godmani* myotoxin II. It is interesting to note that the highest identity of *C. godmani* myotoxin I is shared with the PLA₂-X isoform of *T. flavoviridis*, which is reportedly not myotoxic, but rather acts as a presynaptic neurotoxin [45,48]. A phylogenetic analysis of the PL-X'-PLA₂ of *T. flavoviridis*, which is closely related in sequence to the PLA₂-X isoform, reveals an evolutionary divergence from basic Lys-49 isoforms of the same snake that are closely related to the *B. asper* isoforms [48]. Thus particular structural feature(s) common to these PLA₂s are recognized by γ inhibitors but not by the CRD domains of α

inhibitors. The cloning and determination of the full sequence of *C. godmani* myotoxin I will eventually allow a more systematic analysis of the molecular specificity of γ -type inhibitors.

Instead of CRD domains, γ inhibitors contain two internal repeats of the three-finger motif [21] characteristic of a group of proteins comprised by the u-PAR, the Ly-6 superfamily of proteins, such as the cell-surface antigens Ly-6A/E, Ly6C, ThB and CD59, as well as the family of α -type neurotoxins and the recently described RoBo-1, a putative modulator of bone growth and remodelling that is expressed in rat bone and growth plate cartilage [49]. Recent deletion studies of recombinant forms of the γ -type PLI-I inhibitor of *T. flavoviridis* [40] indicate that such a three-finger motif is required for binding to PLA₂ isoforms from the venom of the same snake. The γ inhibitors from an elapid (*N. n. kaouthia*), a hydrophid (*L. semifasciata*) and a colubrid (*E. quadrigata*) inhibit the PLA₂ activities of all three groups of PLA₂s [22,23,25] with generally stronger affinities towards the PLA₂s from their own venom [25]. On the other hand, the γ inhibitor CNF from the crotalid snake *C. d. terrificus* is reportedly more specific towards the group-II B subunit of crotoxin than to bee venom group-III PLA₂ and the group-I PLA₂ from porcine pancreas [17]. Thus whereas PLIs from crotalid snakes (α -type inhibitors and the γ -type CNF) may have adapted to inhibit the group-II-type PLA₂s characteristic of their own venoms, the γ inhibitors of elapids and hydrophids exhibit a wider range of inhibition, perhaps as a result of the recognition of common motifs among all groups of PLA₂s. To date, no evidence for the presence of α inhibitors in elapids or hydrophids has been reported. Although this does not preclude the presence of CRD-type inhibitors in the blood of these snakes, a Western blot analysis of crude plasma from various Central American vipers as well as the elapids *N. n. kaouthia* and the coral snake (*Micrurus nigrocinctus*) using polyclonal antibodies against BaMIP indicated that a cross-reactive component similar in size to this inhibitor is present only in viperid plasma, thus suggesting the absence of this type of factor in the blood of elapids (S. Lizano, unpublished work). However, evidence at the genetic level is needed to demonstrate that α inhibitors are in fact not encoded or expressed in elapids or hydrophids.

The sequence alignment of CgMIP-I with other γ -type inhibitors (Figure 4) indicates a close relationship (> 85 % sequence identity) among factors belonging to snakes of the subfamily Crotalinae, namely CNF and PLI-I from *T. flavoviridis*. Within this group, the degree of similarity correlates with the geographical distribution of the snakes, as the sequence identity is higher between CgMIP-I and CNF, which belong to vipers of the American continent, than between CgMIP-I and PLI-I from the Habu snake, an Asian viper. Furthermore, the lower homology with the γ inhibitors of *N. n. kaouthia*, an elapid, and *L. semifasciata*, a hydrophid, particularly with two of the subunit types from their respective heteromeric inhibitors, suggest a divergence in the evolution of the γ inhibitors of viperids from those of elapids and hydrophids. In addition, the Asn-157 glycosylation site is conserved among these inhibitors, including CgMIP-I (Figure 4), except with the two least homologous subunits of *L. semifasciata* [22] and *N. n. kaouthia* [20,21]. These observations, coupled with the evidence that CNF shows preference towards a group-II PLA₂ whereas elapid and hydrophid γ inhibitors are not group-specific, may merit a separation of the γ -type inhibitors into two subclasses: heteromeric, mono-glycosylated, PLA₂ group, non-specific γ inhibitors from elapids and hydrophids (γ 1); and homomeric, fully glycosylated, PLA₂ group II, restricted γ inhibitors from crotalid snakes (γ 2).

Finally, the inability of both inhibitors to block the enzymic activity of an acidic PLA₂ fraction from this venom indicates that

they are not effective against all PLA₂ variants of *C. godmani* venom. Thus additional plasma inhibitors and/or alternative mechanisms of toxin resistance may be present in this snake. The recent description of a β -type inhibitor in the blood of *A. b. siniticus* [15] indicates that at least three different types of inhibitor protein are produced in the liver and secreted to the circulation in snakes.

The significance of sequence heterogeneity among γ -type inhibitors and the apparent restriction of α -type inhibitors to viperid snakes are still the subjects of further research as new members of these groups are discovered. Nonetheless, these variations reflect possible adaptations to the diverse PLA₂ composition of snake venoms. Furthermore, the neutralization of myotoxic effects, much of which does not appear to be a direct function of PLA₂ activity in these toxins, opens a new possibility for studying the structure and function of myotoxin action. Although the structural elements of PLA₂s necessary for binding to α and γ inhibitors have yet to be elucidated, the selectivity of these inhibitors to toxic PLA₂s of the same structural classification (group II) and with similar toxic properties provide a unique opportunity to analyse the molecular basis of PLA₂–inhibitor interaction. A combined approach involving deletion studies and site-directed mutagenesis of specific sites within these inhibitors and/or their respective toxic PLA₂ targets should help elucidate the molecular events of myotoxicity as well as of the specific inhibitor–myotoxin interaction.

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