Crotoxin from *Crotalus durissus terrificus* and Crotoxin-Related Proteins: Structure and Function Relationship

1

Grazyna Faure, Dorota Porowinska, and Frederick Saul

Abstract

Snake venom presynaptic phospholipases A_2 (PLA_2s) are β-neurotoxins present in monomeric form or as multimeric complexes with various quaternary structures. Three classes of β-neurotoxins from snake venom have been described. Here in the heterodimeric CACB crotoxin complex, a potent β-neurotoxin from *Crotalus durissus terrificus* venom, and its natural isoforms are presented. Crotoxin and crotoxin-related proteins possess PLA_2 activity and display diverse pharmacological properties. Many of these properties are conferred by regions of the structure not involved in catalysis but directly implicated in protein-protein interactions (PPI) with PLA_2-receptor targets. Mono- and multimeric PLA_2s are involved in various biological functions and can modulate specific disease processes. Numerous attempts have been made to correlate PLA_2 structures with these pharmacological properties and to identify PPI sites. These sites represent potential lead structures for the development of new compounds for modulation of specific disease processes. However, PPI sites are difficult to discover and design in the absence of 3D structural studies (co-crystallization with protein targets), and few structures of PLA_2-receptor

G. Faure (✉)
Département de Neuroscience, Institut Pasteur, Unité Récepteurs-Canaux, CNRS, UMR 3571, Paris, France
e-mail: grazyna.faure-kuzminska@pasteur.fr

D. Porowinska
Département de Neuroscience, Institut Pasteur, Unité Récepteurs-Canaux, CNRS, UMR 3571, Paris, France

Department of Biochemistry, Nicolaus Copernicus University, Torun, Poland
e-mail: porowinska@umk.pl

F. Saul
Institut Pasteur, Plate-forme de Cristallographie, CNRS-UMR 3528, Paris, France
e-mail: frederick.saul@pasteur.fr

P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_7

complexes have been reported. The acidic CA subunit of crotoxin may be considered as a natural target of the basic PLA_2 CB subunit. The 3D structure of the crotoxin CACB complex provides a detailed structural model of the interaction between the CA and CB subunits. Identification of the molecular interface between the two subunits of crotoxin is essential to predict other biologically relevant PPI sites. This chapter is focussed on the structure-function relationship of crotoxin and crotoxin-related proteins and recent investigations to identify new biological targets of crotoxin.

Keywords
Crotoxin • Isoform • Neurotoxic phospholipase A_2 • Snake venom • PLA_2 targets

Contents

Introduction

Snake venom is a complex mixture of components that exhibit numerous pathophysiological effects. The venom of the South American rattlesnake *Crotalus durissus terrificus* contains four proteic components: gyroxin, crotoxin, convulxin, and crotamine, of which crotoxin represents the principal toxic component. Crotoxin is a potent β-neurotoxin that acts primarily at the presynaptic level of the neuromuscular junction and inhibits acetylcholine release; blockage of neuromuscular transmission leads to muscle paralysis and death by asphyxia (Brazil and Excell 1971; Hawgood and Smith 1977). Crotoxin can also act at the postsynaptic level, stabilizing a desensitized form of the acetylcholine receptor (AChR) (Bon et al. 1979; Brazil et al. 2000).

In addition to neurotoxicity, crotoxin exhibits other significant pharmacological properties and biological effects such as myotoxic (Gutiérrez et al. 2008), cardiotoxic (potentiation of L-type Ca^{2+} channel) (Zhang et al. 2010), cytotoxic (chronic seizure effect by release of glutamate from cerebrocortical synaptosomes via N and P/Q Ca^{2+} channel in the central nervous system) (Lomeo Rda et al. 2014), bactericidal (Oliveira et al. 2002; Toyama et al. 2003; Perumal Samy et al. 2006), anti-inflammatory (Cardoso et al. 2001; Sampaio et al. 2005; Zambelli et al. 2008; Nunes et al. 2010), antitumoral (Rudd et al. 1994; Costa et al. 1998; Cura et al. 2002;

Yan et al. 2007), and analgesic (Zhang et al. 2006; Nogueira-Neto Fde et al. 2008) effects and antiviral activity (Muller et al. 2012; Muller et al. 2014).

Crotoxin from *Crotalus durissus terrificus* venom was the first animal neurotoxin isolated and characterized (Slotta and Fraenkel-Conrat 1938). Other crotoxin-like proteins commonly occur in the venoms of *Crotalus* species, e.g., *Crotalus durissus cascavella* (Beghini et al. 2000; Beghini et al. 2004; Rangel-Santos et al. 2004; Fonseca et al. 2006), *Crotalus durissus collilineatus* (Ponce-Soto et al. 2002; Rangel-Santos et al. 2004; Toyama et al. 2005; Ponce-Soto et al. 2007; Salvador et al. 2009), *Crotalus durissus cumanensis* (Pereañez et al. 2009; Cavalcante et al. 2015), *Crotalus durissus ruruima* (Dos-Santos et al. 2005), *Crotalus scutulatus* (Mojave toxin) (Bieber et al. 1975; Gopalakrishnakone et al. 1980), and *Crotalus simus* (Calvete et al. 2010; Castro et al. 2013; Durban et al. 2013), and *Erotalus vegronolis* (Viala et al. 2015). Heterodimeric complexes of crotoxin-like proteins have also been identified in the venom of non-rattlesnake species belonging to Crotalinae subfamily: pit vipers *Gloydius intermedius* (Gintexin) (Yang et al. 2015a, b) and *Bothriechis nigroviridis* (Nigroviriditoxin) (Fernández et al. 2010; Lomonte et al. 2015).

A comparision of the structure, biological properties, and toxicity of crotoxin from *Crotalus durissus terrificus* and other crotoxin-like proteins is presented here. Other biological targets of crotoxin and its basic PLA_2 CB subunit are also described.

Crotoxin

Crotoxin is a heterodimeric protein complex formed by the non-covalent association of an acidic, nontoxic, nonenzymatic CA subunit with a basic, weakly toxic CB subunit with phospholipase A_2 activity (group IIA $sPLA_2$) (Hendon and Fraenkel-Conrat 1971). Individual snake venom contains up to 16 natural crotoxin complexes having different biochemical and biological properties (Faure and Bon 1988). These complexes result from the association of different isoforms of the CA and CB subunits (Faure et al. 1991, 1993). Moreover, the profiles of crotoxin isoforms vary between individual snakes of the same species (Faure and Bon 1987).

CA Subunit

The CA subunit of crotoxin (crotapotin, component A, or crotoxin A) is an acidic, nontoxic protein comprising three disulfide-linked polypeptide chains α, β, and γ. The CA subunit does not possess catalytic activity and in snake venom is present only in complex with the basic CB subunit, enhancing its lethal potency. CA prevents the formation of oligomers between PLA_2 subunits and targets CB to reach the specific crotoxin receptor. CA also inhibits the PLA_2 and anticoagulant activities of CB (Faure et al. 1991, 1993, 2007).

Table 1 Molecular masses of CA subunit isoforms of crotoxin and crotoxin-like proteins

	Molecular mass [Da]	References
Crotalus durissus terrificus		
CA_1	9597	This review
CA_2	9429.9 (±1.4)	This review
CA_3	9645 (±0.2)	This review
CA_4	9687	This review
Gloydius intermedius		
Gintexin-A	9569	Yang et al. (2015a)
	9487	Yang et al. (2015a)
	9947	Yang et al. (2015a)
	9670	Yang et al. (2015a)
Bothriechis nigroviridis		
Nigroviriditoxin A_1	9605.6	Lomonte et al. (2015)
Nigroviriditoxin A_2	9421.5	Lomonte et al. (2015)

Molecular masses were determined by mass spectrometry

Four CA isoforms (CA_{1-4}) have been identified in *Crotalus durissus terrificus* venom. Briefly, crotoxin was isolated from crude venom by gel filtration chromatography, and the CA and CB subunits were separated by ion-exchange chromatography in the presence of 6 M urea. Four CA isoforms were purified from a mixture of isoforms on Mono Q column by an anion-exchange chromatography. The three chains of each purified CA isoforms from *Crotalus durissus terrificus* were separated by reverse-phase (RP)-HPLC (Vydac C18 column) and the sequences determined by Edman degradation (Faure et al. 1991). Several isoforms of an acidic CA-like protein were found in the non-rattlesnake species *Gloydius intermedius* (Yang et al. 2015a) and *Bothriechis nigroviridis* (Lomonte et al. 2015) and were isolated from the crude venoms using directly RP-HPLC in acetonitrile gradient (Yang et al. 2015a; Lomonte et al. 2015). Gel filtration chromatography was performed in the case of Gintexin-A (Yang et al. 2015a). The molecular masses of CA subunit isoforms isolated from *Crotalus durissus terrificus*, *Gloydius intermedius*, and *Bothriechis nigroviridis* are shown in Table 1.

All CA subunit isoforms of crotoxin derive from a pro-CA PLA_2-like precursor protein homologous to nontoxic acidic group IIA $sPLA_2$ and result from post-translational modifications by removal of three peptide segments (Faure et al. 2011) (the enzymes responsible for this process have not yet been identified). During protein maturation a conversion of N-terminal glutamine residues to pyrrolidone carboxylic acid occurs in the β and γ chains. This modification increases protection of the protein from proteolytic degradation. The mature CA protein is composed of three polypeptide chains (α, β, γ) linked by two intra- and five interchain disulfide bonds (Bouchier et al. 1991; Faure et al. 1991; Faure et al. 2011). Differences between CA isoforms of crotoxin and crotoxin-like proteins result from slightly different lengths and amino acid sequences of the

Fig. 1 Multiple sequence alignment of precursors and mature proteins of CA from *Crotalus durissus terrificus* (Faure et al. 1991) and Gintexin-A from *Gloydius intermedius* (Yang et al. 2015a). *pro-CA* precursor of the acidic subunit of crotoxin from *Crotalus durissus terrificus*, *pro-Gintexin-A* precursor of the acidic subunit of the crotoxin-like protein from *Gloydius intermedius*. Amino acid numbering is according to Renetseder et al. 1985. In *blue*, point mutations between sequences; *black boxes*, chain α, β, and γ in mature proteins; *red boxes*, pyrrolidone carboxylyl residues, converted from glutamine by posttranslational modification; underlined, residues determined only by amino acid composition (not sequenced). Sequence alignment was performed with CLC Sequence Viewer 6

individual polypeptide chains (Fig. 1) (Faure et al. 1991). The post-translational maturation of pro-CA is a prerequisite for an appropriate assembly of the three independent CA chains with the basic CB subunit to form the crotoxin complex (Faure et al. 1991).

The crystal structure of only one CA isoform has been solved, the isoform CA_2 in complex with isoform CBb in the 3D structure of crotoxin (PDB 3R0L) (Faure et al. 2011). This structure revealed that the two long α-helices C and D in the positions expected for group IIA $sPLA_2$ are preserved in the CA subunit, but the long α-helix A and the short α-helix B are absent (as expected from posttranslational cleavage of pro-CA) (Faure et al. 2011). Despite the preservation of the amino acid sequence in the predicted Ca^{2+}-binding loop, an extended conformation unlike the canonical Ca^{2+}-binding loop of group IIA PLA_2S structures was revealed, explaining in part why the CA subunit does not perform a catalytic function. The amino acid sequence of the precursor of the acidic subunit from *Gloydius intermedius* (Gintexin-A) displays 76% identity with pro-CA from *Crotalus durissus terrificus* (Fig. 1). Similar to the CA subunit of crotoxin, mature Gintexin-A consists of three polypeptide chains resulting from post-translational modifications of a precursor protein, and the structure is stabilized by seven disulfide bonds (Yang et al. 2015a). The α, β, and γ chains of this protein show high amino acid sequence identity (87% and 69% with $CA_{1,2,4}$ for chain α and 85% and 66% with CA_3 for chain β of crotoxin and 78% for chain γ of CA).

CB Subunit

The CB subunit of crotoxin (component B or crotoxin B) is a basic, weakly neurotoxic protein that possesses PLA_2 activity and catalyzes the hydrolysis of the *sn*-2 ester bond of phospholipids, producing free fatty acids and lysophospholipids (Hendon and Fraenkel-Conrat 1971; Faure and Bon 1988). In snake venom, isoforms of the CB subunit of crotoxin occur in complex with CA. The isolated CB subunit also acts at the presynaptic level of the neuromuscular junction and blocks neuromuscular transmission, but tenfold higher doses of this protein are needed compared to the CACB complex.

Four isoforms of the CB subunits (CBa_2/CB2, CBb, CBc/CB1, and CBd) in *Crotalus durissus terrificus* venom have been identified. These isoforms were purified from crude venom using a four-step chromatographic procedure: (i) gel filtration (isolation of crotoxin), (ii) ion-exchange chromatography in the presence of 6 M urea (separation of the CA and CB subunits each containing a mixture of isoforms), (iii) cation-exchange column using double gradient (0–1 M NaCl and 0–3 M urea, purification of CB isoforms), and (iv) RP-HPLC, Vydac C4 column (additional purification step) (Faure and Bon 1988). The CB isoforms are products of different mRNAs (Bouchier et al. 1991; Faure et al. 2011). However, in *Crotalus durissus terrificus* venom, several additional CB isoforms (CB-like proteins: F15 (Toyama et al. 2003), F16 (Hernandez-Oliveira et al. 2005), F17 (Oliveira et al. 2002), and Intercro (Vieira et al. 2013)) have been identified. Proteins F15, F16, and F17 were isolated from the crotoxin fraction by RP-HPLC. Intercro was eluted from the column as a fraction located between crotamine and crotoxin during gel filtration chromatography. In the case of non-rattlesnake species, the presence of basic CB-like isoforms has also been observed. Two isoforms of Nigroviriditoxin B, a CB-like basic protein, were purified by RP-HPLC from the crude venom of *Bothriechis nigroviridis* (Lomonte et al. 2015). A single isoform of Gintexin-B was isolated from crude venom of *Gloydius intermedius* using gel filtration and RP-HPLC (Yang et al. 2015a). Both rattlesnake and non-rattlesnake CB and CB-like proteins contain 122 amino acid residues with a molecular weight of approximately 14 kDa and pI values around 9 (Table 2).

All CB isoforms from *Crotalus durissus terrificus* are secreted group IIA phospholipases structurally homologous to the inflammatory, non-pancreatic human group IIA $sPLA_2$ (EC 3.1.1.4). The crystal structures of isoforms CBa_2, CBb, and CBc show the presence of highly conserved canonical structural features of group IIA $sPLA_2$ stabilized by seven disulfide bonds (Marchi-Salvador et al. 2008; Faure et al. 2011). The 3D structures of CB-like PLA_2s have not yet been determined experimentally, but as shown in Fig. 2, they share a high degree of sequence homology with CB. CB isoforms and other CB-like proteins isolated from *Crotalus durissus terrificus* venom (F15, F16, F17, and Intercro) show sequence identities of more than 90% (except CBc/F17) (Table 3). The high sequence identity between CBa_2 and Intercro (97.5%) is surprising since the latter is not part of the crotoxin fraction (Vieira et al. 2013). These proteins differ by only three residues (positions CBa_2/Intercro: W70/F, Y117/L, and Y120/F) (Fig. 2), resulting in lower PLA_2

Table 2 Biochemical properties of CB and CB-like proteins from *Crotalus durissus terrificus*, *Bothriechis nigroviridis*, and *Gloydius intermedius*

	Molecular mass [Da]	pI	References
Crotalus durissus terrificus			
CBa_2	4245 (±1)^	8.74	Faure et al. (1994)
CBb	4152 (±1)^	8.74	Faure et al. (1994)
CBc	4186 (±0.9)^	8.74	Faure et al. (1994)
CBd	4234 (±1)^	8.74	Faure et al. (1994)
F17	4664.14^	8.74	Oliveira et al. (2002)
F16	4860^	9.01	Hernandez-Oliveira et al. (2005)
F15	14479.7*	9.16	Toyama et al. (2003)
Intercro	4188^ 4282^	8.75	Vieira et al. (2013)
Gloydius intermedius			
Gintexin-B	14,177^	8.73	Yang et al. (2015a)
Bothriechis nigroviridis			
Nigroviriditoxin B	14,083^ 14,113 (±2)^	8.5	Lomonte et al. (2015)

^ Molecular masses determined by ES mass spectrometry, * based on the protein sequence with the program ProtParam
pI values were determined based on the protein sequence with the use of ProtParam program

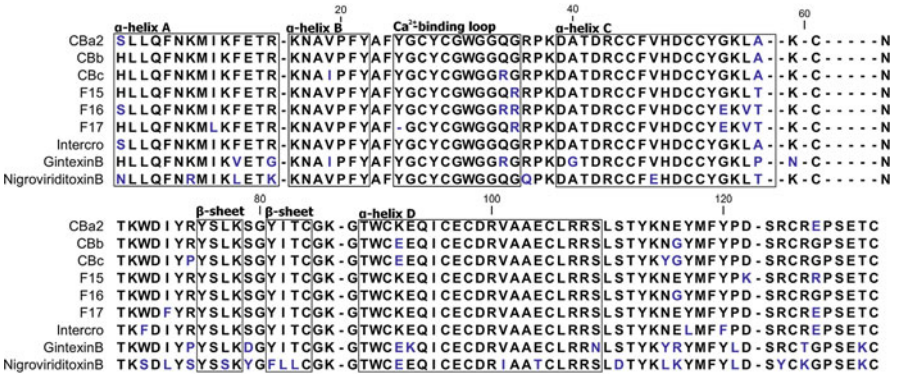

Fig. 2 Multiple sequence alignment of CB isoforms and CB-like proteins F17, F16, F15, and Intercro from *Crotalus durissus terrificus* and non-rattlesnake basic subunits Gintexin-B from *Bothriechis nigroviridis* and Nigroviriditoxin B from *Gloydius intermedius*. Amino acid numbering is according to Renetseder et al. 1985. In *blue*, point mutations occurring in sequences of analyzed proteins; *black boxes*, highly conserved regions of $SPLA_2$-IIA: α-helix A, helix B, Ca^{2+}-binding loop, α-helix C, the β-wing, and α-helix D.

activity and toxicity of Intercro and preventing complex formation with CA (Table 4). The amino acid sequences of CB-like subunits isolated from non-rattlesnake species Gintexin-B and Nigroviriditoxin B reveal the identities up to 90% and 80%, respectively, to all PLA_2s isolated from *Crotalus durissus terrificus*

Table 3 Sequence identities (%) of $sPLA_2$ isoforms from *Crotalus durissus terrificus*, *Bothriechis nigroviridis*, and *Gloydius intermedius* venom

	CBa_2	CBb	CBc	F17	F16	F15	Intercro	Gintexin-B
CBb	96.72	–	–	–	–	–	–	–
CBc	93.44	96.72	–	–	–	–	–	–
F17	94.21	92.56	89.26	–	–	–	–	–
F16	94.26	94.26	92.62	95.04	–	–	–	–
F15	95.9	95.08	91.8	95.04	94.26	–	–	–
Intercro	97.54	94.26	90.98	91.74	91.8	93.44	–	–
Gintexin-B	84.43	86.89	90.16	80.99	83.61	83.61	81.97	–
Nigroviriditoxin B	78.69	80.33	78.69	76.03	77.05	77.87	77.05	77.05

(Table 3). Several sequence differences occur in the C-terminal region often considered to be responsible for the neurotoxicity of the venom PLA_2s. The phylogenetic relationship of the CB and CB-like proteins analyzed here is shown in Fig. 3.

CB isoforms and CB-like proteins in the presence of acidic subunit CA form different classes of crotoxin complexes (see below), confirming that natural mutations in CB isoforms determine different properties of crotoxin complexes (Faure and Bon 1988). The phospholipase A_2 activities of all CB isoforms are quite similar (Table 4) (Faure et al. 1993). However, significant differences appear in the presence of the CA subunit (except for isoform CBa_2 where binding of CA does not influence PLA_2 activity). The crystal structure of the crotoxin CA_2CB_6 complex (Faure et al. 2011) revealed that the natural point mutation at position 1 (Ser1 in CBa_2, His1 in CBb,c) is crucial for the functional differences between crotoxin isoforms. The presence of Ser at position 1 in CBa_2 associated with a displacement of Trp70 and Trp31 prevents their interaction with Asp89 and Asp99 of the CA subunit (chain β), leading to greater substrate access to the catalytic site of CB (His48 and Asp99) (Faure et al. 2011). These structural differences explain the higher PLA_2 activity of the CA_2CBa_2 complex.

For the CBb, CBc, and CBd isoforms, a two- to fourfold decrease in enzymatic activity is observed after CA binding, depending on the CB isoform (Table 4) (Faure et al. 1993). Once again, these differences result from point mutations in the CB subunit (Fig. 2). For isoforms F15, F16, and F17, very weak inhibition of enzymatic activity is observed in complexes with the CA subunit (Table 4). Intercro does not form complexes with CA (Vieira et al. 2013). In agreement with previous results (Faure et al. 2011), the mutation at position 70 (Phe70 in Intercro, Trp70 in CBa_2) explains the absence of binding of Intercro with CA. Nigroviriditoxin B alone shows very similar PLA_2 activity to CB (Lomonte et al. 2015).

The enzymatic activity of all PLA_2s is strictly dependent on the presence of Ca^{2+} ions (Mayer and Marshall 1993; Murakami and Kudo 2002). The calcium binding sites (Cys29, Gly30, Gly32), as well as Trp31 which affects stability in this region, are conserved in all CB isoforms and other crotoxin-like proteins analyzed here

Table 4 Enzymatic activity of CB isoforms and CB-like PLA_2s from *Crotalus durissus terrificus*, *Gloydius intermedius*, and *Bothriechis nigroviridis*

	PLA_2 activity	Influence of CA on PLA_2 activity
Crotalus durissus terrificus		
CBa_2	V_{max} = 24 (±2) μmol/min/mg K_m = 0.06 (±0.02) μM[a*]	For CA_2 V_{max} = 25 (±2) μmol/min/mg K_m = 0.05 (±0.03) μM[a*] For CA_3 V_{max} = 24 (±1) μmol/min/mg K_m = 0.05 (±0.02) μM[a*]
CBb	V_{max} = 22 (±2) μmol/min/mg K_m = 0.07 (±0.02) μM[a*]	For CA_2 V_{max} = 10 (±2) μmol/min/mg K_m = 0.3 (±0.1) μM[a*] For CA_3 V_{max} = 9 (±2) μmol/min/mg K_m = 0.2 (±0.05) μM[a*]
CBc	V_{max} = 18 (±2) μmol/min/mg K_m = 0.07 (±0.02) μM[a*]	For CA_2 V_{max} = 7 (±1) μmol/min/mg K_m = 0.22 (±0.05) μM[a*] For CA_3 V_{max} = 6.6 (±1) μmol/min/mg K_m = 0.2 (±0.05) μM[a*]
CBd	V_{max} = 19.7 (±2) μmol/min/mg K_m = 0.06 (±0.03) μM[a*]	For CA_2 V_{max} = 4.6 (±1) μmol/min/mg K_m = 0.35 (±0.05) μM[a*] For CA_3 V_{max} = 4 (±1) μmol/min/mg K_m = 0.3 (±0.1) μM[a*]
F17	V_{max} = 0.0082 μmol/min/mg K_m = 31.2 mM[b] ^	Slight inhibition of PLA_2 activity[b]
F16	V_{max} = 0.0107 μmol/min/mg K_m = 27.3 mM[c] ^	V_{max} = 0.0098 μmol/min/mg K_m = 53.4 mM[c] ^
F15	V_{max} = 0.0085 μmol/min/mg K_m = 38 mM[d] ^	V_{max} = 0.0082 μmol/min/mg K_m = 58.4 mM[d] ^
Intercro	Activity represent ~40% of CB[e x #]	No complex formation with CA[e]
Gloydius intermedius		
Gintexin-B	nd	nd
Bothriechis nigroviridis		
Nigroviriditoxin B	Activity represent ~80% of CB[f ^ #]	Inhibition of PLA_2 activity: 2x[f]

Substrates: *2 μM 1-palmitoyl-2-(l0-pyrenyldecanoyl)-sn-glycero-3-monomethyl phosphatidic acid, ^ 0.32 mM synthetic 4-nitro-3-octanoylbenzoic acid, [x]1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine, *nd* not determined, # activity given as a fluorescence intensity

[a]Faure et al. (1993)
[b]Oliveira et al. (2002)
[c]Hernandez-Oliveira et al. (2005)
[d]Toyama et al. (2003)
[e]Vieira et al. (2013)
[f]Lomonte et al. (2015)

Fig. 3 Phylogenetic tree of CB and CB-like PLA_2 isoforms F17, F16, F15, and Intercro from *Crotalus durissus terrificus* and non-rattlesnake basic subunits Gintexin-B from *Gloydius intermedius* and Nigroviriditoxin B from *Bothriechis nigroviridis*. The phylogenetic tree was calculated with the UPGMA algorithm.

(Fig. 2). Moreover it has been shown that Mg^{2+} stimulates PLA_2 activity of Intercro (Vieira et al. 2013).

All CB isoforms display neurotoxic properties, CBa_2 being the least toxic (Table 5). Binding of the acidic CA subunit increases the lethal potency of the CB subunit, and this toxic effect is correlated with the stability of CACB complexes (Faure et al. 1993, 2011). CBa_2 in complex with CA forms less stable and less neurotoxic complexes, with affinity to CA sixfold lower compared to the other CB isoforms (Table 6). CBb, CBc, and CBd have comparable affinity to CA (K_D = 3.5–5 nM), and these complexes are at least four times more toxic. However, the stability of these complexes is different. The half-life of CACBd is two times greater than that observed for CACBb (Table 6) (Faure et al. 1993). Slight variations in the neurotoxic activities of these isoforms are shown in Table 5. Differences in the stability and neurotoxicity of crotoxin complexes result from the point mutations in CB isoforms (see below). F15, F16, and F17 PLA_2s in complex with CA reveal much lower toxicity (Oliveira et al. 2002; Toyama et al. 2003; Hernandez-Oliveira et al. 2005), whereas Intercro does not display neurotoxic properties (Vieira et al. 2013). Surprisingly, the toxicity of the Gintexin complex is similar to that observed for crotoxin (Yang et al. 2015a). Uncomplexed Nigroviriditoxin B revealed almost sixfold lower potency in comparison with CB isoforms. The toxicity of the complex (Nigroviriditoxin B with Nigroviriditoxin A) increases only slightly and is 20-fold lower than that determined for crotoxin (Table 5) (Lomonte et al. 2015).

Based on their enzymatic and neurotoxic properties, crotoxin isoforms can be divided into two classes (Faure and Bon 1988). Class I includes complexes formed by CBb, CBc, CBd, and CA isoforms, forming stable complexes with high toxicity and low phospholipase A_2 activity. CBa_2, which belongs to crotoxin complexes of class II, forms less toxic and less stable complexes with higher enzymatic activity.

The F15, F16, F17, and Intercro proteins show very weak or no toxicity and low phospholipase A_2 activity. However, binding of the CA subunit has little effect on their enzymatic activity. Based on these data, complexes of F15, F16, and F17 with CA can be classified as class II crotoxin-like complexes similar to the CA_2CBa_2 crotoxin complex.

Table 5 Neurotoxic properties of CB isoforms and CB-like PLA_2s from *Crotalus durissus terrificus*, *Gloydius intermedius*, and *Bothriechis nigroviridis*

	Neurotoxic activity		
	Chick biventer cervicis preparation	Mouse phrenic nerve-diaphragm preparation	LD_{50} intravenous injection [μg/kg tissue]
Crotalus durissus terrificus			
CBa_2	With CA_2: 50% blockage after 155 (±10) min (dose 35 nM)[a] With CA_3: 50% blockage after 230 (±8) min (dose 35 nM)[a]	Alone: ~30% blockage after 90 min With CA: 50% blockage after 30 min (dose 10 μg/ml)[b]	Alone: 700 (±100)[a] With CA_2: 420 (±70)[a] With CA_3: 450 (±60)[a]
CBb	With CA_2: 50% blockage after 44 (±6) min (dose 35 nM)[a] With CA_3: 50% blockage after 60 (±5) min (dose 35 nM)[a]		Alone: 500 (±100)[a] With CA_2: 110 (±20)[a] With CA_3: 95 (±20)[a]
CBc	With CA_2: 50% blockage after 87 (±11) min (dose 35 nM)[a] With CA_3: 50% blockage after 77 (±5) min (dose 35 nM)[a]		Alone: 480 (±100)[a] With CA_2: 80 (±20)[a] With CA_3: 110 (±45)[a]
CBd	With CA_2: 50% blockage after 67 (±7 min) (dose 35 nM)[a] With CA_3: 50% blockage after 69 (±3) min (dose 35 nM)[a]		Alone: 480 (±120)[a] With CA_2: 70 (±10)[a] With CA_3: 90 (±20)[a]
F17	nd	No blockage (alone or with CA) (dose 10 μg/ml)[c]	nd
F16	Alone – 50% blockage after 26 (±2) min (dose 20 μg/ml)[c] With CA: 50% blockage after 20 (±2) min (dose 20 μg/ml)[c]	Alone – no blockage[c] With CA: 50% blockage after 50.9 (±6.2) min (dose 10 μg/ml)[c]	nd
F15	Alone: 50% blockage after 28.4 (±4.3) min (dose 10 μg/ml)[d]	Alone: 23 (±3.5) % blockage after 120 min (dose 20 μg/ml)[d]	nd
Intercro	nd	No blockage after 90 min (dose 10 μg/ml)[b]	nd
Gloydius intermedius			
Gintexin-B	nd	nd	With Gintexin-A: 40–120[e]
Bothriechis nigroviridis			
Nigroviriditoxin B	nd	nd	Alone: 2900[f] With Nigroviriditoxin A: 2200[f]

nd not determined
[a]Faure et al. (1993)
[b]Vieira et al. (2013)
[c]Hernandez-Oliveira et al. (2005)
[d]Toyama et al. (2003)
[e]Yang et al. (2015a)
[f]Lomonte et al. (2015)

Complexes of CB with Different Protein Targets

The CA and CB subunits of crotoxin are present in snake venom as non-covalent, high (nanomolar) affinity complexes. However, CB isoforms obtained *in vitro* after separation of the two subunits from a mixture of crotoxin isoforms can also

Table 6 Stability of CACB complexes

	Affinity of CB to CA K_D [nM]	Half-life of dissociation of complex with CA_2 [min]
CBa_2	With CA_2: 28 (±3) With CA_3: 22 (±2)	1.4 (±0.4)
CBb	With CA_2: 5 (±2) With CA_3: n.d.	10.5 (±1)
CBc	With CA_2: 5 (±1.5) With CA_3: 3.5 (±1)	Nd
CBd	With CA_2: 5 (±2) With CA_3: 4 (±2)	22 (±3)

nd not determined
Faure et al. (1993)

interact with other protein targets such as human factor Xa (hFXa), CICS (a natural crotoxin inhibitor from snake blood), GLIC pentameric receptor, and CFTR chloride channel, affecting their pharmacological properties and biological functions. The crystal structure of crotoxin (pdb 3R0L) provides a detailed model (Fig. 4) to explore possible modes of binding of CB to different protein targets.

CB Interaction with Human Coagulation Factor Xa

It has been shown that CB can form complexes with human coagulation factor Xa (hFXa) (Faure et al. 2007). This interaction influences the coagulation pathway by inhibiting the prothrombinase complex formation and retarding the formation of thrombin. Surface Plasmon Resonance (SPR) analysis showed that binding affinity depends on the CB isoform. CBc interacts with hFXa with very high affinity ($K_D = 0.6 \pm 0.3$ nM) compared to CBa_2 ($K_D = 52 \pm 4$ nM), which is correlated with anticoagulant activity (stronger inhibition of prothrombinase complex formation) (Faure et al. 2007).

The anticoagulant binding site of CB was identified by mutagenesis, affinity-binding studies, functional assays, and molecular docking calculations (Faure et al. 2007; Faure and Saul 2011). Two adjacent regions were identified. The first region is composed of residues located in helices A and B of the CB subunit and a loop between helix C and the β-wing. The second region includes part of the Ca^{2+} binding loop and the C-terminal region of the protein. Seven regions in hFXa (five in the heavy chain and two in the light chain) are involved in binding with CB. The catalytic sites of both proteins are not involved in these interactions (Faure and Saul 2011). Moreover, analysis of crystallographic structures showed that His1, Arg34, and Gly128 may be essential in the binding of hFXa by isoform CBc. Mutations of these residues in isoform CBa_2 (Ser1, Gln34, and Glu128) lead to conformational changes in adjacent residues and result in lower affinity for hFXa (Faure et al. 2007; Faure and Saul 2011).

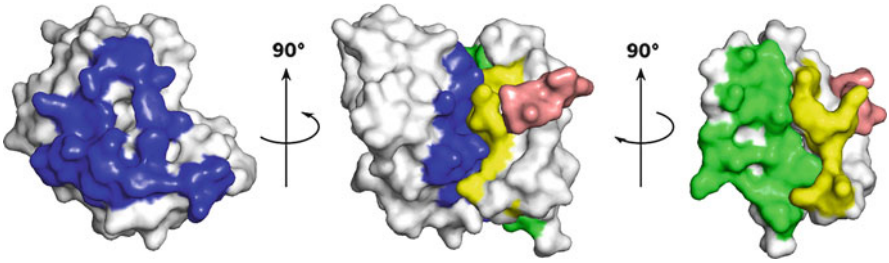

Fig. 4 Molecular binding interface of the CA and CB subunits of crotoxin. The crystal structure of crotoxin (pdb 3R0L) provides a detailed model to explore possible modes of binding of CB to other protein targets. *Left*: surface representation of the CB subunit (*in blue*, amino acid residues in contact with the CA subunit). *Center*: the assembled CACB complex. *Right*: surface representation of the CA subunit (*in yellow*, α-chain residues in contact with CB; *in green*, β-chain residues in contact with CB; *in pink*, the γ-chain of the CA subunit makes no contact with CB)

CB Interaction with CICS

Several natural PLA_2 inhibitors (PLI) have been identified in the blood of venomous snakes (Faure 2000). They can be divided into three types (PLI-α, PLI-β, and PLI-γ) according to their structure and properties. The crotoxin inhibitor from *Crotalus durissus terrificus* serum (CICS) belongs to PLI-γ type inhibitors, homologous to proteins of the Ly-6 superfamily. CICS is a 130 kDa acidic glycoprotein formed by the non-covalent association of six 23–25 kDa subunits (Perales et al. 1995; Faure et al. 2000). SPR analysis revealed a very high affinity of crotoxin and its CB subunit to CICS (nM affinity) (Faure et al. 2000). CICS neutralizes the toxic effect and inhibits the PLA_2 activity of crotoxin by interacting with the CB subunit. Binding of CICS with crotoxin leads to dissociation of the crotoxin complex and the release of CA (Perales et al. 1995).

Using peptide arrays Fortes-Dias and co-workers (Fortes-Dias et al. 2009) identified three possible interaction regions involved in CICS/CNF binding. According to their studies, the CA-CB and CICS-CB interfaces partially overlap.

CB Interaction with Prokaryotic Receptor GLIC

Crotoxin is primarily a presynaptic neurotoxin. However, some studies have shown that it also displays postsynaptic action. Recently, the proton-gated ion channel GLIC from *Gloeobacter violaceus*, a bacterial homolog of pentameric ligand-gated ion channel receptors, was identified as a new protein target of the CB subunit of crotoxin, and a novel function of CB as an inhibitor of proton-gated ion channel activity was reported (Ostrowski et al. 2016). Using SPR studies, it was shown that the interaction PLA_2-GLIC involves the extracellular domain of GLIC and that the enzymatic activity of PLA_2 is enhanced during interaction with GLIC (Ostrowski et al. 2016). Formation of the CB-GLIC complex leads to conformational changes in

the secondary structure of the proteins, and it was proposed that PLA_2 would be a new negative allosteric modulator of GLIC (Ostrowski et al. 2016).

CB Interaction with CFTR

Numerous studies have shown that neurotoxic PLA_2 can enter cells and interact with various intracellular protein targets, resulting in a wide range of pharmacological effects. PLA_2s as multifunctional proteins could potentially interfere as binders with ion channels. It has recently been shown that crotoxin and its CB subunit interact with the nucleotide-binding domain (NBD1) of CFTR (cystic fibrosis transmembrane regulator, a cyclic AMP-regulated chloride channel) and increase its chloride channel currents (Faure et al. 2016). Interestingly, the CB subunit possesses high affinity for both wild type and ΔF508-CFTR (the most frequent mutation associated with cystic fibrosis). CB behaves as a dual modulator of CFTR activity, as a potentiator and as a corrector (Faure et al. 2016).

Conclusion

Knowledge at the molecular level of the crotoxin CACB-binding interface and the 3D structures of natural isoforms of CB and CB-like proteins may help to identify other biologically relevant PPI sites. Two examples cited here (the CB-FXa and CB-CFTR/ΔF508CFTR complexes) open interesting perspectives for the future development of noncompetitive inhibitors of human FXa as anticoagulant agents and new correctors and potentiators for the treatment of cystic fibrosis.

References

Beghini DG, Toyama MH, Hyslop S, Sodek LC. Novello, Marangoni S. Enzymatic characterization of a novel phospholipase A2 from Crotalus durissus cascavella rattlesnake (Maracambóia) venom. J Protein Chem. 2000;19(8):679–84.

Beghini DG, Rodrigues-Simioni L, Toyama MH, Novello JC, da Cruz-Höfling MA, Marangoni S. Neurotoxic and myotoxic actions of crotoxin-like and Crotalus durissus cascavella whole venom in the chick biventer cervicis preparation. Toxicon. 2004;43(3):255–61.

Bieber AL, Tu T, Tu AT. Studies of an acidic cardiotoxin isolated from the venom of Mojave rattlesnake (Crotalus scutulatus). Biochim Biophys Acta. 1975;400(1):178–88.

Bon C, Changeux JP, Jeng TW, Fraenkel-Conrat H. Postsynaptic effects of crotoxin and of its isolated subunits. Eur J Biochem. 1979;99(3):471–81.

Bouchier C, Boulain JC, Bon C, Ménez A. Analysis of cDNAs encoding the two subunits of crotoxin, a phospholipase A2 neurotoxin from rattlesnake venom: the acidic non enzymatic subunit derives from a phospholipase A2-like precursor. Biochim Biophys Acta. 1991; 1088(3):401–8.

Brazil V, Excell BJ. Action of crotoxin and crotactin from the venom of Crotalus durissus terrificus (South American rattlesnake) on the frog neuromuscular junction. J Physiol. 1971; 212(2):34P–5.

Brazil VO, Fontana MD, Heluany NF. Nature of the postsynaptic action of crotoxin at guinea-pig diaphragm end-plates. J Nat Toxins. 2000;9(1):33–42.

Calvete JJ, Sanz L, Cid P, de la Torre P, Flores-Díaz M, Dos Santos MC, Borges A, Bremo A, Angulo Y, Lomonte B, Alape-Girón A, Gutiérrez JM. Snake venomics of the Central American rattlesnake Crotalus simus and the South American Crotalus durissus complex points to neurotoxicity as an adaptive paedomorphic trend along Crotalus dispersal in South America. J Proteome Res. 2010;9(1):528–44.

Cardoso DF, Lopes-Ferreira M, Faquim-Mauro EL, Macedo MS, Farsky SH. Role of crotoxin, a phospholipase A2 isolated from Crotalus durissus terrificus snake venom, on inflammatory and immune reactions. Mediators Inflamm. 2001;10(3):125–33.

Castro EN, Lomonte B, del Carmen Gutiérrez M, Alagón A, Gutiérrez JM. Intraspecies variation in the venom of the rattlesnake Crotalus simus from Mexico: different expression of crotoxin results in highly variable toxicity in the venoms of three subspecies. J Proteomics. 2013;87:103–21.

Cavalcante WL, Ponce-Soto LA, Marangoni S, Gallacci M. Neuromuscular effects of venoms and crotoxin-like proteins from Crotalus durissus ruruima and Crotalus durissus cumanensis. Toxicon. 2015;96:46–9.

Costa LA, Miles H, Araujo CE, Gonzales S, Villarrubia VG. Tumor regression of advanced carcinomas following intra- and/or peritumoral inoculation with VRCTC-310 in humans: preliminary report of two cases. Immunopharmacol Immunotoxicol. 1998;20:15–25.

Cura JE, Blanzaco DP, Brisson C, Cura MA, Carbol R, Larrateguy L, et al. Phase I and pharmacokinetics study crotoxin (cytotoxic PLA2 NSC-624 244) in patients with advanced cancer. Clin Cancer Res. 2002;8:1033–41.

Dos-Santos MC, Assis EB, Moreira TD, Pinheiro J, Fortes-Dias CL. Individual venom variability in Crotalus durissus ruruima snakes, a subspecies of Crotalus durissus from the Amazonian region. Toxicon. 2005;46(8):958–61.

Durban J, Pérez A, Sanz L, Gómez A, Bonilla F, Rodríguez S, Chacón D, Sasa M, Angulo Y, Gutiérrez JM, Calvete JJ. Integrated "omics" profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, Crotalus simus simus. BMC Genomics. 2013;14:234.

Faure G. Natural inhibitors of toxic phospholipases A2. Biochimie. 2000;82:833–40.

Faure G, Bon C. Several isoforms of crotoxin are present in individual venoms from the South American rattlesnake Crotalus durissus terrificus. Toxicon. 1987;25(2):229–34.

Faure G, Bon C. Crotoxin, a phospholipase A2 neurotoxin from the South American rattlesnake Crotalus durissus terrificus: purification of several isoforms and comparison of their molecular structure and of their biological activities. Biochemistry. 1988;27(2):730–8.

Faure G, Saul F. Structural and functional characterization of anticoagulant, FXa-binding *Viperidae* snake venom phospholipases A2. Acta Chim Slov. 2011;58:671–7.

Faure G, Guillaume JL, Camoin L, Saliou B, Bon C. Multiplicity of acidic subunit isoforms of crotoxin, the phospholipase A2 neurotoxin from Crotalus durissus terrificus venom, results from posttranslational modifications. Biochemistry. 1991;30(32):8074–83.

Faure G, Harvey AL, Thomson E, Saliou B, Radvanyi F, Bon C. Comparison of crotoxin isoforms reveals that stability of the complex plays a major role in its pharmacological action. Eur J Biochem. 1993;214(2):491–6.

Faure G, Choumet V, Bouchier C, Camoin L, Guillaume JL, Monegier B, Vuilhorgne M, Bon C. The origin of the diversity of crotoxin isoforms in the venom of Crotalus durissus terrificus. Eur J Biochem. 1994;223(1):161–4.

Faure G, Villela C, Perales J, Bon C. Interaction of the neurotoxic and nontoxic secretory phospholipases A2 with the crotoxin inhibitor from Crotalus serum. Eur J Biochem. 2000; 267(15):4799–808.

Faure G, Gowda VT, Maroun RC. Characterization of a human coagulation factor Xa-binding site on Viperidae snake venom phospholipases A2 by affinity binding studies and molecular bioinformatics. BMC Struct Biol. 2007;7:82.

Faure G, Xu H, Saul FA. Crystal structure of crotoxin reveals key residues involved in the stability and toxicity of this potent heterodimeric β-neurotoxin. J Mol Biol. 2011;412(2):176–91.

Faure G, Bakouh N, Lourdel S, Odolczyk N, Premchandar A, Servel N, Hatton A, Ostrowski M, Xu H, Saul F, Moquereau C, Bitam S, Pranke I, Planelles G, Teulon J, Herrmann H, Zielenkiewicz P, Dadlez M, Lukacs GL, Sermet-Gaudelus I, Ollero M, Corringer P-J, Edelman A. Rattlesnake phospholipase A2 as a novel dual-acting modulator of ΔF508 cystic fibrosis transmembrane regulator dysfunction. J Mol Biol. 2016;428:2898–2915.

Fernández J, Lomonte B, Sanz L, Angulo Y, Gutiérrez JM, Juan J. Calvete. snake venomics of bothriechis nigroviridis reveals extreme variability among Palm pitviper venoms: different evolutionary solutions for the same trophic purpose. J Proteome Res. 2010;9:4234–41.

Fonseca FV, Antunes E, Morganti RP, Monteiro HS, Martins AM, Toyama DO, Marangoni S, Toyama MH. Characterization of a new platelet aggregating factor from crotoxin Crotalus durissus cascavella venom. Protein J. 2006;25(3):183–92.

Fortes-Dias CL, Santos RM, Magro AJ, Fontes MR, Chávez-Olórtegui C, Granier C. Identification of continuous interaction sites in PLA(2)-based protein complexes by peptide arrays. Biochimie. 2009;91(11–12):1482–92.

Gopalakrishnakone P, Hawgood BJ, Holbrooke SE, Marsh NA. Santana De Sa S, Tu AT. Sites of action of Mojave toxin isolated from the venom of the Mojave rattlesnake. Br J Pharmacol. 1980;69(3):421–31.

Gutiérrez JM, Ponce-Soto LA, Marangoni S, Lomonte B. Systemic and local myotoxicity induced by snake venom group II phospholipases A2: comparison between crotoxin, crotoxin B and a Lys49 PLA2 homologue. Toxicon. 2008;51(1):80–92.

Hawgood BJ, Smith JW. The mode of action at the mouse neuromuscular junction of the phospholipase A-crotapotin complex isolated from venom of the South American rattlesnake. Br J Pharmacol. 1977;61(4):597–606.

Hendon RA, Fraenkel-Conrat H. Biological roles of the two components of crotoxin. Proc Natl Acad Sci U S A. 1971;68(7):1560–3.

Hernandez-Oliveira S, Toyama MH, Toyama DO, Marangoni S, Hyslop S, Rodrigues-Simioni L. Biochemical, pharmacological and structural characterization of a new PLA2 from Crotalus durissus terrificus (South American rattlesnake) venom. Protein J. 2005;24(4):233–42.

Lomeo Rda S, Gonçalves AP, da Silva CN, de Paula AT, Costa Santos DO, Fortes-Dias CL, Gomes DA, de Lima ME. Crotoxin from Crotalus durissus terrificus snake venom induces the release of glutamate from cerebrocortical synaptosomes via N and P/Q calcium channels. Toxicon. 2014;85:5–16.

Lomonte B, Mora-Obando D, Fernández J, Sanz L, Pla D, Gutiérrez JM, Calvete JJ. First crotoxin-like phospholipase A(2) complex from a New World non-rattlesnake species: nigroviriditoxin, from the arboreal Neotropical snake Bothriechis nigroviridis. Toxicon. 2015;93:144–54.

Marchi-Salvador DP, Corrêa LC, Magro AJ, Oliveira CZ, Soares AM, Fontes MR. Insights into the role of oligomeric state on the biological activities of crotoxin: crystal structure of a tetrameric phospholipase A2 formed by two isoforms of crotoxin B from Crotalus durissus terrificus venom. Proteins. 2008;72(3):883–91.

Mayer RJ, Marshall LA. New insights on mammalian phospholipase A2(s); comparison of arachidonyl selective and -nonselective enzymes. FASEB J. 1993;7:339–48.

Muller VD, Russo RR, Cintra AC, Sartim MA, Alves-Paiva Rde M, Figueiredo LT, Sampaio SV, Aquino VH. Crotoxin and phospholipases A_2 from Crotalus durissus terrificus showed antiviral activity against dengue and yellow fever viruses. Toxicon. 2012;59(4):507–15.

Muller VD, Soares RO, dos Santos Jr NN, Trabuco AC, Cintra AC, Figueiredo LT, Caliri A, Sampaio SV, Aquino VH. Phospholipase A2 isolated from the venom of Crotalus durissus terrificus inactivates dengue virus and other enveloped viruses by disrupting the viral envelope. PLoS One. 2014;9(11):e112351.

Murakami M, Kudo I. Phospholipase A2. J Biochem. 2002;131(3):285–92.

Nogueira-Neto Fde S, Amorim RL, Brigatte P, Picolo G, Ferreira Jr WA, Gutierrez VP, Conceição IM, Della-Casa MS, Takahira RK, Nicoletti JL, Cury Y. The analgesic effect of crotoxin on

neuropathic pain is mediated by central muscarinic receptors and 5-lipoxygenase-derived mediators. Pharmacol Biochem Behav. 2008;91(2):252–60.

Nunes FP, Zychar BC, Della-Casa MS, Sampaio SC, Gonçalves LR, Cirillo MC. Crotoxin is responsible for the long-lasting anti-inflammatory effect of Crotalus durissus terrificus snake venom: involvement of formyl peptide receptors. Toxicon. 2010;55(6):1100–6.

Oliveira DG, Toyama MH, Novello JC, Beriam LO, Marangoni S. Structural and functional characterization of basic PLA2 isolated from Crotalus durissus terrificus venom. J Protein Chem. 2002;21(3):161–8.

Ostrowski M, Porowinska D, Prochnicki T, Prevost M, Raynal B, Baron B, Sauguet L, Corringer PJ, Faure G. Neurotoxic phospholipase A2 from rattlesnake as a new ligand and new regulator of prokaryotic receptor GLIC (proton-gated ion channel from G. violaceus). Toxicon. 2016;116:63–71.

Perales J, Villela C, Domont GB, Choumet V, Saliou B, Moussatché H, Bon C, Faure G. Molecular structure and mechanism of action of the crotoxin inhibitor from Crotalus durissus terrificus serum. Eur J Biochem. 1995;227(1–2):19–26.

Pereañez JA, Núñez V, Huancahuire-Vega S, Marangoni S, Ponce-Soto LA. Biochemical and biological characterization of a PLA2 from crotoxin complex of Crotalus durissus cumanensis. Toxicon. 2009;53(5):534–42.

Perumal Samy R, Pachiappan A, Gopalakrishnakone P, Thwin MM, Hian YE, Chow VT, Bow H, Weng JT. In vitro antimicrobial activity of natural toxins and animal venoms tested against Burkholderia pseudomallei. BMC Infect Dis. 2006;20(6):100.

Ponce-Soto LA, Toyama MH, Hyslop S, Novello JC, Marangoni S. Isolation and preliminary enzymatic characterization of a novel PLA2 from Crotalus durissus collilineatus venom. J Protein Chem. 2002;21(3):131–6.

Ponce-Soto LA, Lomonte B, Rodrigues-Simioni L, Novello JC, Marangoni S. Biological and structural characterization of crotoxin and new isoform of crotoxin B PLA(2) (F6a) from Crotalus durissus collilineatus snake venom. Protein J. 2007;26(4):221–30.

Rangel-Santos A, Dos-Santos EC, Lopes-Ferreira M, Lima C, Cardoso DF, Mota I. A comparative study of biological activities of crotoxin and CB fraction of venoms from Crotalus durissus terrificus, Crotalus durissus cascavella and Crotalus durissus collilineatus. Toxicon. 2004;43 (7):801–10.

Renetseder R, Brunie S, Dijkstra BW, Drenth J, Sigler PB. A comparison of the crystal structures of phospholipase A2 from bovine pancreas and Crotalus atrox venom. J Biol Chem. 1985;260:11627–34.

Rudd CJ, Viskatis LJ, Vidal JC, Etcheverry MA. In vitro comparison of cytotoxic effects of crotoxin against three human tumors and a normal human epidermal keratinocyte cell line. Invest New Drugs. 1994;12(3):183–4.

Salvador GH, Fernandes CA, Corrêa LC, Santos-Filho NA, Soares AM, Fontes MR. Crystallization and preliminary X-ray diffraction analysis of crotoxin B from Crotalus durissus collilineatus venom. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2009;65(10):1011–3.

Sampaio SC, Rangel-Santos AC, Peres CM, Curi R, Cury Y. Inhibitory effect of phospholipases A2 isolated from Crotalus durissus terrificus venom on macrophage function. Toxicon. 2005;45:671–6.

Slotta KH, Fraenkel-Conrat HL. Schlangengifte. III. Mitteilung: Reinigung und Kristallisation des Klapperschlangen-Giftes. Ber Dtsch Chem Ges. 1938;71:1076–81.

Toyama MH, de Oliveira DG, Beriam LO, Novello JC, Rodrigues-Simioni L, Marangoni S. Structural, enzymatic and biological properties of new PLA(2) isoform from Crotalus durissus terrificus venom. Toxicon. 2003;41(8):1033–8.

Toyama MH, Toyama DO, Joazeiro PP, Carneiro EM, Beriam LO, Marangoni LS, Boschero AC. Biological and structural characterization of a new PLA2 from the Crotalus durissus collilineatus venom. Protein J. 2005;24(2):103–12.

Viala VL, Hildebrand D, Fucase TM, Sciani JM, Prezotto-Neto JP, Riedner M, Sanches L, Nishimura PJ, Oguiura N, Pimenta DC, Schlüter H, Betzel C, Arni RK, Spencer

PJ. Proteomic analysis of the rare Uracoan rattlesnake Crotalus vegrandis venom: evidence of a broad arsenal of toxins. Toxicon. 2015;107:234–51.

Vieira LF, Magro AJ, Fernandes CAH, de Souza BM, Cavalcante WLG, Palma MA, Rosa CS, Fuly AL, Fontes MRM, Gallacci M, Butzke DS, Calderon LA, Stábeli RG, Giglio JR, Soares AM. Biochemical, functional, structural and phylogenetic studies on Intercro, a new isoform phospholipase A2 from Crotalus durissus terrificus snake venom. Biochimie. 2013;95:2365–75.

Yan CH, Yang YP, Qin ZH, Gu ZL, Reid P, Liang ZQ. Autophagy is involved in cytotoxic effects of crotoxin in human breast cancer cell line MCF-7 cells. Acta Pharmacol Sin. 2007;28(4):540–8.

Yang ZM, Guo Q, Ma ZR, Chen Y, Wang ZZ, Wang XM, Wang YM, Tsai IH. Structures and functions of crotoxin-like heterodimers and acidic phospholipases A2 from Gloydius intermedius venom: insights into the origin of neurotoxic-type rattlesnakes. J Proteomics. 2015a;112:210–23.

Yang ZM, Yang YE, Chen Y, Cao J, Zhang C, Liu LL, Wang ZZ, Wang XM, Wang YM, Tsai IH. Transcriptome and proteome of the highly neurotoxic venom of Gloydius intermedius. Toxicon. 2015b;107:175–86.

Zambelli VO, Sampaio SC, Sudo-Hayashi LS, Greco K, Britto LR, Alves AS, Zychar BC, Gonçalves LR, Spadacci-Morena DD, Otton R, Della-Casa MS, Curi R, Cury Y. Crotoxin alters lymphocyte distribution in rats: involvement of adhesion molecules and lipoxygenase-derived mediators. Toxicon. 2008;51(8):1357–67.

Zhang HL, Han R, Chen ZX, Chen BW, Gu ZL, Reid PF, Raymond LN, Qin ZH. Opiate and acetylcholine-independent analgesic actions of crotoxin isolated from crotalus durissus terrificus venom. Toxicon. 2006;48(2):175–82.

Zhang P, Lader AS, Etcheverry MA, Cantiello HF. Crotoxin potentiates L-type calcium currents and modulates the action potential of neonatal rat cardiomyocytes. Toxicon. 2010;55(7):1236–43.