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Interaction of the neurotoxic and nontoxic secretory phospholipases A_2 with the crotoxin inhibitor from *Crotalus* serum

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Crotalus durissus terrificus snakes possess a protein in their blood, named crotoxin inhibitor from *Crotalus* serum (CICS), which protects them against crotoxin, the main toxin of their venom. CICS neutralizes the lethal potency of crotoxin and inhibits its phospholipase A_2 (PLA_2) activity. The aim of the present study is to investigate the specificity of CICS towards snake venom neurotoxic PLA_2s (β-neurotoxins) and nontoxic mammalian PLA_2s. This investigation shows that CICS does not affect the enzymatic activity of pancreatic and nonpancreatic PLA_2s, bee venom PLA_2 and *Elapidae* β-neurotoxins but strongly inhibits the PLA_2 activity of *Viperidae* β-neurotoxins. Surface plasmon resonance and PAGE studies further demonstrated that CICS makes complexes with monomeric and multimeric *Viperidae* β-neurotoxins but does not interact with nontoxic PLA_2s. In the case of dimeric β-neurotoxins from *Viperidae* venoms (crotoxin, Mojave toxin and CbICbII), which are made by the noncovalent association of a PLA_2 with a nonenzymatic subunit, CICS does not react with the noncatalytic subunit, instead it binds tightly to the PLA_2 subunit and induces the dissociation of the heterocomplex. *In vitro* assays performed with *Torpedo* synaptosomes showed a protective action of CICS against *Viperidae* β-neurotoxins but not against other PLA_2 neurotoxins, on primary and evoked liberation of acetylcholine. In conclusion, CICS is a specific PLA_2 inhibitor of the β-neurotoxins from the *Viperidae* family.

Keywords: snake venom; serum antitoxic protein; neurotoxin neutralization; phospholipase A_2 inhibitor; surface plasmon resonance.

Snake venom phospholipases A_2 (EC 3.1.1.4) (PLA_2) exhibit a wide variety of pharmacological and physiopathological effects [1]. In addition to their role in the digestion of the prey, they can be neurotoxic, myotoxic, able to interfere with coagulation processes, etc. Mammalian and venom PLA_2s have been classified into 10 different structural groups [2]. However, snake venom PLA_2s belong only to groups I and II. Group I includes mammalian pancreatic and snake venom PLA_2s from *Elapidae* and *Hydrophiidae*, while group II includes mammalian nonpancreatic and snake venom enzymes from *Crotalidae* and *Viperidae*.

On the other hand, several snake venom PLA_2s evolved to become potent neurotoxins, developing selectivity for neuronal structures and recognizing specific protein acceptors. Neurotoxic PLA_2s from snake venom, also called β-neurotoxins, cause death by respiratory failure. They act on the neuromuscular junction, primarily at a presynaptic level, inhibiting the release of neurotransmitter acetylcholine (ACh) [3–5]. The β-neurotoxins differ in their quaternary structures and three classes have been distinguished on this basis [4]. As shown in Table 1, the first class of β-neurotoxins comprises single chain polypeptides of 13–15 kDa. It includes monomeric *Elapidae* β-neurotoxins, belonging to PLA_2s group I, such as notexin (Ntx) from *Notechis scutatus scutatus* venom and monomeric *Viperidae* β-neurotoxins, belonging to PLA_2s group II, such as agkistrodotoxin (Agtx) from *Agkistrodon blomhoffii brevicaudus* and ammodytoxin (Atx) from *Vipera ammodytes ammodytes*. The second class of β-neurotoxins (Table 1), includes multichain neurotoxic PLA_2s, that are made of several noncovalently linked polypeptide subunits, at least one of them being enzymatically active. This class includes multimeric *Viperidae* β-neurotoxins such as crotoxin (CACB) from the venom of the South-American rattlesnake *Crotalus durissus terrificus*, Mojave toxin from *Crotalus scutelatus scutelatus* venom and CbICbII from *Pseudocerastes fieldi* venom, which belong to PLA_2s group II, and multimeric *Elapidae* β-neurotoxins such as taipoxin (Tpx) from *Oxyuranus scutellatus scutellatus* venom which belong to PLA_2s group I. Crotoxin is a heterodimer made of a basic and weakly toxic PLA_2 (CB) and of an acidic nontoxic and nonenzymatic subunit CA [6–11] while Tpx is composed of three PLA_2 subunits [12]. The main toxic component of *Pseudocerastes fieldi* venom is a neurotoxic heterodimer composed of a basic subunit CbII and an acidic subunit CbI [13]. The third class of β-neurotoxins includes β-bungarotoxin (β-Btx) from *Bungarus multicinctus* venom which covalently associates a PLA_2 subunit (group I of PLA_2s) with a nontoxic polypeptide, homologous to Kunitz type serine proteinase inhibitor or dendrotoxins [14] (Table 1). Although β-neurotoxins belonging to different

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Abbreviations: Agtx, agkistrodotoxin; Atx, ammodytoxin; β-Btx, β-bungarotoxin; CACB, crotoxin, made of CA and CB subunits; CbICbII, β-neurotoxin from *Pseudocerastes fieldi* venom, made of CbI and CbII subunits; CICS, crotoxin inhibitor from *Crotalus* serum; EDC, *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide; $HsPLA_2$, human group II secretory phospholipase A_2; NHS, *N*-hydroxysuccinimide; Ntx, notexin; PLA_2, phospholipase A_2; PVDF, poly(vinylidene difluoride); 1Pam(PyrDec)Gro*P*Gro, 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol; SPR, surface plasmon resonance; Tpx, taipoxin.
Enzyme: phospholipase A_2 (EC 3.1.1.4).

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Table 1. Classification of β-neurotoxins and nontoxic PLA_2s used in this investigation.

PLA_2	Group I	Group II	Group III
Class I (Monomeric β-neurotoxins)	*Elapidae* Notexin	*Viperidae* Agkistrodotoxin, Ammodytoxin	
PLA_2 subunits (from class II β-neurotoxins)		*Viperidae* CB, CbII, CB Mojave	
Class II (Multimeric β-neurotoxins)	*Elapidae* Taipoxin	*Viperidae* Crotoxin, CbICbII, Mojave toxin	
Class III (β-neurotoxins)	*Elapidae* β-bungarotoxin		
Non-toxic PLA_2	Mammalian pancreatic PLA_2	Mammalian non-pancreatic PLA_2	PLA_2 from bee venom

structural classes have similar electrophysiological effects on the neuromuscular junction [4], their targets are different. High affinity binding proteins were identified in various neuronal tissues and several distinct types of PLA_2 acceptors have been proposed for neurotoxic PLA_2s [14–22].

Venomous snakes are resistant to their own venom and several natural neutralizing proteins have been identified in their plasma [23]. In particular, PLA_2 inhibitors have been isolated from various snake sera and their primary structures have been determined [23–34]. Ohkura and coworkers [27] have classified the PLA_2 inhibitors into three distinct types (α, β and γ) based on their structural characteristics.

α-type PLA_2 inhibitors include proteins such as PLI-A and PLI-B isolated from the plasma of *Trimeresurus flavoviridis* [24,25], PLIα from *Agkistrodon blomhoffii siniticus* [26,27] and BaMIP from *Bothrops asper* [28]. They are characterized by the presence of a carbohydrate recognition domain (CRD) in their structure. They are 75–120 kDa glycoproteins non-covalently associating three or five 20–25 kDa subunits [24–27]. Interestingly, these α-type inhibitors have similarities with the mammalian proteins containing C-type CRDs such as: mannose-binding protein, pulmonary surfactant protein SP-A, M-type receptor for secretory PLA_2s [25,32] or DEC-205 receptor expressed by dendritic cells [35].

β-type PLA_2 inhibitors include the protein isolated from *Agkistrodon blomhoffii siniticus* plasma (PLIß) [27,29]. This inhibitor is a 160-kDa glycoprotein made of three 50 kDa subunits homologous to the human leucin-rich domain of α_2-glycoprotein [27,29].

γ-type PLA_2 inhibitors comprise proteins isolated from *Crotalus durissus terrificus* [30,31], *Naja naja kaouthia* [32–33], PLIγ from *Agkistrodon blomhoffii siniticus* [27] and PLI-I from *Trimeresurus flavoviridis* [34]. They are 90–130 kDa acidic glycoproteins made of the noncovalent association of three to six 24–31 kDa subunits [30–33] structurally homologous to urokinase-type plasminogen activator receptor (u-PAR) and to cell surface antigens belonging to the Ly-6 superfamily, CD59 [33] and bone specific protein called RoBo-1 [36]. Interestingly, these proteins have evolutionary relationships with 'three finger' neurotoxins and cytotoxins from *Elapidae* snake venom [37] and xenoxins, from frog skin [38].

In the case of *Crotalus durissus terrificus* snake, we previously demonstrated that its serum contains a PLA_2 inhibitor of γ-type, called 'crotoxin inhibitor from *Crotalus* serum' (CICS), that neutralizes the toxicity and inhibits the enzymatic activity of its physiological ligand crotoxin [31]. In the present work we investigate the specificity of CICS, examining its interaction with several β-neurotoxins and nontoxic secretory PLA_2s from mammals and snake venom. This investigation indicates that CICS is a specific inhibitor of the monomeric and multimeric β-neurotoxins from *Viperidae* family.

MATERIALS AND METHODS

Torpedo marmorata fishes were purchased from the Station de Biologie Marine (Roscoff, France) and kept alive in artificial oxygenated seawater. The fluorescent PLA_2 substrate analog: 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol (Pam(PyrDec)Gro*P*Gro) was obtained from Interchim (Montluçon, France). Fatty acid-free bovine serum albumin, acetylcholinesterase, Luminol and ethylene-bis(oxyethylene-nitrilo)tetra(acetic acid), (EGTA) were supplied by Sigma Co (St. Louis, MO, USA). Choline oxidase from *Arthrobacter globiformis* was purchased from Waco (Osaka, Japan). Reagents including sensor chips CM5, surfactant P20, the amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide (EDC) and ethanolamine hydrochloride were supplied by Biacore (Biacore AB, Uppsala, Sweden). All other chemicals and solvents of the highest available purity were obtained either, from Merck A.G. (Darmstad, Germany), Prolabo (Paris, France) or from Sigma Co (St. Louis, MO, USA).

CbICbII from *Pseudocerastes fieldi* venom was a gift from A. Bdolah (Department of Zoology, Tel Aviv University, Israel). Tpx from *Oxyuranus scutelatus scutelatus* venom, β-bungarotoxin and porcine pancreatic PLA_2 were purchased from Sigma. PLA_2 from *Apis mellifera* venom, Ntx from *Notechis scutatus* snake venom and Atx from *Vipera ammodytes ammodytes* were purchased from Latoxan (Valence, France). Recombinant human group II secretory phospholipase A_2 ($hsPLA_2$) was kindly furnished by C. Mounier [39] (Unité des Venins, Institut Pasteur, Paris). L. Smith (Dept. of Immunology and Mol. Biology, Fort Detrick, Frederick, USA) kindly supplied rabbit antiserum produced against Tpx and Ntx. Monoclonal antibody B-65.5 was kindly furnished by V. Choumet [40] (Unité des Venins, Institut Pasteur, Paris).

Purification of CICS and preparation of toxins

CICS was purified from *Crotalus durissus terrificus* serum and its concentrations were determined spectrophotometrically, as previously described by Perales *et al.* [31]. Crotoxin, its isolated subunits CA and CB and its isoforms were isolated from *Crotalus durissus terrificus* venom as previously reported

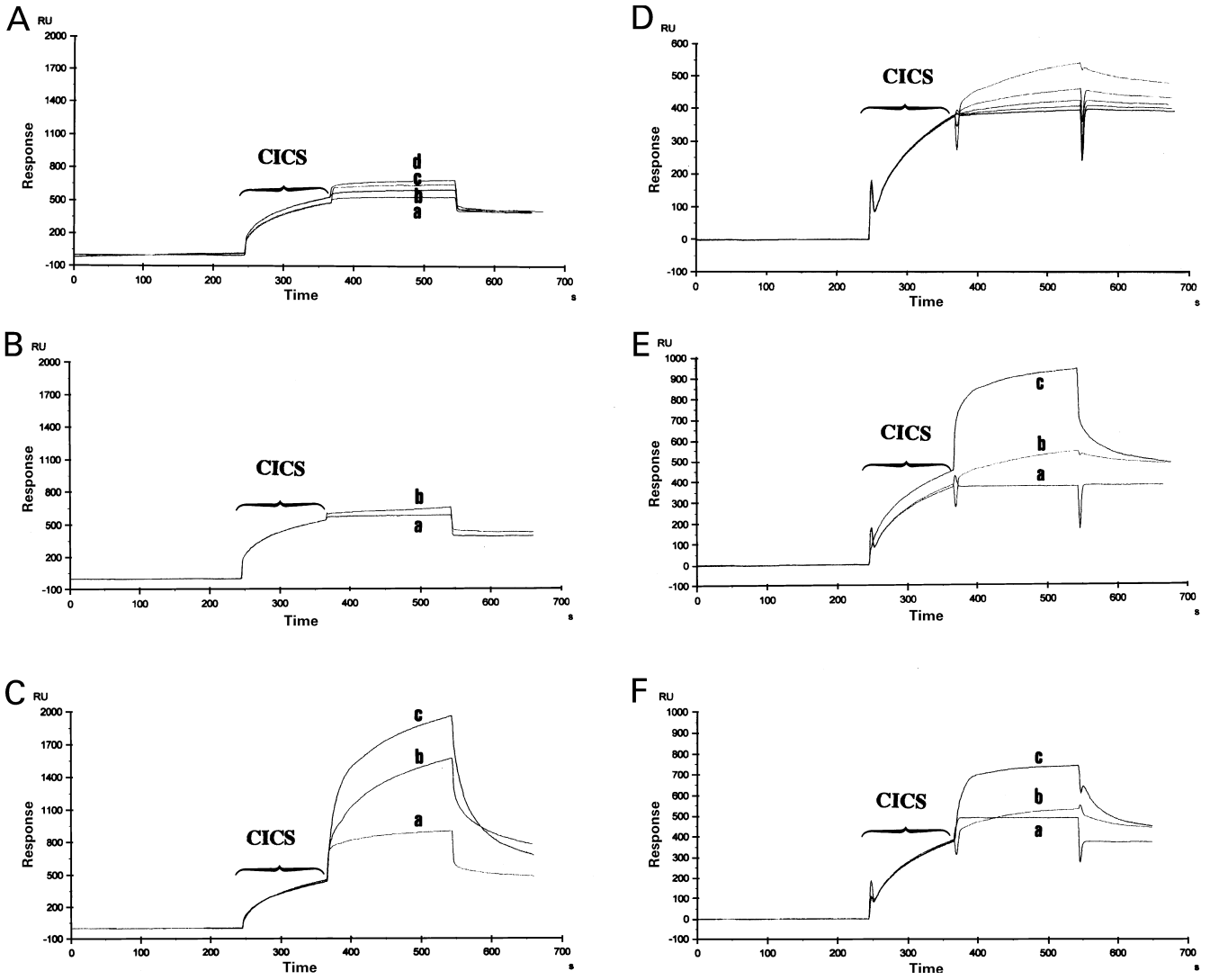

Fig. 1. Analysis by SPR of the interaction of CICS with β-neurotoxins and nontoxic. CICS (30 $\mu g \cdot mL^{-1}$) was injected for 3 min at a flow rate of 10 $\mu L\ min^{-1}$ on a CM5 sensor chip in which rabbit anti-CICS IgG were covalently immobilized then β-neurotoxin or nontoxic PLA_2 were injected for a further 3 min. The experiment was carried out at 25 °C and at the end of each run, a 30 s injection of 0.05 M glycine/HCl, pH 2.0 was performed to restore the complete binding capacity of the sensor chip. (A) β-Btx (a), porcine pancreatic PLA_2 (group I) (b), hs-PLA_2 (group II) (c), bee venom PLA_2 (group III) (d), at concentration 100 $\mu g \cdot mL^{-1}$. (B) Tpx (a) and Ntx (b) (100 $\mu g \cdot mL^{-1}$). (C) Atx (80 $\mu g \cdot mL^{-1}$) (a), Agtx (40 $\mu g \cdot mL^{-1}$) (b) or CB subunit of Mojave toxin (20 $\mu g \cdot mL$) (c). (D) Crotoxin (isoform CA_2CB_d) at concentration: 5, 10, 20, 40 and 80 $\mu g \cdot mL^{-1}$. (E) Acidic subunit CA of crotoxin (isoform CA_2) (25 $\mu g \cdot mL^{-1}$) (a), whole complex crotoxin (isoform CA_2CB_d) (40 $\mu g \cdot mL^{-1}$) (b) and basic subunit CB of crotoxin (isoform CB_d) (5 $\mu g \cdot mL^{-1}$) (c). (F) Acidic subunit CbI (25 $\mu g \cdot mL^{-1}$) (a), whole β-neurotoxin from *Pseudocerastes fieldi*, CbICbII (40 $\mu g \cdot mL^{-1}$) (b) and basic subunit CbII (5 $\mu g \cdot mL^{-1}$) (c).

[41,42]. The protein concentration of crotoxin and of its isolated subunits was determined spectrophotometrically as previously reported [41]. Agtx was purified from the venom of *Agkistrodon blomhoffii brevicaudus* as described by Chen *et al*. [43].

Preparation of anti-CICS antiserum and specific anti-CICS Ig

New Zealand white rabbits (1.5 kg body weight) were injected intradermally with 100 μg of CICS in 100 μL of 150 mM sodium chloride emulsified with 100 μL of complete Freund's adjuvant. Three subcutaneous booster injections of the same materials, in incomplete Freund's adjuvant, were given at two-week intervals. The antibody level was monitored weekly by ELISA. Antisera were collected 1–2 months after the end of immunization protocol. Specific anti-CICS immunoglobulins were purified by immunoaffinity on an AH-Sepharose 4B column coupled with CICS [44].

Analysis of PLA_2-CICS complexes by PAGE

The formation of the complexes was analyzed at pH 6.5 in the absence of detergent and reducing agents by PAGE in 20% acrylamide gels, using a PhastSystem (Pharmacia). CICS was incubated for 1 h at 37 °C with PLA_2 or toxins to be tested then aliquots were applied to the gel. Proteins were stained with Coomassie Brilliant blue R-250 or by immunoblotting as previously described [31].

Analysis of PLA_2-CICS interaction by surface plasmon resonance (SPR)

Studies were performed using a BIACORE® 2000 system (Biacore AB). The running and dilution buffer in all experiments was Hepes buffer (HBS; 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4).

Immobilization of anti-CICS IgG on the sensor chip surface. Immunopurified rabbit anti-CICS IgG was covalently coupled via primary amino groups on a CM5 sensor chip surface according to Jonsson *et al*. [45]. Briefly, the carboxymethylated dextran matrix was activated with 30 μL of an EDC/NHS (1/1) mixture, 10 μL of rabbit anti-CICS IgG (20 $\mu g \cdot mL^{-1}$) in 10 mM sodium acetate (pH 4.8) were injected and unreacted groups were blocked with 30 μL ethanolamine (pH 8.5). The immobilization run was performed at a flow of 5 $\mu L \cdot min^{-1}$ at 25 °C. One independent flow cell of the same sensor chip was used as a control flow cell by subjecting it to a 'blank immobilization', i.e. with no rabbit anti-CICS IgG added. The SPR signal for immobilized rabbit anti-CICS IgG was found to be 4047 $pg \cdot mm^{-2}$.

CICS-PLA2 interaction. First CICS samples (30 $\mu g \cdot mL^{-1}$), and then β-neurotoxins or nontoxic PLA_2s (0–100 $\mu g \cdot mL^{-1}$) were successively injected into the control and assay cells and their binding was monitored. Data were analyzed using the BIA EVALUATION 2.1 software (Biacore).

Effect of CICS on the enzymatic activity of PLA_2s

The PLA_2 activity of different neurotoxic and nontoxic secretory PLA_2s was measured by a fluorimetric method [46] using a fluorescent labeled phospholipid analog [1Pam(Pyr-Dec)Gro*P*Gro] as substrate. To analyze the effect of CICS on PLA_2 activity, a fixed concentration (1 μg in 20 μL NaCl/P_i) of tested PLA_2 was incubated with various concentrations of CICS at the indicated molar ratios. Incubation was carried out for 1 h at 37 °C in NaCl/P_i. After 1/20 dilution, 1–10 μL, were introduced into the cuvette containing 1 mL of the fluorescent phospholipid PG (2 μM), for the measurement of PLA_2 activity.

Effect of CICS on primary and evoked Ach release induced by tested β-neurotoxins

Purely cholinergic nerve terminals (synaptosomes) were isolated from the electronic organ of *Torpedo marmorata* as described by Israel *et al*. [47]. A fixed concentration (1 μg in 20 μL NaCl/P_i) of tested β-neurotoxin was preincubated for 1 h at 37 °C with various concentrations of CICS, then aliquot (100 nM of each tested toxin) was applied to the synaptosomes for 3 min at room temperature according to the procedure previously described [48]. Primary release was triggered by the addition of Ca^{2+} (final concentration, 5 mM) and evoked ACh release was triggered by adding a high concentration of K^+ ions (final concentration, 90 mM) [48] and ACh was continuously monitored by the chemiluminescent technique [49].

RESULTS

Interaction between CICS and neurotoxic and nontoxic PLA_2

The interaction of CICS with neurotoxic and nontoxic PLA_2 was first studied in real-time by SPR. Due to the fact that CICS is an acidic, oligomeric glycoprotein, its direct immobilization with the dextran matrix was not possible. Consequently, immunopurified anti-CICS antibodies were prepared and covalently linked to the dextran matrix. The experiments were initiated by injecting CICS, allowing it to bind to the anti-CICS antibodies. Then the PLA_2 to be tested was injected and its interaction with CICS was examined as shown in Fig. 1.

β-Btx (class III of β-neurotoxins) and nontoxic PLA_2s that we tested (i.e. human nonpancreatic PLA_2, group II, porcine pancreatic PLA_2, group I) and bee venom PLA_2, group III were unable to interact with CICS (Fig. 1A), even when injected at

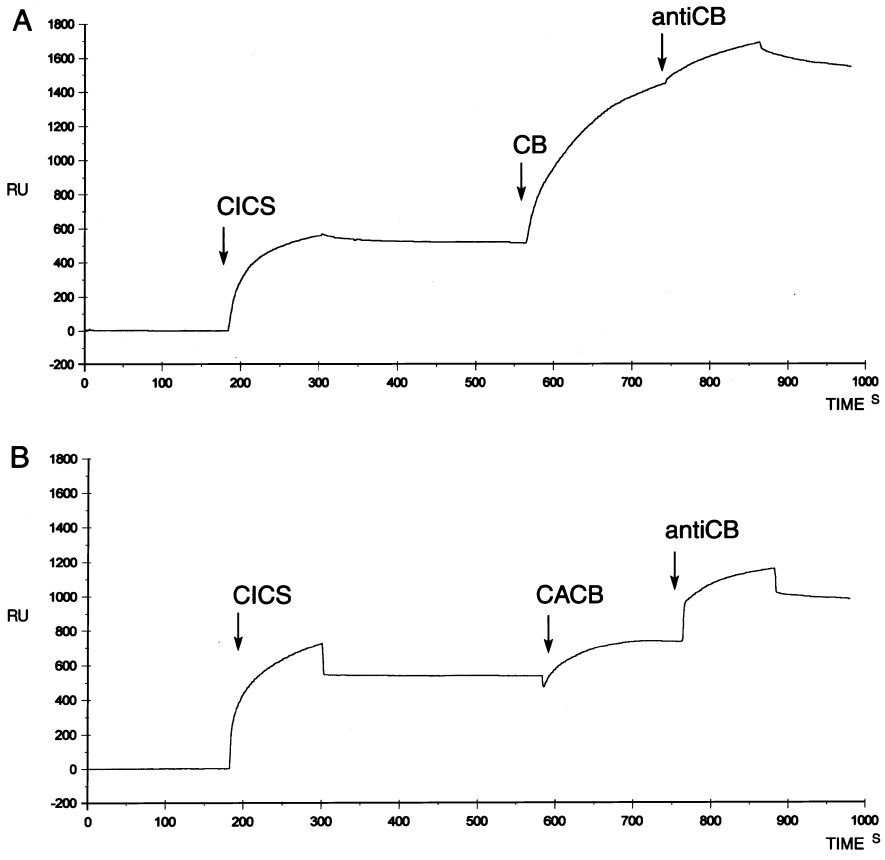

Fig. 2. Analysis of the complex composition using a monoclonal anti-CB antibody. CICS (30 $\mu g \cdot mL^{-1}$) was first attached to the CM_5 sensor chip surface through immunopurified anti-CICS antibodies, then CB (isoform CB_d of crotoxin) (5 $\mu g \cdot mL^{-1}$) (A) or crotoxin complex (isoform CA_2CB_d) (40 $\mu g \cdot mL^{-1}$) (B) were injected. Finally, the presence of CB bound to CICS was determined by injecting monoclonal anti-CB antibody (B−65.5) (20 $\mu g \cdot mL^{-1}$).

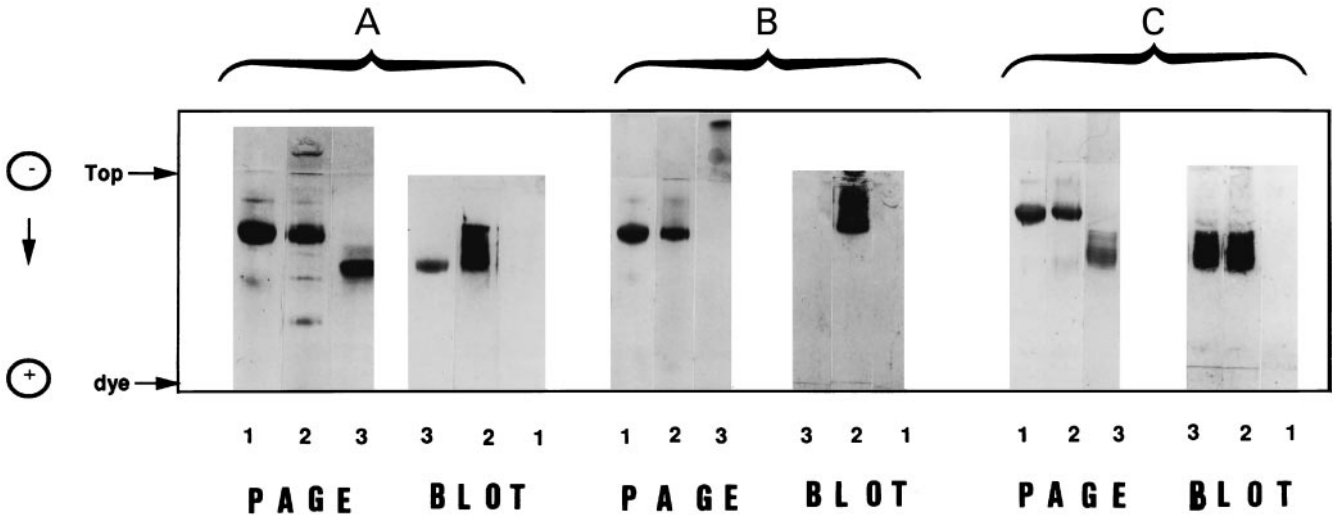

Fig. 3. PAGE and immunoblotting analysis of the interaction of CICS with β-neurotoxins. CICS (35 μg) and 20 μg of Agtx (A), Ntx (B) or Tpx (C) were preincubated in 40 μL of water, for 1 h at 37 °C. Aliquots of 1 μL were applied to the 20% acrylamide precast gels and nondenaturing PAGE were performed. Proteins were stained with Coomassie Brilliant Blue R-250 or electrotransferred to PVDF membrane and visualized using a polyclonal antiserum against Agtx (A) against Ntx (B) or against Tpx (C). Lane 1: CICS alone; Lane 2: CICS plus toxin; Lane 3: toxin alone.

high concentrations (100 $\mu g \cdot mL^{-1}$). Similarly, monomeric and multimeric β-neurotoxins from *Elapidae* snake family (group I PLA_2) did not interact with or weakly bind to CICS, as shown in Fig. 1(B), for Tpx and Ntx. On the other hand, β-neurotoxins from *Viperidae* snake family (group II PLA_2) strongly associated to CICS. These interactions were examined in detail in the case of crotoxin and CbICbII and of their isolated subunits (Fig. 1C–F). Monomeric β-neurotoxins from *Viperidae*, Atx and Agtx less effectively bound to CICS than the isolated PLA_2 subunits of multimeric β-neurotoxins from *Viperidae* (Fig. 1C). Basic subunit CB alone, as well as associated to CA in crotoxin interacted with CICS, while acidic subunit CA alone did not bind to CICS (Fig. 1E). The same behavior was observed in the case of CbICbII where only basic subunit CbII and whole toxin associated with CICS (Fig. 1F). Furthermore, SPR experiments performed with a monoclonal antibody directed against the catalytic subunit CB of crotoxin demonstrated that the PLA_2 subunit remains bound to CICS (Fig. 2). A quantitative study (Table 2, Fig. 1D) determined the kinetic parameters of the interaction between CICS and crotoxin: the dissociation rate constant k_{off} 1.7×10^{-4} s^{-1}, the association rate constant k_{on} 1.3×10^{4} $M^{-1} \cdot s^{-1}$ and the apparent dissociation constant $K_d^{app} = k_{off}/k_{on}$ of 13 nM. Similar kinetic parameters were observed with CbICbII and

Table 3. Effect of CICS on ACh release induced by β-neurotoxins on *Torpedo* synaptosomes. A fixed concentration of tested β-neurotoxin (1 μg in 20 μL NaCl/P_i) was incubated for 1 h at 37 °C with the indicated molar excess of CICS, then the aliquot (100 nM) was applied to the synaptosomes for 3 min at room temperature. ACh release was triggered by the addition of 5 mM $CaCl_2$ and expressed in arbitrary units. Finally ACh release was evoked with 90 mM KCl. Data are the mean ± SE values from three experiments.

		ACh release (mV)	
Origin and class	β-neurotoxin	$CaCl_2$ (5 mM)	KCl (90 mM)
	None	5 ± 4	220 ± 20
	CICS	7 ± 3	195 ± 25
Elapidae (Class I)	Ntx	130 ± 15	128 ± 10
	Ntx/CICS 1/16	92 ± 6	140 ± 15
Elapidae (Class II)	Tpx	287 ± 15	132 ± 20
	Tpx/CICS 1/16	160 ± 8	128 ± 15
Viperidae	CBd	575 ± 20	20 ± 5
(Class II PLA_2 subunits)	CBd/CICS 1/16	10 ± 5	220 ± 15
	CbII	625 ± 20	15 ± 3
	CbII/CICS 1/16	10 ± 5	156 ± 10
	CA2CBd	140 ± 20	76 ± 6
Viperidae	CA2CBd/CICS 1/16	30 ± 5	212 ± 15
(Class II)	CbICbII	250 ± 30	40 ± 5
	CbICbII/CICS 1/16	15 ± 4	196 ± 12
Viperadae (Class I)	Atx	520 ± 30	39 ± 10
	Atx/CICS 1/16	20 ± 6	160 ± 25

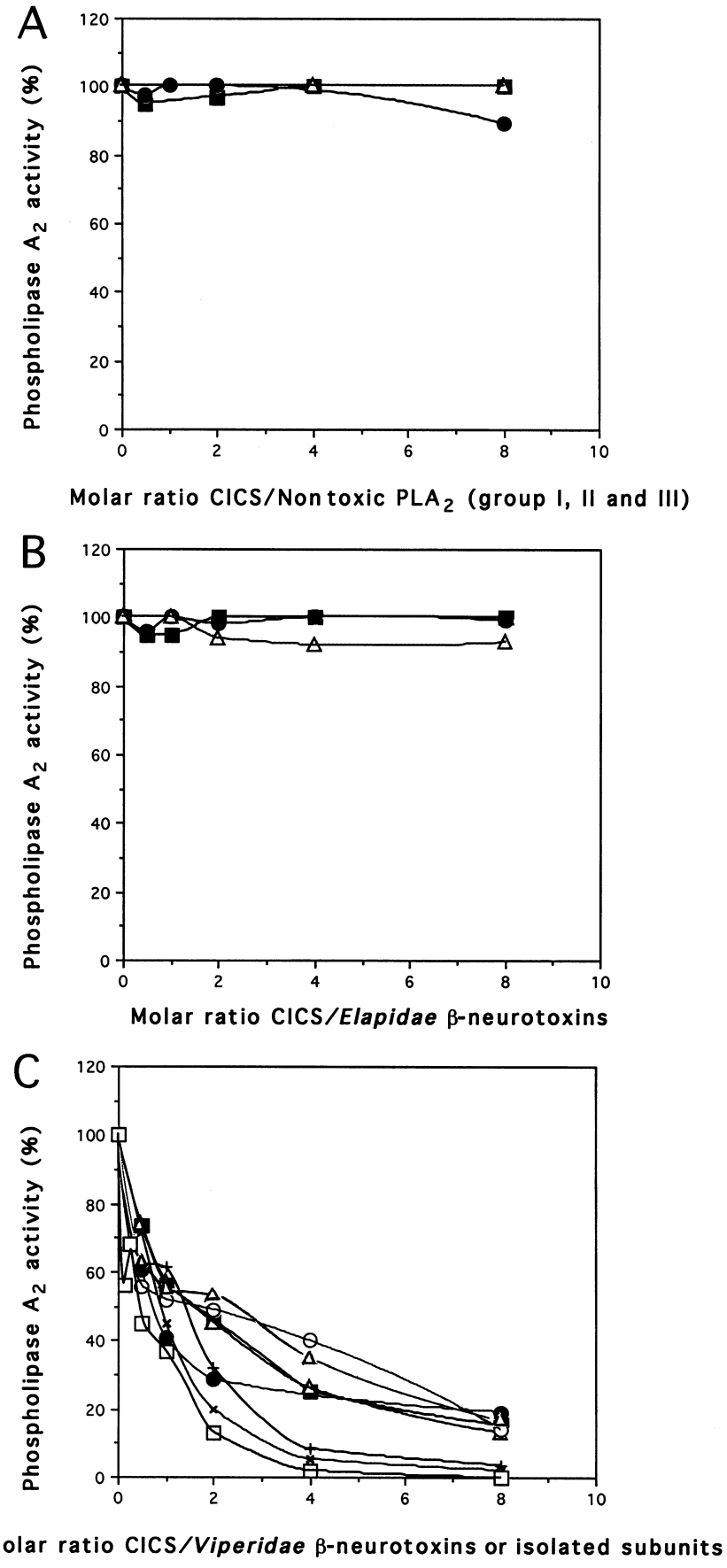

Fig. 4. Effect of CICS on the PLA_2 activity of the neurotoxic and nontoxic PLA_2s. Fixed concentrations of β-neurotoxins (1 μg in 20 μL of NaCl/P_i) were preincubated for 1 h at 37 °C with different concentrations of the CICS at the indicated molar ratio. After 1/20 dilution, 1–10 μL from this solution was introduced into the spectrophotometer cuvette containing 1 mL of the fluorescent phospholipid 1Pam(PyrDec)Gro*P*Gro (2 μM), then the residual PLA_2 activity was assayed by fluorescence according to Radvanyi *et al.* [46]. (A) Nontoxic PLA_2: porcine pancreatic PLA_2 (group I) (■), $hsPLA_2$ (group II) (△), bee venom PLA_2 (group III) (●). (B) *Elapidae* β-neurotoxins (PLA_2s group I): Ntx (■), Tpx (●) and β-Btx (△). (C) *Viperidae* β-neurotoxins (PLA_2s group II): Agtx (■), Atx (△), crotoxin, isoform CA_2CB_d (●), CbICbII (○), Toxin Mojave (▲) and isolated subunit, CBd (□), CbII (+), CB Mojave (×).

Table 2. SPR determination of k_{on} and k_{off} rate constants of CICS interaction with crotoxin and its CB subunit.

	k_{on} (10^4 M^{-1}·s^{-1})	k_{off} (10^{-4} s^{-1})	$K_d^{app} = k_{off}/k_{on}$ (nM)
Crotoxin	1.3 ± 0.3	1.7 ± 0.1	13
CB	12 ± 2	16 ± 2	13

toxin Mojave (results not shown). Interestingly, the PLA_2 subunits (CB, CB Mojave and CbII) when isolated from their complexes bind to CICS with a 10-fold increase in k_{on} and a nearly 10-fold increase in k_{off}, leading to a similar K_d^{app} (Table 2).

PAGE was used to analyze further the formation of complexes between CICS and β-neurotoxins. The various β-neurotoxins were preincubated with CICS, then submitted to PAGE and the gels were stained with Coomassie blue. Almost all tested monomeric (class I) and multimeric (class II) β-neurotoxins from *Viperidae* venom (PLA_2 group II, Table 1) or their isolated PLA_2 subunits formed complexes with CICS, characterized by a slower mobility than that of CICS alone (results not shown), as previously reported in the case of crotoxin [31]. However, this reduction in the mobility of CICS in the presence of Agtx, Ntx and Tpx was not detected and the composition of possible toxin-inhibitor complexes was further analyzed by Westernblot using polyclonal antibody anti-Agtx, anti-Ntx and anti-Tpx (Fig. 3). Westernblot performed with polyclonal antibodies directed against Agtx (Fig. 3A, panel 2 BLOT) shows that anti-Agtx detected free Agtx and also Agtx present in a protein band that migrates at CICS position. This indicates that this band is in fact a complex made of CICS and of Agtx. This complex co-migrates with CICS because the molecular mass/electric charge ratio of the CICS-Agtx complex is therefore very similar to that of CICS (we performed PAGE in the absence of SDS and reducing agents). In the case of the experiments performed with Ntx, free Ntx (very basic and hydrophobic protein) remains in the stacking gel (Fig. 3B, panel 3 PAGE). It cannot therefore be detected by Westernblot because the stacking zone cannot be removed from the solid support used to performed PAGE (Fig. 3B, panel 3 BLOT). The proportion of free Ntx and Ntx in a complex with CICS can be determined from a full panel (positions 2 and 3 PAGE) after staining with Coomassie blue. Free Ntx which remains in the stacking zone (Fig. 3B, panel 3 PAGE) was not detected in this zone after incubation with CICS (Fig. 3B, panel 2 PAGE). We conclude therefore that the majority of Ntx is present in the complex and this was confirmed with anti-Ntx antibodies where a new band corresponding to CICS/Ntx complex was labeled (Fig. 3B, panel 2 BLOT). On the other hand SPR experiments showed a very small signal of association of Ntx with CICS, most probably because these experiments were performed in the presence of 0.005 % polysorbate 20 (v/v) which reduce the formation of hydrophobic complexes. In contrast, the anti-Tpx antibody only detected free Tpx and no complex between CICS and Tpx can be labeled with anti-Tpx antibodies (Fig. 3C, panel 2 BLOT). In agreement with BIACORE® results, incubation between CICS and a multimeric *Elapidae* β-neurotoxin did not produce any complex.

Effect of CICS on the enzymatic activity of nontoxic PLA_2 and β-neurotoxins

The PLA_2 activity was measured by recording the changes in fluorescence intensity following hydrolysis of a substrate analogue. No significant change was observed in enzymatic activity of nontoxic secretory PLA_2s (porcine pancreatic PLA_2, group I; hsPLA_2, group II and bee venom PLA_2, group III), preincubated and tested in the presence of CICS (Fig. 4A). A similar lack of effect was observed with *Elapidae* β-neurotoxins (PLA_2 group I) i.e. (monomeric Ntx (class I), multimeric Tpx (class II) and β-Btx (class III) (Fig. 4B). On the other hand, mono and multimeric β-neurotoxins from *Viperidae* (group II PLA_2) were strongly inhibited by CICS, as well as their isolated enzymatic subunit. As shown in Fig. 4C, the PLA_2 activity of Agtx, CB, CB Mojave and CbII was reduced to 55 %, 90 %, 80 % and 70 %, respectively, in the presence of a twofold molar excess of CICS, while an eightfold molar excess of CICS is required for 80 % inhibition in the case of crotoxin, CbICbII and Mojave toxin (Fig. 4C).

Effect of CICS on the pharmacological properties of β-neurotoxins

Synaptosomes isolated from the electric organ of *Torpedo marmorata* provided a purely cholinergic model of the presynaptic part of the neuromuscular junction to study the neurotoxic action of β-neurotoxins. These isolated nerve terminals were still able to respond to depolarization with high concentrations of K^+ ions (90 mM) by releasing ACh. CICS alone did not affect this physiological response (Table 3). In the presence of Ca^{+2} (5 mM), β-neurotoxins induce a dose-dependent primary ACh release from synaptosomes [48]. When tested β-neurotoxins were incubated for 1 h with increasing amounts of CICS and compared to the control (the same β-neurotoxin without CICS), a dose dependent inhibition of primary ACh release was observed (data not shown). This release was almost totally prevented after preincubation of multimeric *Viperidae* β-neurotoxins or of their PLA_2 subunits with CICS at the molar ratio of 1/16 (toxin/inhibitor) (Table 3). This inhibitory effect of CICS on primary ACh release induced by *Elapidae* β-neurotoxins was less evident (Table 3).

After treatment with β-neurotoxins *Torpedo* synaptosomes were also tested for their ability to release ACh on depolarization by 90 mM KCl. As shown in Table 3, the crotoxin and CbICbII (100 nM) affected the release of ACh, by reducing their ability to respond to high K^+ levels. This reduction was inverted after application of increasing amounts of CICS. In the case of *Elapidae* β-neurotoxins that weakly reduced K^+ evoked ACh release, CICS did not affect this response.

DISCUSSION

Three types of PLA_2-inhibitors (α, β and γ) were described in the serum of various snakes. Numerous studies demonstrated their protective role against the PLA_2s of their own venom but their specificity for nontoxic and neurotoxic PLA_2s has not yet been well investigated. In this study we analyzed the specificity of CICS for neurotoxic and nontoxic secretory PLA_2s and examined the molecular mechanism of the interactions between CICS and neurotoxins belonging to the three structural groups of β-neurotoxins.

Our results show that CICS, which presents a sequence homology with neurotoxin-like inhibitors and therefore belonging to the γ-type PLA_2 inhibitors [33], exhibits a pattern of PLA_2 inhibition different from other members of this inhibitor family. In fact, CICS inhibits the PLA_2 activity and neutralizes the pharmacological action of crotoxin, the main neurotoxin from *Crotalus durissus terrificus* venom. It also inhibits and neutralizes other monomeric and multimeric PLA_2 β-neurotoxins from

Viperidae snake venom, but does not act on PLA_2 β-neurotoxins from *Elapidae* venom or the toxic or nontoxic PLA_2s from other sources. This observation is in accordance with the recent report of Fortes-Dias *et al*. [50], showing that CNF (CNF and CICS being different denominations of the same protein) is able to inhibit several PLA_2s isolated from the venom of the *Viperidae* snake *Lachesis muta muta*. Our conclusion contrasts, however, with the wider specificity of the other members of the γ type family of PLA_2 inhibitors. For example, it has been reported that the PLA_2 inhibitor isolated from the *Elapidae* (*Naja naja kaouthia*) plasma and PLIγ isolated from *Agkistrodon blomhoffii siniticus* plasma not only inhibited PLA_2s from their own venoms but also PLA_2s from other sources and belonging to groups I, II and III [27,33].

In a previous investigation [31] we suggested that CICS might be used as an alternative to horse antiserum in the antivenin treatment of human envenomations by *Crotalus durissus terrificus*. Because of its larger specificity, CICS might be therefore useful in the treatment of envenomations by all the *Viperidae* snakes. In this context, it is important to note that CICS has no effect on mammalian pancreatic (group I) and mammalian nonpancreatic (group II) PLA_2s, indicating that it should not interfere with the biological function of these human enzymes.

In this investigation, we further demonstrated using PAGE and SPR that CICS forms complexes with PLA_2 subunits of multimeric toxins such as CbII from CbICbII, CB Mojave from Mojave toxin and CB from crotoxin. The PLA_2 subunits were very sensitive to CICS. The isolated PLA_2 subunits bind to CICS more rapidly than the whole complex (the k_{on} value was found to be 10-fold higher). In agreement with the former, only a twofold molar excess of CICS is sufficient for almost total inhibition of the PLA_2 activity of PLA_2 subunits, while an eightfold molar excess is required for the same level of inhibition in the case of whole toxin. Further, SPR experiments performed with monoclonal antibodies specific for the catalytic subunit of β-neurotoxins showed that this subunit remains bound to CICS (Fig. 2). This observation is in agreement with previous results showing that CICS induces the dissociation of multimeric β-neurotoxins, the PLA_2 subunit remaining bound to CICS [31]. This dissociation of the complex explains how CICS neutralizes the neurotoxicity of these multimeric toxins. A similar mechanism has been described with a monoclonal antibody, A-56.36, directed to the CA subunit of crotoxin which neutralizes the lethal potency of crotoxin by dissociating the complex [51]. However, it should be emphasized that, at variance with CICS, the monoclonal antibody interacts with CA subunit releasing the CB subunit into solution which is less toxic than the whole crotoxin. Therefore, because of the residual toxicity of CB, the mechanism of neutralization by CICS is more efficient than that obtained with the monoclonal antibody A-56.36.

We observed a strong protective effect of CICS against the neurotoxic action of β-neurotoxins from *Viperidae* venoms on primary and evoked ACh release from *Torpedo* synaptosomes. β-neurotoxins cause a PLA_2 dependent primary release of ACh and the partial blockage of K^+-evoked ACh release on isolated nerve terminals from the electric organ of *Torpedo* [48]. As shown in Table 3, CICS inhibited the primary effect of *Viperidae* β-neurotoxins and also inhibited their blockage of K^+-evoked ACh release. This inhibition by CICS of the *in vitro* effect of *Viperidae* β-neurotoxins could correspond to its neutralizing effect demonstrated *in vivo* by lethality assays, in the case of crotoxin [31]. A possible explanation for the protective effect of CICS might be that it behaves as a false soluble receptor for β-neurotoxins, preventing their binding to their pharmacologically active receptors on synaptosomes, as previously suggested [31]. In contrast with PLA_2 β-neurotoxins from *Viperidae,* we did not observe any inhibitory action of CICS either on the enzymatic or the pharmacological activities of PLA_2 β-neurotoxins from *Elapidae* venoms. The functional activity of Ntx-CICS complex is not biologically relevant as Ntx remains enzymatically and pharmacologically active in the complex.

In conclusion, CICS specifically inhibits monomeric and multimeric β-neurotoxins from *Viperidae* family. The formation of PLA_2-CICS complexes is responsible for the inhibition of PLA_2 activity and for the protective effect of CICS against the neurotoxic action of these β-neurotoxins.

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